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Research Article

MiR-26a enhances the radiosensitivity of glioblastoma multiforme cells through targeting of ataxia–telangiectasia mutated

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ABSTRACT

Glioblastoma multiforme (GBM) is notoriously resistant to radiation, and consequently, new radiosensitizers are urgently needed. MicroRNAs are a class of endogenous gene modulators with emerging roles in DNA repair. We found that overexpression of miR-26a can enhance radiosensitivity and reduce the DNA repair ability of U87 cells. However, knockdown miR-26a in U87 cells could act the converse manner. Mechanistically, this effect is mediated by direct targeting of miR-26a to the 3′UTR of ATM, which leads to reduced ATM levels and consequent inhibition of the homologous recombination repair pathway. These results suggest that miR-26a may act as a new radiosensitizer of GBM.

Introduction

Glioblastoma multiforme (GBM, WHO grade IV) is the most common and lethal primary brain tumor type in adults, with an overall median survival of less than one year after diagnosis [1,2]. Surgical removal followed by radiation therapy (RT) and chemotherapy represents the standard treatment for GBM [3–5]. Radiotherapy significantly contributes to the prolongation of patient survival time; however, resistance of tumor cells to the effects of irradiation limits the success of treatment. To improve the response of glioblastoma to radiotherapy, laboratory investigations have aimed to define the determinants of radiosensitivity and to evaluate radiosensitizers using well-established glioblastoma cell lines [6,7]. The identification of new and more effective radiosensitizing agents for the treatment of malignant glioma is an emerging focus of research.

DNA double strand breaks (DSBs) are one of the most deleterious types of damage caused by radiation, and inducing DSBs in tumor cells is one of the major cancer therapy approaches [8]. However, the efficient repair of DNA DSBs by many tumors makes them radioresistant, which frequently prevents successful treatment [9,10]. In mammalian cells, two major DNA repair pathways play a critical role after DSBs: homologous recombination repair (HRR) and non-homologous end-joining (NHEJ) [11,12]. The

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critical components of these pathways are conserved from yeast to mammals. ATM (ataxia–telangiectasia mutated), a serine/threonine protein kinase, functions as a transducer of the DNA damage signal to the downstream effector machinery involved in the HRR-mediated DSBs repair pathway [13,14]. In response to DSBs, ATM is transformed from an inactive dimer to an active monomer in a process involving autophosphorylation of serine (ser1981) [15,16]. Subsequently, its hyperphosphorylation activates the downstream effector molecules, CHK2, p53 and H2AX, which leads to cell cycle arrest and the formation of discrete nuclear foci at the site of damaged DNA [17–19]. The critical role of ATM in the response to radiation is underscored by ATM-deficient human cell lines (A–T cells), which are sensitive to ionizing radiation (IR) [20,21]. ATM-deficient mice also display radiosensitivity, neurologic dysfunction, defects in T lymphocyte maturation and predisposition to cancer, with particularly increased levels of hematopoietic malignancy [22]. These phenotypic manifestations in both AT cells and ATM-deficient mice demonstrate the pleiotropic function of ATM kinase in various biological processes, including DNA repair, cell cycle checkpoints, gene regulation and telomere maintenance [23]. Consistent with these functions, mutation within ATM and repression of ATM have been associated with multiple cancer types, such as breast cancer, pancreatic cancer, leukemia and lymphomas [24–27]. Hence, understanding the molecular mechanisms underlying the regulation of ATM in cancer has received much attention.

MicroRNAs (miRNAs) are a class of short non-coding RNAs of 18–23 nucleotides that function as regulators of gene expression [28]. MiRNAs promote the targeted degradation of mRNAs or block the translation of mRNAs by base pairing with the 3′-untranslated region (UTR) of the targeted mRNAs [29]. Most mammalian mRNAs are conserved targets of miRNAs, and it is reasoned that identifying the miRNAs that target DNA DSB repair genes could be a new way of sensitizing tumors to IR. Several miRNAs were reported to affect the radiosensitivity of cancer cells, such as let-7, including miR-21, miR-101, miR-421, and miR-181a [30–34]. However, further identification of miRNAs that substantially increase the vulnerability of glioblastoma to radiation therapy is still lacking. MiR-26a, which is a frequent target of the 12q13.3–14.1 amplicon, has received much attention in recent years [35]. In GBM, miR-26a promotes decreased expression of the tumor suppressors PTEN and RB1 and inhibits expression of MAP3K2/MEKK2 [35]. Therefore, we hypothesized that miR-26a may target ATM. Our results demonstrate that in silico analysis using the publically available algorithm miRanda supports this hypothesis. Furthermore, we show that overexpression of miR-26a downregulates ATM expression by directly targeting the ATM-3′-UTR in GBM cells, consistently resulting in reduced DNA repair. However, knockdown miR-26a in U87 cells could act the converse manner. These results suggest that miR-26a plays an important role in the regulation of ATM and may represent a therapeutic target for glioblastoma multiforme and other diseases that are dependent on DSBs.

Materials and methods

Cell culture

Human U87 and U251 glioblastoma cells were obtained from the China Academic Sinica Cell Repository, Shanghai, China. All cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Los Angeles, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and were incubated at 37 °C in 5% CO_2 atmosphere.

Plasmid construction

The sequence of pre-has-miR-26a-2 was identified in the miRBase and confirmed in the genome. The pri-miRNA sequence and the sequences 100 bp before and after this site were amplified using the following primers flanked by restriction sequences: has-miR-26a-2-F (*Xho* I): CCGCTCGAGATTGAGGGGAAAAAGTCACTTCTCC; and has-miR-26a-2-R (*BamH*I): CGGGATCCAGGCTTCCAATGGATCAGTGGT.

To construct the miR-26a-shRNA vector, the following pair of oligonucleotides were synthesized, annealed and cloned into the *Xho*I and *BamH*I sites of the pGMLV-SB1 vector: Top Strand: 5′-gatccGACGGCGCTAGGATCATCAACGAAACAAGTAATATCTCAAGA-ATAGGCAAGTATTCTGGTCACAGAATACAACGAAACAAGTAATATCTCA-AGAATAGGCAAGATGATCCTAGCGCCGTCTTTTTTg-3′; Bottom Strand: 5′-aattcAAAAAAGACGGCGCTAGGATCATCTTGCCTATTCTTGAGATATT-ACTTGTTTCGTTGTATTCTGTGACCAGAATACTTGCCTATTCTTGAGATAT-TACTTGTTTCGTTGATGATCCTAGCGCCGTCg-3′. To construct the pmirGLO-ATM-3′UTR-WT plasmid, a wild type 3′-UTR segment of the human ATM mRNA (GenBank accession number: NM_000051) containing the putative miR-26a binding sequence was amplified and cloned into the *Sac*I and *Xho*I sites downstream of the luciferase reporter gene in pmirGLO vector (Promega). pmirGLO-ATM-3′UTR-MUT, which carries a mutated sequence in the complementary site for the seed region of miR-26a, was generated based on pmirGLO-ATM-3′UTR-WT plasmid by site-specific mutagenesis using the following primers: forward primer: 5′-TTTGAATGTTGGTTTTAAAAG-TAGTT-3′; reverse primer: 5′-GTGATTAAAACTACTATTAA-3′.

Quantitative reverse transcription (qRT)-PCR

Total miRNA from cultured GBM cells was extracted using TRIzol (Invitrogen). cDNA was synthesized from 5 ng of total RNA using the Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), and the expression levels of miR-26a were quantified using a specific TaqMan MiRNA Assay Kit (Applied Biosystems). Real-time PCR was performed using the Applied Biosystems 7500 Sequence Detection system. The expression of miR-26a was defined based on the threshold cycle (Ct), and relative expression levels were calculated as $2^{-[(Ct\ of\ miR\text{-}26a)-(Ct\ of\ RNU48)]}$ by normalization to the expression of RNU48 small nuclear RNA.

Western blotting

Western blot assays were performed as previously described using antibodies against ATM (2873, Cell Signaling, Danvers, MA, USA), p-ATM (5883, Cell Signaling, Danvers, MA, USA), p-CHK2(2661, Cell Signaling, Danvers, MA, USA), p53(2527, Cell Signaling, Danvers, MA, USA), and anti-γ-H2AX (ab22551, Abcam, Cambridge, MA, USA). The membranes were stripped and re-probed with an anti-GAPDH antibody (sc-25778, Santa Cruz Biotechnology) as a loading control.

Clonogenic formation assay

Cells were trypsinized to generate single-cell suspensions and plated at 200 cells per 60-mm^3 dish in triplicate. After allowing