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miR-375 targets the p53 gene to regulate cellular response to ionizing radiation and etoposide in gastric cancer cells

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ABSTRACT

MicroRNAs (miRNAs) offer a new approach for molecular classification and individual therapy of human cancer due to their regulation of oncogenic pathways. In a previous report, elevated miR-375 was found in recurring gastric cancer, and it was predicted that miR-375 may be a regulator of p53 gene. However, its biological role and mechanism of actions remain unknown. In this study, we characterized the expression level of miR-375 in gastric cancer cell lines – BGC823, MGC803, SGC7901, AGS, N87, MKN45 – using RT-PCR. We found that exogenous expression of miR-375 promoted the growth of AGS cells in both liquid and soft agar media. In agreement with the previous report, overexpression of miR-375 in AGS cells reduced the p53 protein expression level. A luciferase assay demonstrated that miR-375 down-regulated p53 expression through an interaction with the 3′ UTR region of p53. In addition, the expression of miR-375 desensitizes cells to ionizing radiation and etoposide. Flow cytometry analyses showed that miR-375 abrogated the cell cycle arrest and apoptosis after DNA damage. These results demonstrate that miR-375 targets p53 to regulate the response to ionizing radiation and etoposide treatment.

1. Introduction

microRNAs (miRNAs) are a class of endogenous, non-coding RNAs that negatively regulate gene expression by binding to the 3′ untranslated region (UTR) of mRNAs. It is known that the aberrant expression of miRNAs is involved in many human diseases, notably cancers [1,2]. Multiple miRNA expression profiles of cancer patients show the correlation with stage, progression and prognosis [3–5]. Yanaihara et al. [6] found that the abnormal miRNA expression associated with survival and disease stage of lung adenocarcinomas. Kuo et al. [7] performed miRNA expression profiles and demonstrated that miR-29a/c could be a biomarker for early recurrence of colorectal cancer. Previously, we have identified 17 differential miRNAs within a recurrence group and ascertained a combination of miR-375 and miR-142-5p as predictors of recurrence risk in gastric cancer patients [8]. The frequent aberrant expression and functional implications of miRNAs in cancers have captured the attention of the research community for their potential to serve as prognosis biomarkers or drug targets. However, before drugs can be used as therapies, an understanding of the mechanisms and identification of the genes targeted by miRNAs is required. One potential target that has already emerged is the p53 gene.

p53 is a tumor suppressor gene whose function is frequently lost in cancers [9,10]. It is usually activated in response to toxicity stress signals to prevent replication of damaged DNA [11,12]. To a large extent, this nature of p53 is mediated by its ability to function as a transcription factor of target genes and induce the inhibition of cell growth in response to DNA damage, through the activation of either cell cycle arrest or apoptotic cell death [13]. Therefore, abnormal expression of p53 would result in both loss of tumor suppressor activities and gain of functions that contribute to tumor formation. The regulation of p53 expression is complex and mainly occurs at both transcriptional and post-translational levels. In addition to regulation by transcription factors such as AP1, p53 undergoes various post-translational modifications such as phosphorylation, ubiquitination, methylation, acetylation and neddylation [14].

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In the present study, we report a novel regulation of p53 by miR-375, and propose a cascade of events leading to an alterative response to ionizing radiation (IR) and etoposide in gastric cancer cells. To explore the biological role of miR-375, we transfected AGS cells with miR-375 and measured their growth ability in regular and soft agar medium. We also investigated the effect of miR-375 on p53 protein expression in the tumor cell lines, AGS, MCF-7 and A549. Using a luciferase assay we demonstrated that miR-375 could interact with the 3′ UTR region of p53 to down-regulate its protein level. Furthermore, we detected that the presence of miR-375 abrogated the response to IR and etoposide through regulation of the p53-mediated cell cycle arrest and apoptosis. We further confirmed that miR-375 impaired cell toxicity through inactivation of the p53 pathway.

2. Materials and methods

2.1. Cell culture

The cell lines AGS, A549 and MCF-7 used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). AGS and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS); A549 cells were cultured in RPMI-1640 medium (Invitrogen), supplemented with 5% FBS. All cell lines were maintained at 37 °C in 5% CO_2.

2.2. Plasmid construction and cell transfection

The expression plasmid containing miR-375 was created by PCR amplification, using human genomic DNA as a template. The following primers were used: 5′-CCGCTCGAGCCTCCTCTCCCACCCCGTACGGTT-3′ (F); 5′-CGGGATCCTGAGGGCGGAGGCTAGCGGG-3′ (R). The PCR product (270 bp containing pri-miRNA) was cloned into the pcDNA3.1(−) expression vector (Invitrogen) and confirmed by DNA sequencing. The empty vector was used as a control. To establish a stable cell line with ectopic miR-375 expression, miR-375 expression vectors were transfected into AGS cells, and selected with G418 (400 μg/ml) for 3–4 weeks.

miR-375 mimics, a nonspecific miR control, anti-miR-375, a nonspecific anti-miR control, p53 siRNA oligonucleotides, and the control siRNA oligonucleotides were purchased from Invitrogen. These were transfected at a concentration of 100 nmol/L using Lipofectamine 2000 reagent (Invitrogen).

2.3. Luciferase reporter assay

The pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA; E1910) was used for construction of the luciferase reporter vector. Double-stranded oligonucleotides containing the wild type (wt-3′ UTR) or mutant (mt-3′ UTR) miR-375 binding sites in the p53 3′ UTR were synthesized (Sangon-Biotech, Shanghai, China) and inserted into the XhoI and SbfI restriction sites of the pmirGLO plasmid (Promega). The sequences of inserted fragments were confirmed by sequencing.

Cells were transfected with the luciferase reporter vectors in 24-well plates together with miR-375, or control vector and anti-miR375 or a nonspecific anti-miR control by Lipofectamine 2000. Luciferase activities were measured 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized against Renilla luciferase activities.

2.4. RT-PCR and realtime PCR analysis

Total RNA was extracted with TRIzol (Invitrogen) and reverse transcribed for 30 min at 42 °C, followed by 5 min at 85 °C, using EasyScript First-Strand cDNA Synthesis SuperMix (Transgen, Beijing, China). Stem-loop reverse transcription (RT) primers for miR-375 and U6 were TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGC and AACGCTTCACGAATTTGCGT. The resulting cDNA was used for PCR. The thermal cycling program consisted of an initial denaturation step at 95 °C for 5 min followed by 28 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s. The primer pairs used for amplification of miR-375 and U6 were: 5′-CAGGGTCCGAGGTATT-3′ (F) and 5′-CTGCTTTGTTCGTTCG-3′ (R), and 5′-CTCGCTTCGGCAGCACA-3′ (F) and 5′-AACGCTTCACGAATTTGCGT-3′ (R), respectively. The internal RT-PCR control (U6) was done on all specimens simultaneously.

Quantification of miR-375 and U6 expression was conducted with an RT-Cycler 436 system (CapitalBio Corp, Beijing, China). The Transtart Green qPCR Supermix (Transgen) was used according to the manufacturer's protocol, to detect mature miRNAs using the SYBR Green method. The results were normalized against the U6 gene.

2.5. Western blot analysis

Fifty micrograms cell line protein was separated by 12% Tris-glycine polyacrylamide gels and transferred to polyvinylidene fluoride membranes (General Electric Healthcare, Buckinghamshire, UK). Membranes were incubated with mouse monoclonal antibodies against human p53, p21, MDM2, Bcl-2 and Bax (sc-126, sc-6246, sc-965, sc-492, sc-65532, respectively; Santa Cruz, CA, USA) followed by horseradish peroxidase-labeled goat-anti-mouse IgG (Proteintech Group Inc., Chicago, IL, USA). β-actin was used as a loading control (Sigma, St. Louis, MO, USA). Protein intensity was quantified using the LANE 1D Analyzer software (Sage Creation, Beijing, China).

2.6. Flow cytometry analysis of cell cycle and apoptosis

The transfected AGS cells (1×10^5) were harvested by trypsin digestion, washed with PBS, and then fixed in cold 75% ethanol at 4 °C overnight. After staining with propidium iodide (PI) solution for 30 min, cell cycle analysis was performed by fluorescence activated cell sorting to determine the percentage and distribution of cells in the G1, S and G2/M phases.

For detection of phosphatidylserine externalization, trypsinized cells were double-stained with fluorescein isothiocyanate-conjugated AnnexinV (20 μg/ml) and PI (50 μg/ml) according to the manufacturer's protocol (Biosea, Beijing, China). Ten thousand cells were collected and analyzed on a FACSorter flow cytometer, and the cell cycle profiles were analyzed using the Cell Quest software (Becton–Dickinson, Mountain View, CA, USA).

2.7. MTT assay

AGS cells with stable ectopic miR-375 expression or controls (containing the empty vector) were plated into 96-well culture plates, and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltertrazolium bromide (MTT) was added at 24, 48, 72 and 96 h. After 4 h incubation at 37 °C in 5% CO_2, 200 μL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan product. The absorbance at 570 nm was determined using a microplate reader (Bio-Rad, Hercules, CA, USA).