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Down-regulation of 5S rRNA by miR-150 and miR-383 enhances c-Myc–rpL11 interaction and inhibits proliferation of esophageal squamous carcinoma cells

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ABSTRACT

5S rRNA plays an important part in ribosome biology and is over-expression in multiple cancers. In this study, we found that 5S rRNA is a direct target of miR-150 and miR-383 in esophageal squamous cell carcinoma (ESCC). Overexpression of miR-150 and miR-383 inhibited ESCC cell proliferation in vitro and in vivo. Moreover, 5S rRNA silencing by miR-150 and miR-383 might intensify rpL11–c-Myc interaction, which attenuated role of c-Myc as an oncogenic transcriptional factor and dysregulation of multiple c-Myc target genes. Taken together, our results highlight the involvement of miRNAs in ribosomal regulation during tumorigenesis.

1. Introduction

As one of the most common and fatal malignancies in the world, esophageal squamous cell carcinoma (ESCC) shows a relatively high incidence in Asian including China [1]. Cigarette smoking, heavy alcoholic consumption, micro-nutrient deficiency as well as dietary carcinogen exposure have been identified as main environmental etiological factors of ESCC [1–3]. However, only a part of exposed individuals eventually developed ESCC, indicating that host genetic differences may also contribute to ESCC carcinogenesis [4–6].

Ribosome is composed of both ribosomal RNA (rRNA) and ribosomal proteins (RPs). Deregulation of ribosomal biogenesis has been related to cell cycle alterations and cell growth arrest [7]. Cancer, anemia, and aging have been clearly linked to abnormal ribosome biogenesis [8]. The highly conserved 5S rRNA is transcribed from multiple loci in the genome by RNA polymerase III and makes a great contribution to stabilizing the structure of the large ribosomal subunit [9]. 5S rRNA is involved in cell cycle control through assembly of 5S rRNA-rpL11-rpL5 RNA–protein (RNP) complex [10–12]. The breakdown of this RNP complex by depletion of 5S rRNA could release free-bound rpL11, which in turn caused cell cycle arrest [13,14]. The deregulations of MDMX (also known as MDM4) are common in many human cancers, through inhibiting P53 tumor suppressor functions [15]. Interestingly, interaction between 5S rRNA and MDMX was responsible for MDMX stability [16], which also supports the involvement of 5S rRNA in tumorigenesis.

MicroRNAs (miRNAs) are a kind of small RNA molecules which are about 21–23 nucleotides (nt). Mature miRNAs could be

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incorporated into RNA-induced silencing complex (RISC), which is composed of catalytic subunit Ago2 and many other proteins [17]. The precise genomic mapping of Ago2, identified by Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP), provides us a clue for identifying binding loci of Ago2 [18]. Interestingly, bioinformatics data-mining of PAR-CLIP data indicate that there are widespread regulatory interactions between miRNA and non-coding RNAs (ncRNAs) [19,20].

In this study, we found for the first time that 5S rRNA could be directly targeted by miR-150 and miR-383 in ESCC cells. Overexpression of miR-150 and miR-383 could significantly down-regulate 5S rRNA expression, which in turn led to growth inhibition of ESCC cells. The decreased 5S rRNA expression may result in rpL11 release from 5S rRNA-rpL11-rpL5 RNP complex, enhancement of c-Myc–rpL11 interaction and, then, inhibition of c-Myc oncogene activity.

2. Materials and methods

2.1. Quantitative real-time PCR

Total RNA was isolated from ESCC cells using Trizol reagent (Invitrogen). RNA samples were reverse transcribed (RT) into cDNA with random RT primers using ReverTra Ace kit (TOYOBO). Real-time qPCR primers used for detecting 5S rRNA, CDK4, CCND1, P21 and β-actin were described previously [16,21]. The primers for human U6 snRNA in qPCR were: forward 5′-CTCGCTTCGGCAG CACA-3′ and reverse 5′-AACGCTTCACGAATTTGCGT-3′, respectively. Transcript level expression of genes or miRNAs were quantified against β-actin or U6 RNA by qPCR using the SYBR®Green I fluorogenic dye (TOYOBO).

2.2. Plasmid construction

The fragment corresponding to the 5S rRNA was synthesized and inserted between the *Xba* I restriction site of the pGL3-control reporter plasmid (Promega). The plasmid was named as pGL3-5S. For construction of 5S rRNA reporter gene plasmids with mutant miR-150 binding site or mutant miR-383 binding site, QuikChange Site-Directed Mutagenesis kit (Stratagene) was used with mutation primers were shown in Supplementary Table 1. These mutant plasmids were named as pGL3-mut150 or pGL3-mut383. Wild-type and mutant inserts were confirmed by Sanger sequencing.

2.3. Dual luciferase reporter gene assays

A firefly luciferase reporter plasmid (pGL3-5S, pGL3-mut150 or pGL3-mut383), a Renilla luciferase vector (pRL-SV40) (Promega) plus small RNAs (miR-150 mimics, miR-383 mimics, 5S rRNA siRNA duplex or negative control RNAs (NC) (Genepharma, China) were co-transfected into KYSE30 or KYSE510 cells with Lipofectamine® 2000 (Invitrogen). The sequence for 5S rRNA siRNA (si-5S) or si-5S-2 was 5′-GGGAAUACCGGGUGCUGUAUU-3′ as reported previously [16] or 5′-GGUUAGUACUUGGAUGGGAUU-3′. Three independent transfection experiments were performed and each was done in triplicate. Firefly luciferase activities derived from pGL3-control derived plasmids were normalized to Renilla luciferase activity from pRL-SV40 using a Dual Luciferase Assay system (Promega) as reported previously [22–24].

2.4. Cell proliferation assays

ESCC cells were seeded in a 12-well plate at a density of 1×10^5 cells per well. Cells were transfected with 20 nM miR-150 mimics, miR-383 mimics, si-5S or negative control (NC) RNA (Genepharma) combined with Lipofectamine® RNAi Max (Invitrogen). Cells were harvested by trypsin digestion, washed by cold PBS twice, dyed with trypan blue and counted under microscopy at 24 hours (h) and 48 h after transfection.

2.5. Flow cytometry

KYSE30 cells were transfected with 20 nM miR-150, miR-383 or NC RNA. Cell apoptosis was measured using Dead Cell Apoptosis Kit with Annexin V FITC and propidium iodide (PI) (Invitrogen). Cell cycle changes were examined using PI staining. Cell apoptosis and cell cycle were then analyzed by flow cytometry.

2.6. Colony formation assays

A total of 4000 KYSE450 cells were transfected with 20 nM NC RNA, miR-150 mimics, miR-383 mimics or si-5S rRNA. After 14 days, cells were washed with cold PBS twice and fixed with 3.7% formaldehyde. Cells were dyed with crystal violet and the colony number of each well was counted.

2.7. Western blotting

Total cellular proteins were harvested from KYSE30, KYSE150, KYSE180, KYSE450, KYSE510 and TE-1 cell lines. KYSE510 cells were transfected with 20 nM miR-150, miR-383, NC RNA or si-5S. Cells were harvested at 48 h after transfection and cell lysates were immunoblotted as previously reported [25–28]. Antibodies against MDMX (MILLIPORE, clone 8C6), P21 (Cell signaling technology, US, 12D1), P53 (Santa Cruz, DO-1) or β-actin (Santa Cruz, C-4) were used. The expression level of MDMX, P21 and P53 was scanned by Image J and normalized to β-actin expression.

2.8. MiR-150, miR-383 and 5S rRNA expression in ESCC specimens

Twenty-four pairs of ESCC tissues and normal tissues adjacent to the tumors were obtained from surgically removed specimens of ESCC patients in Shandong Cancer Hospital, Shandong Academy of Medical Sciences. Total RNA was extracted from tissue samples using TRIZOL. For quantification of miR-150 or miR-383, cDNA was synthesized from total RNA using specific stem-loop RT primers (Ribobio, China) according to the manufacturer's protocol. For quantification of 5S rRNA, cDNA was synthesized from total RNA using random primers. U6 RNA and β-actin were used as endogenous controls for miRNAs and 5S rRNA detection, respectively.

2.9. Immuno-precipitation (IP) assays

A total of 2×10^7 KYSE150 cells were transfected with 20 nM miR-150 mimics, miR-383 mimics, si-5S or NC RNA. Cells were treated with cell lysis buffer for Western blotting and IP (Beyotime, China) supplemented with 1 mM PMSF at 48 h after transfection. Cell lysates were pre-incubated with protein A/G plus beads (Santa Cruz) and normal rabbit IgG (Santa Cruz) at 4 °C for 1 h on a vertical mix device. After cell lysates were centrifuged, supernatants were collected. The supernatants were incubated with c-Myc antibody (Santa Cruz, N262) or normal rabbit IgG and protein A/G plus beads at 4 °C for 12 h on a vertical mix device. The proteins bound on the beads were eluted with the SDS loading buffer, incubated for 10 min at 70 °C. 10 μl each samples were separated on a SDS–PAGE, transferred to a PVDF membrane, blocked by 5% BSA, probed by c-Myc antibody or primary rpL11 antibody (Abcam, ab79352) and corresponding secondary antibodies.

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