

microRNA-299-3p inhibits laryngeal cancer cell growth by targeting human telomerase reverse transcriptase mRNA

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Received February 19, 2014; Accepted October 24, 2014

DOI: 10.3892/mmr.2015.3287

Abstract. Aberrant microRNA (miRNA) expression has been linked to cancer development. In this study, we aimed to investigate whether the anti-cancer effect of miRNA-299-3p on laryngeal cancer Hep-2 cells is mediated through targeting human telomerase reverse transcriptase (hTERT). The expression of miR-299-3p in laryngeal cancer Hep-2 cells and human osteosarcoma U2OS cells was quantified by stem-loop-mediated reverse transcription quantitative polymerase chain reaction. miR-299-3p mimic was transfected into Hep-2 cells to induce overexpression of miR-299-3p. A CCK-8 assay was performed to identify the effects of miR-299-3p overexpression on the proliferation of Hep-2 cells. Western blot analysis was carried out to determine the expression of hTERT protein. A significant decrease was noted in the expression of miR-299-3p in Hep-2 cells compared with that of U2OS cells ($P < 0.05$). Overexpression of miR-299-3p resulted in a notable inhibition of cellular proliferation ($P < 0.05$), as well as downregulation of hTERT mRNA and protein in Hep-2 cells ($P > 0.05$). The expression of miR-299-3p is downregulated in human laryngeal cancer Hep-2 cells. Overexpression of miR-299-3p inhibits Hep-2 cell growth by targeting the 3'-untranslated region of hTERT mRNA.

Introduction

MicroRNAs (miRNAs), a class of highly conserved small non-coding RNAs, play crucial roles in regulating the expression of target genes in a sequence-specific manner (1,2). It is acknowledged that the binding of a specific miRNA to the

3'-untranslated region (3'-UTR) of target mRNA may inhibit the expression of target genes. To date, several classes of miRNAs have been reported to play significant regulatory roles in various cellular processes through translational repression, including cell proliferation, apoptosis and developmental timing (3). Previous studies have revealed that aberrant expression of miRNA is associated with the development of human cancer, and its expression profiling has been identified as a signature of the diagnostic and prognostic characteristics of specific carcinomas (4,5). Li *et al* demonstrated that downregulation of miR-1297 in Hep-2 cells may inhibit proliferation of cancer cells, migration and tumor genesis *in vitro* (6). Simultaneously, these authors discovered that miR-1297 may be directly involved in the downregulation of phosphatase and tensin homolog in Hep-2-cells and solid tumors. Moreover, Wu *et al* demonstrated that overexpression of miR-16 contributed to the cell migration of Hep-2 laryngeal cancer cells and suppression of intercellular adhesion. Additionally, zyxin, which was negatively regulated by miR-16, was confirmed to be a direct target gene of miR-16 (7). On this basis, we predicted that miRNA-mediated regulation of cellular and genetic expression may be an appropriate treatment option for malignant tumors.

Human telomerase reverse transcriptase (hTERT), a catalytic subunit of telomerase, is considered to be the most significant component of the telomerase complex together with the telomerase RNA component (8,9). Telomerase activity is crucial in the immortality of cancer cells (e.g. Hep-2 cancer cells) through continuously adding the telomeric repeats onto the G-overhang of the 3' termini of telomeres (10). Research reveals that only one study has investigated the correlation between the expression of miRNA and hTERT (11), in which miR-138 induced a reduction in hTERT protein in human anaplastic thyroid carcinoma cell lines. Therefore, it is still unclear which of the miRNAs could exert a strong suppressive effect on the expression of hTERT in cancer cells.

In the present study, hTERT expression was found to be inversely correlated with the endogenous expression of miR-299-3p in Hep-2 and U2OS cells; moreover, overexpression of miR-299-3p inhibited the expression of hTERT mRNA and protein by directly targeting the 3'-UTR of hTERT mRNA. Furthermore, downregulation of hTERT mediated by miR-299-3p mimic inhibited Hep-2 cell growth. The results of

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Key words: microRNA, microRNA-299-3p, human telomerase reverse transcriptase, laryngeal cancer, Hep-2 cell

this study may be of use in the treatment of human laryngeal cancer.

Materials and methods

Cell culture and transfection. Hep-2 cells and U2OS cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Sigma-Aldrich) at 37°C in 5% CO₂ at a humidity of 95%. Transfection of Hep-2 cells was performed with Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Transfections were performed in triplicate for each experiment.

Prediction of miRNAs targeting hTERT. To predict the miRNAs that target hTERT and the conserved sites bound by the seed region of these miRNAs, different target prediction programs were used, including TargetScan (<http://www.targetscan.org/>), miRanda (<http://www.microrna.org/microrna/home.do>) and starBase (<http://starbase.sysu.edu.cn/>).

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted from Hep-2 cells or U2OS cells using TRIzol reagent (Invitrogen Life Technologies), strictly following the instructions. The synthesis of cDNA was carried out using the RevertAid First strand cDNA synthesis kit (Fermentas; Thermo Fisher Scientific, Waltham, MA, USA). qPCR was performed using TaqMan miRNA assay kits (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 7500 thermal cycler. The expression of miRNA was normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers for hTERT were 5'-CTTCCACTCCCCACATAGGA-3' (hTERT-F) and 5'-GTA CAGGGCACACCTTTGGT-3' (hTERT-R). The primers for GAPDH were 5'-AGAAGGCTGGGGCTCATTG-3' (GAPDH-F) and 5'-AGGGGCCATCCACAGTCTTC-3' (GAPDH-R). PCR amplification was conducted in a volume of 20 µl containing 3 µl RT product, 10 µl 1X SYBR Select master mix and 7 µl all-in-one miRNA qPCR primer. The reaction conditions were as follows: 50°C for 2 min and 95°C for 20 sec, followed by 40 cycles of 95°C for 10 sec, 60°C for 34 sec and 70°C for 10 sec. Melting analysis was carried out at the end of the amplification cycle to verify non-specific amplification. The expression level was quantified using the 2^{-ΔΔCt} method.

Construction of luciferase reporter plasmids and luciferase assays. The 3'-UTR segments of hTERT containing the miR-299-3p binding sites were amplified by PCR from Hela cell genomic DNA. The primers were 5'-CCGCTCGAGTGG CCACCCGCCACAG-3' (hTERT-F) and 5'-GTTTAGCGG CCGCTTTACTCCCACAGCACCTCC-3' (hTERT-R). Next, the PCR product was cloned into the *XhoI/NotI* sites downstream of the *Renilla* luciferase (Rluc) reporter gene pmiR-RB-ReportTM vector (RiboBio, Guangzhou, China), which was named pmiR-hTERT-3'-UTR-wt. Mutations in the predicted miR-299-3p binding sites were established using pmiR-hTERT-3'-UTR-wt as a template, and was named

pmiR-hTERT-3'-UTR-mut. The pmiR-RB-Report vector containing a synthetic *Renilla* luciferase (hRluc) gene and a synthetic firefly luciferase (hluc) gene encoding firefly luciferase was used as the internal control. The recombinant plasmid, designated as pmiR-RB-hTERT-3'-UTR, was confirmed by restriction enzyme digestion and DNA sequencing. Hela cells were co-transfected in 96-well plates with miR-299-3p mimic or negative control (RiboBio), pmiR-hTERT-3'-UTR-wt or pmiR-hTERT-3'-UTR-mut by Lipofectamine 2000. Forty-eight hours after transfection, luciferase activity was measured using the dual luciferase assay system (Promega, Heidelberg, Germany). The firefly luciferase activity of each sample was normalized to *Renilla* luciferase activity.

Western blot analysis. Western blot analysis was performed as routinely described. Briefly, the protein was homogenized in modified RIPA buffer containing 0.5% sodium dodecyl sulfate (SDS) in the presence of proteinase inhibitor cocktail (Complete mini; Roche Diagnostics, Basel, Switzerland). Proteins (40 µg) were separated by electrophoresis on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Subsequently, the membrane was blocked in 5% non-fat milk and incubated with rabbit anti-hTERT antibody (1:1000 dilution; Epitomics, Burlingame, CA, USA) at 4°C overnight, followed by incubation with a secondary antibody (1:5000; LI-COR, Lincoln, NE, USA) for 4 h at room temperature. The same membrane was probed for GAPDH (1:1000 dilution; Epitomics) as the loading control. The relative density of hTERT to GAPDH was analyzed with an Odyssey[®] infrared imaging system (LI-COR).

CCK-8 assay. Hep-2 Cells were seeded in a 96-well plate at a concentration of 10³ cells per well. Following transfection, the cells were maintained at 37°C for 24, 48 and 72 h, respectively. The cells were treated with CCK-8 (Dojindo Inc., Kumamoto, Japan) for 4 h at 37°C. Subsequently, the medium was removed and the pellet was dissolved in 100 µl DMSO. Finally, the number of cells was determined using a microplate reader at a wavelength of 450 nm.

Statistical analysis. SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) was used for the data analysis. Student's t-test was performed to analyze the differences between the data obtained from three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

hTERT expression is inversely correlated with endogenous expression of miR-299-3p in Hep-2 and U2OS cells. Fig. 1 shows the correlation between miR-299-3p expression and hTERT protein in Hep-2 and U2OS cells. Western blot analysis revealed that hTERT was positively expressed in the Hep-2 cells, while it was negatively expressed in U2OS cells (Fig. 1B). In addition, qPCR was used to measure the expression of miR-299-3p in the two cell lines. As shown in Fig. 1C, higher levels of miR-299-3p were observed in U2OS cell lines compared with those in Hep-2 cells. An inverse correlation was observed between the expression of miR-299-3p and hTERT

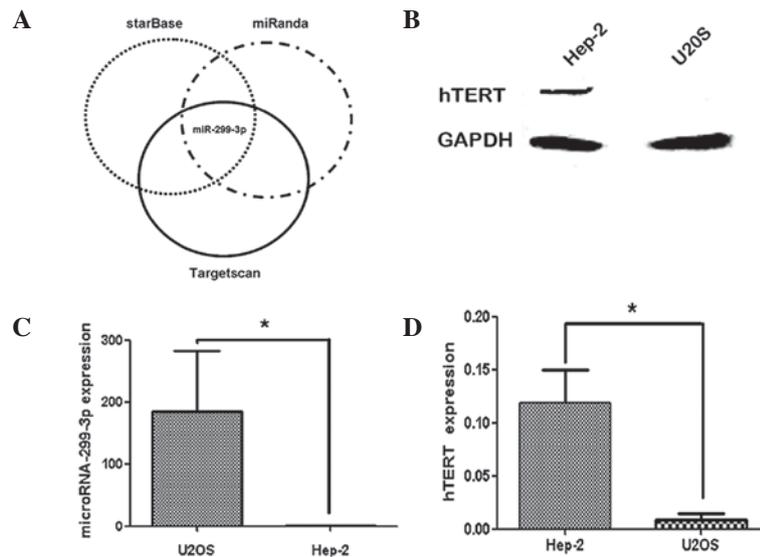


Figure 1. Correlation between miR-299-3p expression and human telomerase reverse transcriptase (hTERT) protein in Hep-2 and U2OS cells. (A) hTERT-targeting microRNA computationally predicted using starBase, miRanda and TargetScan software. (B) Western blot analysis of hTERT protein in Hep-2 and U2OS cells. For each lane, 40 μ g protein was added. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. (C) Comparison of miR-299-3p expression in U2OS cells and Hep-2 cells as indicated by reverse transcription-quantitative polymerase chain reaction. (D) Expression of hTERT protein in U2OS cells compared with that in Hep-2 cells. The data shown are the means \pm SEM of three independent experiments. *P<0.05.

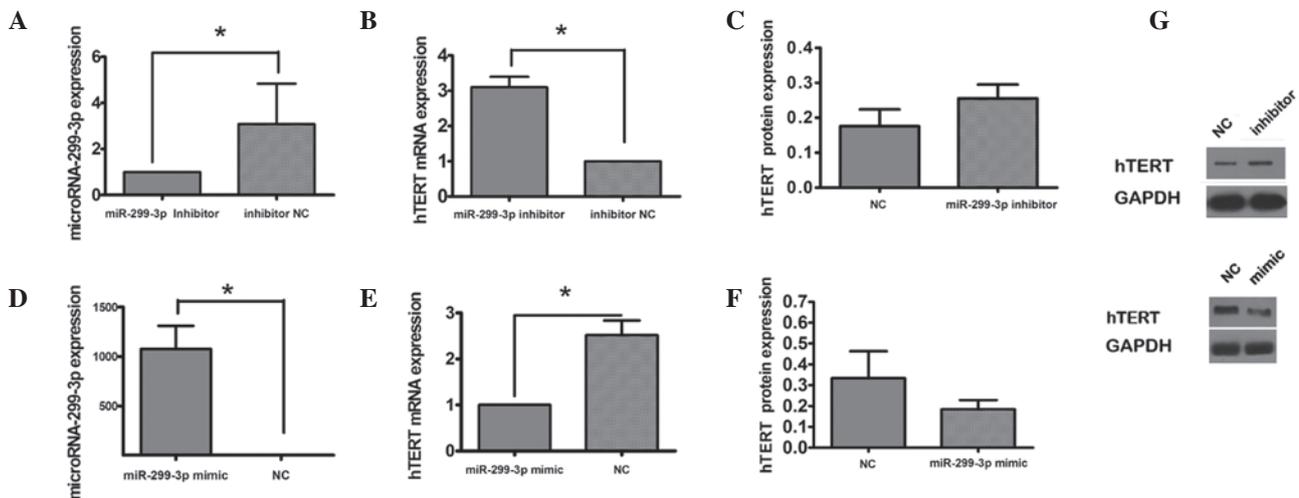


Figure 2. Western blot analysis and reverse transcription-quantitative polymerase chain reaction amplification results. The expression of human telomerase reverse transcriptase (hTERT) protein was increased in the presence of miR-299-3p downregulation, and overexpression of miR-299-3p inhibited the expression of hTERT mRNA and protein. (A) Expression of miR-299-3p in Hep-2 cells following transfection with miR-299-3p inhibitor. (B) Elevated expression of hTERT mRNA was noted following transfection with miR-299-3p inhibitor for 48 h. (C) Expression of hTERT protein was increased following transfection with miR-299-3p inhibitor for 48 h. (D) Expression of miR-299-3p in Hep-2 cells following transfection with miR-299-3p mimic. (E) miR-299-3p contributed to the downregulation of hTERT mRNA in Hep-2 cells. (F) miR-299-3p mimic inhibited the protein expression of hTERT. (G) The protein expression of hTERT was analyzed by western blot analysis 48 h after transfection with miR-299-3p inhibitor, miR-299-3p mimic or miRNA NC, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The data shown are the means \pm SEM from three independent experiments. NC, negative control. *P<0.05.

protein in these two cell lines. These results indicated that the high expression of hTERT in Hep-2 cell lines was inversely correlated with the endogenous expression of miR-299-3p.

miR-299-3p regulates the expression of hTERT in Hep-2 cells. To investigate the association between miR-299-3p and hTERT, the expression of hTERT was measured following knockdown of miR-299-3p *in vitro* (Fig. 2A). RT-qPCR analysis demonstrated that transfection of Hep-2 cells with

miR-299-3p inhibitor contributed to the expression of hTERT mRNA compared with the control group transfected with a scrambled inhibitor negative control (Fig. 2B). In addition, the expression of hTERT protein was increased in the presence of miR-299-3p downregulation (Fig. 2C). The effect of overexpression of miR-299-3p induced by miR-299-3p mimic on the expression of hTERT protein was investigated by RT-qPCR and western blot analysis (Fig. 2D-F). It was observed that upregulation of miR-299-3p reduced the expression of hTERT

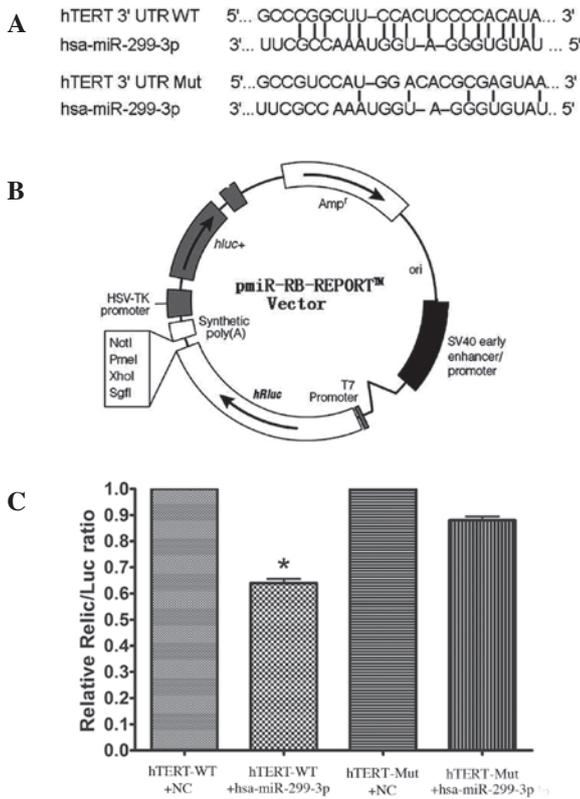


Figure 3. miR-299-3p regulates the expression of endogenous human telomerase reverse transcriptase (hTERT) by directly targeting the 3'-UTR of hTERT mRNA. (A) Sites of miR-299-3p seed matched in the 3'-UTR of hTERT. (B) Schematic diagram of luciferase reporter vector (pmiR-RB-Report). Putative miR-299-3p regulatory elements predicted by bioinformatics were cloned into the 3'-UTR of the luciferase gene at the XhoI/NotI sites. (C) Luciferase reporter constructs were transfected into HeLa cells transfected with miR-299-3p. Luciferase activity was determined 48 h after transfection. The ratio of the normalized sensor to control luciferase activity is shown. Data are expressed as the mean \pm standard deviation of three independent experiments. NC, negative control. *P<0.01, compared with control group.

mRNA and protein. These results indicated that miR-299-3p negatively regulated hTERT expression.

hTERT is a direct target of miR-299-3p. In HeLa cells co-transfected with the reporter plasmids and miR-299-3p mimic or negative control duplex, the activity of the luciferase reporter containing wild-type 3'-UTR was significantly suppressed by miR-299-3p mimic. To be precise, the luciferase activity declined by 36% in comparison with the negative control (P<0.05). However, the luciferase activity of the mutant reporter recovered by 24% compared with the wild type, indicating that miR-299-3p may suppress the expression of hTERT by binding with the 3'-UTR of hTERT mRNA. Together, the results revealed that miR-299-3p regulated the expression of endogenous human hTERT by directly targeting the 3'-UTR of hTERT mRNA (as shown in Fig. 3).

miR-299-3p affects the growth of Hep-2 cancer cells. To investigate the effects of miR-299-3p overexpression on the growth of Hep-2 cancer cells, CCK-8 assay was performed on Hep-2 cells 24, 48 and 72 h after treatment with miR-299-3p mimic, inhibitor and negative control, respectively. As demonstrated

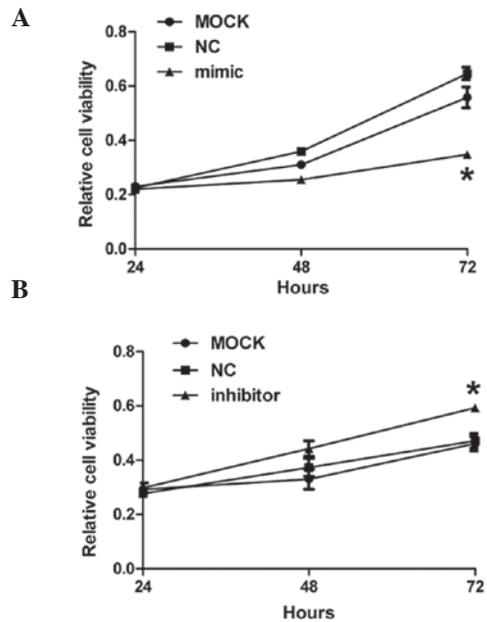


Figure 4. *In vitro* cell growth analysis. Overexpression of miR-299-3p resulted in notable inhibition of cellular proliferation. (A) Hep-2 cells were transfected with Lipofectamine 2000 (MOCK), miRNA negative control (NC) or miR-299-3p mimic. (B) Hep-2 cells were transfected with MOCK, miRNA NC or miR-299-3p inhibitor. Relative cell growth was determined at the indicated time points using CCK-8 assay. The data shown are the means \pm standard deviation of three separate experiments. *P<0.05 compared with MOCK group.

in Fig. 4A, the growth of Hep-2 cancer cells notably decreased 72 h after transfection of miR-299-3p mimic compared with the mock and negative control. However, downregulation of miR-299-3p contributed to a significant increase in cell growth compared with the other two groups 72 h after transfection with miR-299-3p inhibitor. All of these results demonstrated that miR-299-3p affected the growth of Hep-2 cancer cells.

Discussion

Our study firstly demonstrated that miR-299-3p may significantly downregulate the expression of hTERT in human Hep-2 cells. Compared with the hTERT-negative U2OS cells, overexpression of miR-299-3p was noted in hTERT-positive Hep-2 cells. Thus, miR-299-3p may act as a tumor suppressor in Hep-2 cancer cells.

As the core catalytic subunit of the enzyme telomerase, hTERT protein has a significant effect on tumorigenesis. Previous studies have shown that telomerase is activated at various levels, including the transcriptional and post-transcriptional levels of hTERT (8,12). In addition, a positive correlation has been reported between hTERT expression and telomerase activity in a variety of cancer cell lines (13,14). To our knowledge, several protein kinases were involved in the post-transcriptional modification of hTERT protein; for instance, AKT kinase and protein kinase C have been reported to mediate the phosphorylation of hTERT protein, leading to activation of telomerase activity (15,16). In addition, certain accessory proteins (e.g. 14-3-3 protein and nuclear factor- κ B) may modulate telomerase activity by interacting with hTERT protein (17,18). miRNA has always been considered to be

one of the key factors in the post-transcriptional mechanism. However, few studies have noted the suppressive effects of miRNA on hTERT expression (11). To identify a miRNA that can directly target hTERT in human laryngeal cancer, we examined the expression of miR-299-3p and its effects on hTERT in human Hep-2 laryngeal cancer cells.

Computational algorithms reveal that miR-299-3p may directly target the binding site within the 3'-UTR of hTERT through base pairing, as it revealed high complementarity to the 3'-UTR of hTERT mRNA. To confirm that hTERT was a target of miR-299-3p, we cloned the hTERT 3'-UTR containing the target sequence, or a fragment with several mutations in the target site, into a luciferase reporter vector, respectively. Our results demonstrated that miR-299-3p suppressed the luciferase activity of pmiR-hTERT-3'-UTR-wt by 36% compared with the negative control. The suppressive effect was blocked when treating with the vector integrated with mutation of the miR-299-3p binding sites. Moreover, the expression of endogenous hTERT mRNA and protein decreased in miR-299-3p mimic-transfected Hep-2 cells, suggesting that miR-299-3p may regulate hTERT expression by inducing mRNA degradation and translational suppression. Finally, downregulation of miR-299-3p promotes the proliferation of Hep-2 cells. All of these results demonstrate that overexpression of miR-299-3p inhibits Hep-2 cell growth by targeting the 3'-UTR of the hTERT mRNA.

Taken together, our study suggests that miR-299-3p has crucial roles in the progression of Hep-2 cancer cells by interacting with hTERT mRNA. Therefore, miR-299-3p may act as a useful diagnostic tool, and its application in gene therapy in laryngeal cancer is promising.

Acknowledgements

This study was funded by the National Natural Science Foundation of China (nos. 30901662 and 81172569) and the Science and Technology Program of Hubei Province of China (no. 302161991).

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