

MicroRNA-218 inhibits the migration, epithelial-mesenchymal transition and cancer stem cell properties of prostate cancer cells

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Abstract. MicroRNA (miRNA) is a class of non-coding single-stranded RNA, able to regulate tumor-associated genes via binding the 3'-UTR of the target gene mRNA. Previous publications have demonstrated that miRNA-218 (miR-218) acts as a tumor-suppressive miRNA in various types of human cancer, including prostate cancer (PCa). However, the role of miR-218 in regulating PCa cell stemness and epithelial-mesenchymal transition remains unknown and requires further research. In the present study, it is demonstrated that miR-218 was downregulated in 2 PCa cell lines and could suppress cell migration, EMT and the exhibition of cancer stem cell-like properties. The expression of Gli family zinc finger 1 (Gli1) was inhibited by miR-218 overexpression, suggesting miR-218-suppression of Gli1 as a potential mechanism for the tumor-suppressive effect of miR-218. Overall, the results indicate that miR-218 served a critical role in the inhibition of PCa development. This may provide new insight for elucidating the mechanisms of PCa oncogenesis and suggests that miR-218 may be a novel therapeutic target for PCa.

Introduction

Prostate cancer (PCa) is the most common form of cancer among males in developed countries, with an estimated 648,400 new cases and 136,500 mortalities in 2008 (1). Currently, androgen-deprivation therapy is a key treatment for metastatic PCa. However, many patients develop castration-resistant PCa, which is a major cause of male mortality in developed countries (2). Further research is urgently required to develop more effective therapies for this disease.

MicroRNA (miRNA) is a class of non-coding single-stranded RNA that regulates tumor-associated genes via binding the 3'-UTR of target gene mRNA (3-5). Previous research has reported that miRNAs (miRs) serve important roles in human cancer biological processes, including initiation, development, migration and metastasis (6-8). In terms of the development of PCa, miR-34a inhibits prostate cancer stem cell (CSC) features and metastasis by directly repressing CD44 (9). Upregulation of miR-132/212 expression inhibits TGF- β -mediated epithelial-mesenchymal transition (EMT) of PCa cells by targeting SOX4 (10). Tumor-suppressive miR-29 inhibits cancer cell migration and invasion via targeting LAMC1 in PCa (11). Previous studies have also reported that miR-218 serves a tumor-suppressive role in PCa. However, the role of miR-218 in regulating PCa stemness and EMT remains uncharacterized.

In the present study, the expression of miR-218 in PCa cell lines was investigated, and the impact of miR-218 on tumor migration, EMT and CSC properties in PCa was investigated *in vitro*. The results demonstrate that miR-218 was downregulated in PCa cell lines and was able to suppress PCa cell migration, EMT and cancer stem cell properties. The expression of Gli1 was inhibited by miR-218 overexpression, suggesting a role for this protein in the mechanism of tumor-suppression of miR-218 in PCa. Altogether, the present study indicates that miR-218 served a critical role in inhibiting PCa development, providing new insights into clarifying the potential mechanisms of PCa oncogenesis and revealing that miR-218 may be a novel therapeutic target for PCa.

Materials and methods

Cell lines and cell culture. PCa cell lines LNCaP and C4-2 were obtained from the American Type Culture Collection (Manassas, VA, USA). BPH-1 cells were provided by Dr Jer-Tsong Hsieh (University of Texas Southwestern Medical Center, Dallas, TX, USA). These three cell lines were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) in a humidified chamber at 37°C in 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from harvested

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cells using TRIzol (Thermo Fisher Scientific, Inc.) and reverse transcribed to cDNA using the miScript II RT kit (Qiagen GmbH, Hilden, Germany), according to the manufacturers' protocols. The CFX96 PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with SYBR-Green PCR Master Mix (Takara Bio, Inc., Otsu, Japan) was used to detect the transcriptional expression of miR-218. The thermocycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, and then 60°C for 30 sec. U6 was used as an internal control, and relative gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method (12). The primer sequences used were as follows: miR-218 forward, 5'-CGAGTGCATTTG TGCTTGATCTA-3' and reverse, 5'-TAATGGTCGAACGCC TAACGTC-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3'.

Lentivirus transfection. LNCaP and C4-2 cells were seeded and cultured for 24 h and to 40-50% confluence. The lentiviral vector 3 (LV3)-miR-218, constructed by GenePharma Co., Ltd. (Shanghai, China), was used to transfect cells in an overnight incubation. LV3 scrambled lentiviral vector (LV3-NC; GenePharma Co., Ltd.) was used as a negative control. At 48 h after infection, the stable clones were maintained by puromycin (2-3 μ g/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany)-resistant culturing.

Transwell migration assay. Complete growth medium (RPMI-1640 with 10% FBS, 1 ml) was added to each lower chamber as a chemoattractant. Transwell inserts with a pore diameter of 8- μ m were used (Millipore; Merck KGaA). Cells, suspended in serum-free medium at 5-8x10⁴ cells/ml, were seeded into the upper chamber at 400 μ l/well. After an incubation of 20 h, the upper surface of the insert was wiped and cells that had migrated to the lower surface were fixed using 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 20 min at room temperature. Cell number was counted in 6 random fields per well (magnification, x200).

Western blot assay. Cells were washed 3 times in PBS before the protein was extracted using radioimmunoprecipitation assay buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40 and 0.5% sodium deoxycholate] with protease inhibitors. The concentration of protein was detected by Bradford assay protein quantitation kit (Abcam, Cambridge, UK). Proteins (30 μ g) were separated by 12% SDS-PAGE and transferred into nitrocellulose membranes. Following blocking in 5% skim milk at room temperature for 1 h, the membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies used were as follows: GAPDH (1:10,000; cat. no. KC-5G4; Kangchen Bio-tech Co., Ltd., Shanghai, China); E-cadherin (1:1,000; cat. no. sc-8426; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); Vimentin (1:200; cat. no. sc-6260; Santa Cruz Biotechnology, Inc.); CD44 (1:800; cat. no. 3570; Cell Signaling Technology, Inc., Danvers, MA, USA); Oct4 (1:500; cat. no. ab18976; Abcam); Nanog (1:200; cat. no. ab21624; Abcam); and Gli1 (1:1,000; cat. no. 2643; Cell Signaling Technology, Inc.). The membranes were then washed in Tris-buffered saline with 0.1% Tween and incubated with horseradish peroxidase-conjugated secondary antibodies

[goat anti-rabbit IgG (1:2,000; cat. no. ZB-2301); and goat anti-mouse IgG (1:2,000; cat. no. ZB-2305) (all from OriGene Technologies, Inc., Beijing, China) for 1 h at room temperature. Protein bands were visualized using a Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories).

Colony-forming and tumor sphere formation assays. For the colony-forming assay, 2x10³ cells were seeded into each well of a 6-well plate and incubated for 10-14 days. Following 3 washes in PBS, cells were fixed using 4% paraformaldehyde for 30 min at room temperature and stained with 0.1% crystal violet for 20 min at room temperature. The tumor sphere formation assay was performed by seeding 1x10⁴ cells to each well of low-adhesion 6-well plate in serum-free Dulbecco's modified Eagle/F12 medium supplemented with 20 ng/ml epidermal growth factor, 10 ng/ml basic fibroblast growth factor and 2% B27 (all Invitrogen; Thermo Fisher Scientific, Inc.). After 2 weeks, plates were analyzed for tumor sphere formation using an inverted microscope (magnification, x200).

Statistical analysis. All statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences between 2 groups were compared using the Student's t-test. For comparisons of ≥ 3 groups, one-way analysis of variance followed by Tukey's post hoc test was used. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-218 expression is downregulated in PCa cells. RT-qPCR was performed to compare the expression of miR-218 in PCa cells with that in healthy prostate epithelial cells. This revealed that the expression of miR-218 was notably downregulated in PCa cell lines, LNCaP and C4-2, compared with the normal prostate epithelial cells (BPH-1; Fig. 1A).

Construction of miR-218-overexpressing PCa cells. In order to reveal the effects of miR-218 on PCa migration, EMT and CSC properties, miR-218-overexpressing LNCaP/C4-2 cells were constructed by transfecting the cells with LV3-miR-218 lentiviral vectors. Subsequently, cells were analyzed by RT-qPCR to confirm miR-218-overexpression (Fig. 1B).

Overexpression of miR-218 inhibits PCa cell migration and EMT. The Transwell migration assays demonstrated that the migration of LNCaP/C4-2 cells was suppressed by miR-218 overexpression (Fig. 2). Western blotting of EMT markers demonstrated that miR-218 overexpression caused a slight increase in the expression of E-cadherin and a significant decrease in the expression of vimentin (Fig. 3A). These results suggest that overexpression of miR-218 inhibits PCa cell migration and EMT.

Overexpression of miR-218 diminishes PCa cell stemness properties. Protein expression profiling of cancer stemness markers was performed by western blotting. The results indicate that overexpression of miR-218 downregulated the expression of CD44, Oct4 and Nanog (Fig. 3B). Colony forming assays were performed to assess the self-renewal capacity of PCa

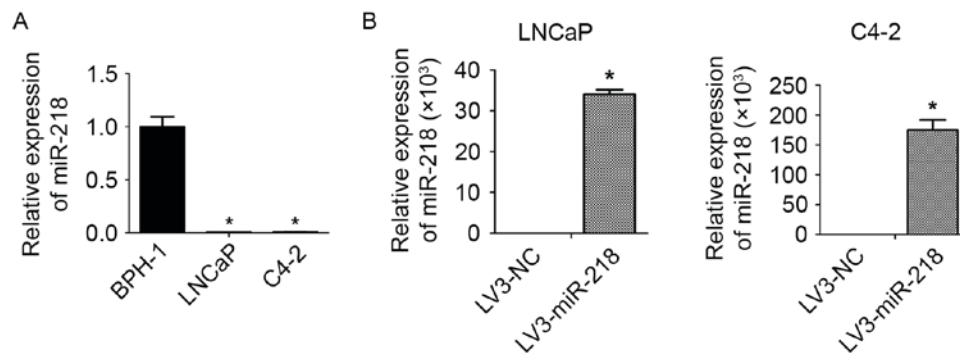


Figure 1. The expression of miR-218 in prostate cancer cells and construction of miR-218 overexpressing prostate cancer cells. (A) reverse transcription-quantitative polymerase chain reaction analysis (normalized using U6) demonstrates the significant downregulation of miR-218 expression in prostate cancer cells compared with the 'normal' prostate epithelial cells. * $P < 0.05$ vs. BPH-1. (B) Validation of the miR-218-overexpressing prostate cancer cell lines. These data are representative of 3 independent experiments. * $P < 0.05$ vs. control. miR-218, microRNA-218.

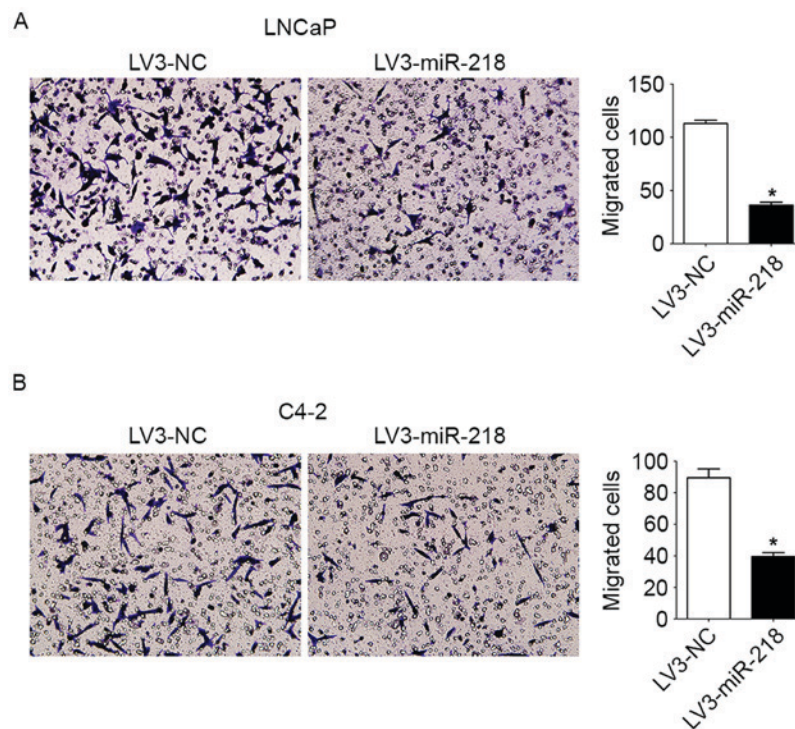


Figure 2. The effects of miR-218 on the migratory capability of prostate cancer cells. (A) The migration of LNCaP cells was suppressed by miR-218 overexpression. (B) A similar result was observed of C4-2 cells. These data are representative of 3 independent experiments (magnification, x200). * $P < 0.05$ vs. LV3-NC. miR-218, microRNA-218; LV3, lentivirus vector 3; NC, negative control.

cells. It was demonstrated that the miR-218-overexpressing LNCaP cells formed fewer and smaller colonies than the control cells (Fig. 4A). Similar results were obtained from the clonogenic assay performed in C4-2 cells (Fig. 4B), indicating that miR-218 may serve a critical role in tumor growth inhibition. The tumor sphere formation assay is well established for measuring the self-renewing capability of stem cells. In these experiments, the control cells generated more tumor-spheres than the miR-218-overexpressing cells in both cell lines (Fig. 4C and D). Therefore, these results suggest that overexpression of miR-218 diminished PCa cell stemness properties.

Gli1 expression is downregulated by miR-218 overexpression. Numerous studies have indicated that the Hedgehog-Gli

signaling pathway serves a critical role in cancer cell EMT occurrence and CSC generation (13-15). In the preset study, western blot analysis indicated that the expression of Gli1 was inhibited by miR-218 overexpression (Fig. 5), indicating that miR-218 suppression of Gli1 may be a mechanism for the anticancer effect of miR-218 in PCa.

Discussion

Advances have been made in PCa diagnosis and treatment in recent years (16). However, management of PCa remains a challenge. It has been widely reported that EMT and CSCs are critical for cancer initiation and development (17-21). However, the molecular mechanisms by which EMT and CSCs execute their effects require further investigation.

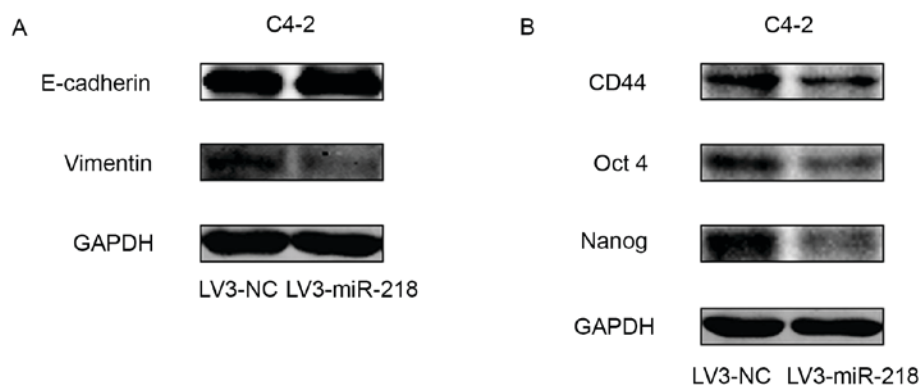


Figure 3. Effects of miR-218 on epithelial-mesenchymal transition and cancer stem cell biomarker expression in prostate cancer cells. (A) Western blot analysis revealed that the overexpression of miR-218 increased the expression of E-cadherin and decreased the expression of Vimentin (EMT biomarkers markers). (B) Western blot analysis also revealed that the overexpression of miR-218 downregulated the expression of cluster of differentiation 44, octamer-binding protein 4 and Nanog (stemness biomarkers). These data are representative of 3 independent experiments and GAPDH was used as a loading control. miR-218, microRNA-218; LV3, lentivirus vector 3; NC, negative control; CD44, cluster of differentiation 44; Oct 4, octamer-binding protein 4.

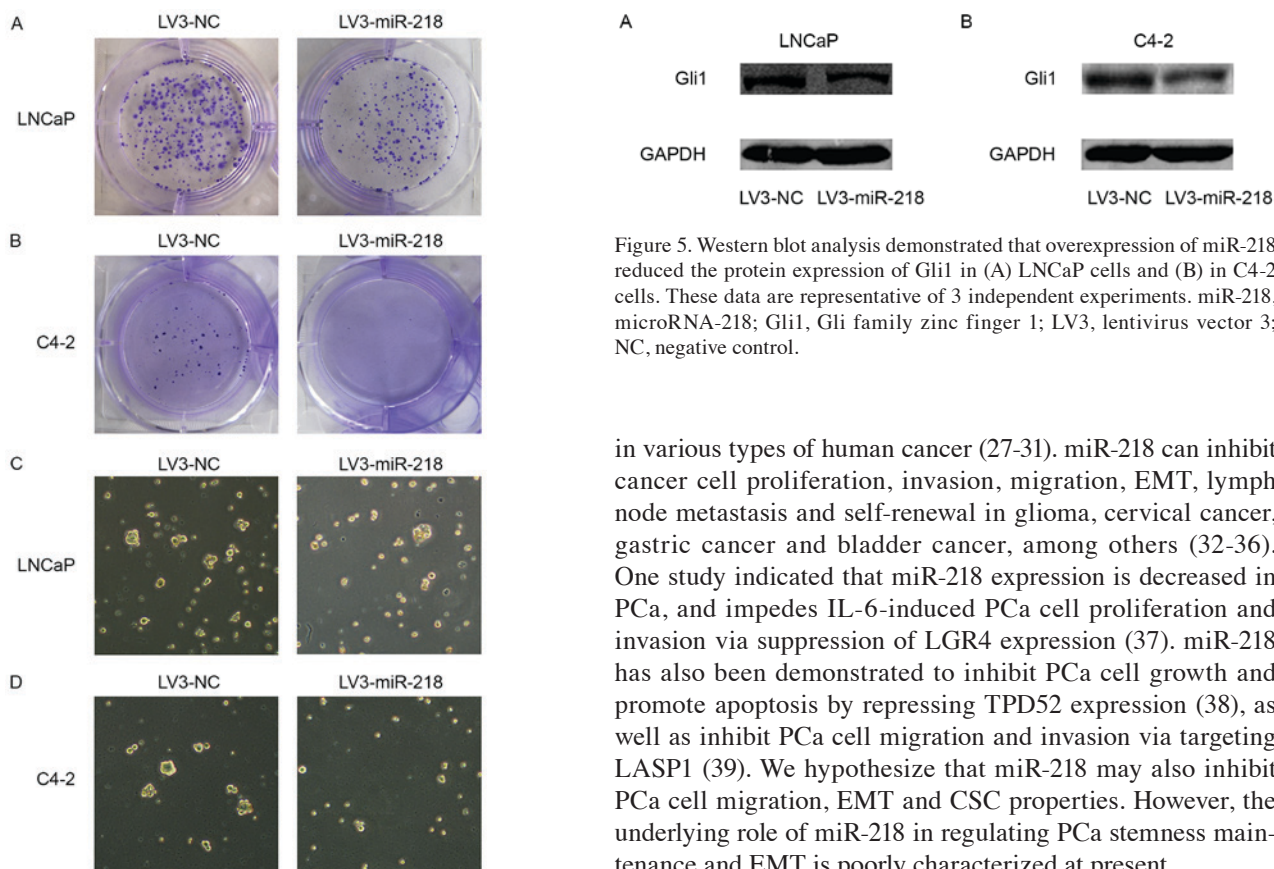


Figure 4. Overexpression of miR-218 suppressed the growth and self-renewal capacities of prostate cancer cells. (A) In colony-forming assays, LNCaP-LV3-miR-218 cells formed fewer and smaller colonies compared with LV3-NC control cells. (B) A similar observation was made of C4-2 cells. (C) Tumor sphere formation assay was used to measure the self-renewal ability of cells. The LV3-NC control cells generated more tumor-spheres than LV3-miR-218 cells in LNCaP cells and (D) C4-2 cells (magnification, x200). These data are representative of 3 independent experiments. miR-218, microRNA-218; LV3, lentivirus vector 3; NC, negative control.

Previous studies have demonstrated that miRNAs serve important roles in human cancer biological processes, including initiation, development, migration and metastasis (22-26). miR-218 can act as a tumor suppressor and is downregulated

Figure 5. Western blot analysis demonstrated that overexpression of miR-218 reduced the protein expression of Gli1 in (A) LNCaP cells and (B) in C4-2 cells. These data are representative of 3 independent experiments. miR-218, microRNA-218; Gli1, Gli family zinc finger 1; LV3, lentivirus vector 3; NC, negative control.

in various types of human cancer (27-31). miR-218 can inhibit cancer cell proliferation, invasion, migration, EMT, lymph node metastasis and self-renewal in glioma, cervical cancer, gastric cancer and bladder cancer, among others (32-36). One study indicated that miR-218 expression is decreased in PCa, and impedes IL-6-induced PCa cell proliferation and invasion via suppression of LGR4 expression (37). miR-218 has also been demonstrated to inhibit PCa cell growth and promote apoptosis by repressing TPD52 expression (38), as well as inhibit PCa cell migration and invasion via targeting LASP1 (39). We hypothesize that miR-218 may also inhibit PCa cell migration, EMT and CSC properties. However, the underlying role of miR-218 in regulating PCa stemness maintenance and EMT is poorly characterized at present.

In the present study, the expression of miR-218 was notably downregulated in PCa cells and the LNCaP/C4-2 cell constructs overexpressing miR-218 indicated that miR-218 inhibited PCa cell migration. Results of western blotting revealed that the overexpression of miR-218 downregulated the expression of vimentin, CD44, Oct4 and Nanog. In colony-forming assays, miR-218-overexpressing cells formed fewer and smaller colonies than the control cells. Consistently, the control cells generated more tumor-spheres than miR-218-overexpressing cells, suggesting that overexpression of miR-218 inhibited PCa stemness properties. Copious evidence has indicated that the Hedgehog-Gli signaling pathway serves a critical role in cancer cell EMT and CSC generation (13-15,40). In the present study, the expression of Gli1 was inhibited by miR-218

overexpression. This indicates that miR-218 suppression of Gli1 may serve in the mechanism by which miR-218 inhibits EMT and stemness maintenance in PCa.

The regulatory mechanisms of human CSC maintenance and EMT are very complex. If miR-218 targets Gli1 by binding its 3'-UTR, or other molecular mediators between miR-218 and Gli1, the mechanism requires further investigation. In conclusion, the present study indicates that miR-218 served a critical role in inhibiting the migration, EMT and CSC properties of PCa cells. This provides a new insight for clarifying the potential mechanisms of PCa oncogenesis, and indicates that miR-218 may be a potential therapeutic target for PCa.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YD and DH designed and supervised all experiments and contributed to the manuscript preparation. BG performed the experiments, analyzed the data and contributed to the manuscript preparation. LM and JT contributed to the cell culture and lentivirus transfection. LZ and KW contributed to the data analysis. SX and XW contributed to western blot assay and manuscript preparation.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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