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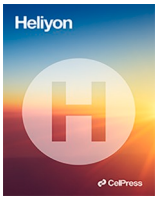

Research article

Dihydroartemisinin inhibits HNSCC invasion and migration by controlling miR-195-5p expression

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ABSTRACT

Objectives: Dihydroartemisinin (DHA), an artemisinin derivative extracted from the traditional Chinese medicinal herb *Artemisia annua*, has the potential to suppress head and neck squamous cell carcinoma (HNSCC) progression. However, the mechanisms underlying these effects remain unclear. Therefore, we aimed to examine the mechanisms underlying the effects of DHA on tumor invasion and migration.

Methods: Human HNSCC cell lines CAL-27 and FaDu were exposed to varying DHA concentrations (0, 5, 20, and 80 μM) for 24 h. Cell proliferation, invasion, and migration were assessed using CCK8, transwell, and wound-healing assays, respectively. Quantitative real-time PCR, western blotting, and immunofluorescence were used to assess the expression levels of the target genes and proteins.

Results: DHA suppressed the invasion and migration of CAL-27 and FaDu cells. Additionally, miR-195-5p suppressed the invasion and migration of HNSCC cells. This study revealed significant differences in the expression of miR-195-5p and TENM2 between clinical samples and multiple public databases. DHA treatment and miR-195-5p overexpression significantly reduced TENM2 expression in HNSCC cells, which suggested that miR-195-5p overexpression enhanced the inhibitory effect of DHA on TENM2.

Conclusions: This study provides the first evidence that DHA inhibits cell invasion and migration by regulating the miR-195-5p/TENM2 axis in HNSCC cells, suggesting it as a potentially effective treatment strategy for HNSCC.

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) stands as a primary form of head and neck cancer, ranking seventh in global cancer incidence [1,2]. Despite recent advancements in treatments such as surgery, radiochemotherapy, and immunotherapy, the five-year survival rate for patients with advanced HNSCC remains relatively low [3,4]. The advancement of HNSCC is driven mainly by invasion and migration. Therefore, delving into the molecular mechanisms driving its invasion and metastasis is essential to develop improved treatment modalities.

Dihydroartemisinin (DHA), derived from artemisinin, is extracted from the traditional Chinese medicinal plant Artemisia annua. WHO suggests it as a substitute for antimalarial medications [5,6]. Multiple studies have reported the anticancer effects of DHA on different tumor types. For instance, DHA inhibits cell migration and invasion in gallbladder cancer by suppressing TCTP and reducing the activation of cell division control protein 42 homolog (Cdc42) [7]. Additionally, it suppresses the metastasis of non-small-cell lung cancer by inhibiting the NF-κB signaling pathway [8] and inhibits the proliferation and metastasis of breast cancer cells by suppressing the TGF-β1/SMAD signaling pathway [9]. In a previous study, we discovered that DHA plays a negative role in HNSCC by selectively blocking the JAK2/STAT3 signaling pathway, suggesting its association with the migration and invasion of HNSCC cells [10]. However, the pathways by which DHA hinders the migration and invasion in HNSCC cells remain elusive and warrant further exploration.

MiRNAs, crucial regulators of human developmental processes, are composed of approximately 22-nucleotide sequence and can bind to the target messenger RNA. This interaction can lead to RNA cleavage or translational inhibition. Downregulated or absent miRNAs often act as tumor suppressors, repressing oncogenes and proliferation-related genes. For instance, microRNA-195-5p is associated with various cancers, including glioblastoma [11], osteosarcoma [12], hepatocellular carcinoma [13], colon cancer [14], esophageal cancer [15], prostate cancer [16], and thyroid cancer [17]. Decreased expression of miR-195-5p is associated with poor prognosis in HNSCC [18] and occurrence of tongue carcinomas and is a promising candidate for prognostic and therapeutic monitoring in plasma samples from patients with HNSCC [19]. Additionally, reduced miR-195 expression causes poor overall survival in HNSCC patients [20]. MiR-195 inhibits cell invasion and migration in other cancer types [21–23]. However, the precise molecular mechanisms underlying the inhibition of invasion and migration of HNSCC by miR-195-5p remain unclear.

In this study, we predicted that TENM2 is one of the miR-195-5p target. TENM2 (Teneurin transmembrane protein 2) is coding for membrane protein that mainly involved in embryonic, neuronal development and cancer progression [24]. We found the over-expression of TENM2 in human HNSCC tissues was positively correlated with clinicopathological parameters. DHA effectively hindered the invasion and migration of HNSCC cells by modulating the miR-195-5p/TENM2 axis. These findings provide experimental evidence for the potential use of DHA in HNSCC treatment and offer insights into improving the clinical outcomes among patients with HNSCC.

2. Materials and methods

2.1. Cell culture

Human HNSCC CAL27 (CL-0265) and FaDu cells (CL-0083) were purchased by Procell Life Science & Technology Co., Ltd. (Wuhan, China). HNSCC cells were cultured using DMEM (HyClone, USA) with 1 % penicillin/streptomycin (Beyotime, China) and 10 % fetal bovine serum (Lonsera, Uruguay) in 5 % CO_2 at 37 °C.

2.2. Patients and tissue samples

Tissue samples were collected from four patients who underwent surgery at Chongqing General Hospital from July 2021 to January 2022. The research protocol received approval from the Ethics Committee of Chongqing General Hospital (approval number: S2020-110-01) and was conducted in compliance with the Helsinki Declaration of 1975. All participants provided written informed consent and the collection of clinical information was approved by the Institutional Review Board. Three adjacent tissue specimens were obtained from the patients to serve as paired controls.

2.3. Cell transfection

To investigate the effects of miR-195-5p mimic, miR-195-5p inhibitor, and their respective negative controls on CAL-27 and FaDu cells, we transfected 10 μM of each molecule, along with their respective negative controls, into 2×10^5 cells/well in 6-well microplates. The transfection was carried out using 9 μL of Lipofectamine2000 (Thermo Fisher Scientific, USA), with an siRNA to Lipofectamine2000 ratio of 5:6. After transfection, cells were cultured under standard conditions at 37 °C for 6 h. Transfection efficiency was confirmed through qRT-PCR analysis, and further experiments were conducted following the 6-h transfection period.

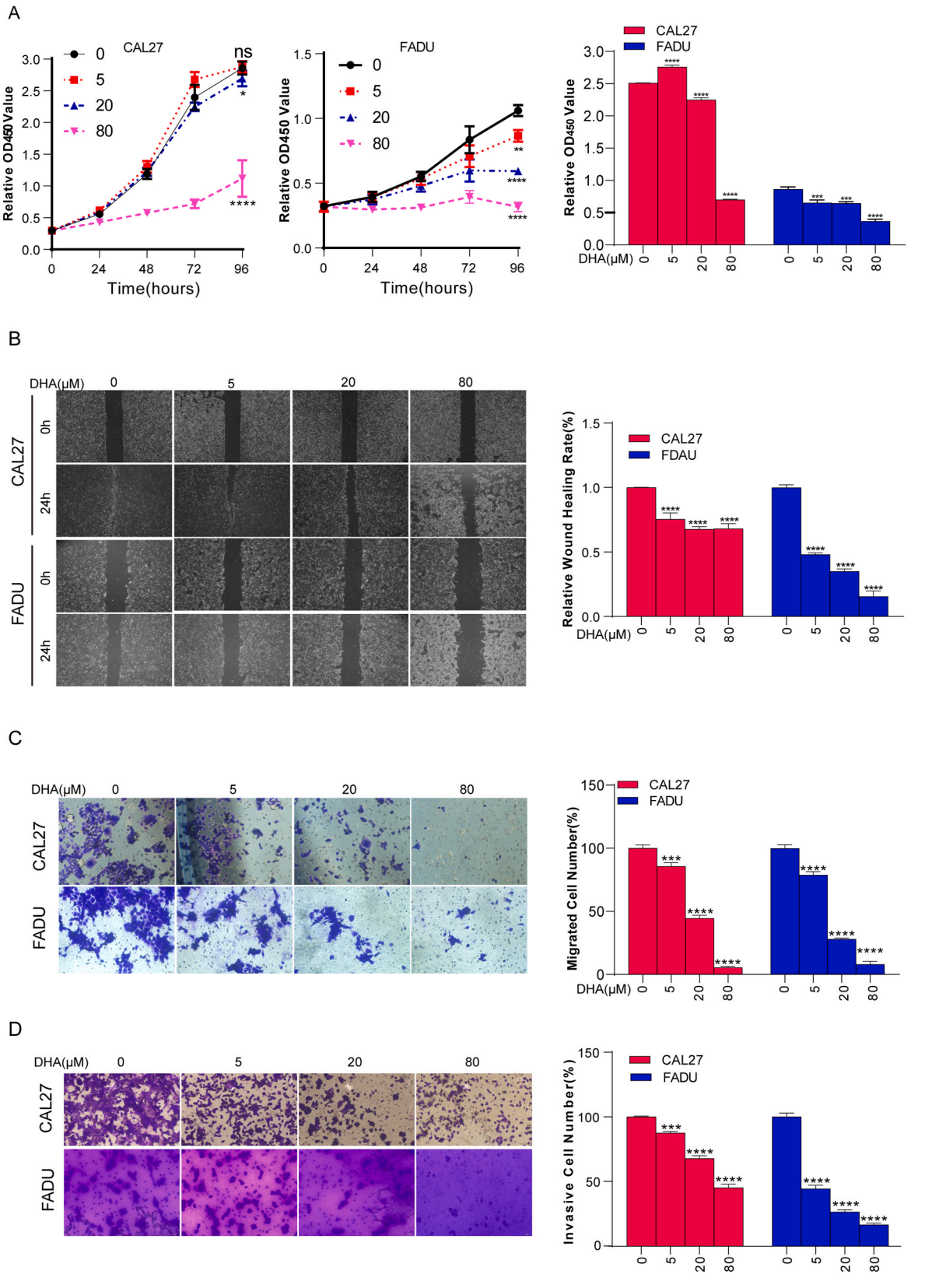

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Fig. 1. Effects of DHA on the proliferation, migration, and invasion of CAL-27 and FaDu cells. A, Proliferation of CAL-27 and FaDu cells after DHA treatment assessed by CCK8 assay. B, Reduced wound-healing rates in CAL-27 and FaDu cells after 0 and 24 h of DHA treatment. C, Transwell assay performed in DHA-treated CAL-27 and FaDu cells. *P < 0.05, **P < 0.01, ***P < 0.001.

2.4. Cell viability assays

The CAL-27 and FaDu cells were planted in 96-well plates, 24 h before incubation, at a density of 2×10^3 cells/well, followed by DHA treatment at various concentrations (0, 5, 20, and 80 μM) for varying periods (0, 24, 48, 72, and 96 h). Cell viability was detected using CCK8 (Bioground, China) and optical density was measured at 450 nm using Synergy H1 Hybrid Multi-Mode Reader (BioTek, USA).

2.5. Transwell assay

A 24-well chamber was used for the transwell experiment. Cells (2×10^5 cells/well) suspended in 500 μL DMEM (no FBS) were added in the apical chamber, while the basolateral chamber containing 200 μL complete DMEM (10 % FBS) served as the chemoattractant. Different concentrations of DHA (0, 5, 20, and 80 μM) were administrated in each well for 24 h at 37 °C; thereafter, the cells in the apical chamber were removed. In the lower chamber cavity, migrated cells were fixed in 4 % paraformaldehyde for 30 min, followed by immersion in crystal violet solution for 10 min. The cells were visualized using a microscope (Olympus, Tokyo, Japan). Matrigel (Corning Life Sciences, USA) was used for the transwell invasion assay to precoat the apical chamber. Cell suspensions containing 2×10^5 cells were added to the apical chamber and the following assay was conducted as described above.

2.6. Wound-healing assay

The exponentially growing CAL-27 and FaDu cells were seeded into 6-well plates at a density of 3×10^5/well and cultured until 90 % confluence. A perpendicular scratch was made on the surface of plate using a 200 μL pipette tip. Cells were then incubated in 10 % FBS-DMEM and treated with varying DHA concentrations (0, 5, 20, and 80 μM) for 24 h. At 0, 12, and 24 h after the scratch, photographic images were acquired under a microscope (Olympus, Tokyo, Japan) and analyzed using the ImageJ software to calculate the healing percentage.

2.7. qRT-PCR

After 24 h of transfection, we extracted total RNAs from the cells and tissues using TRIzol reagent (Invitrogen). RNA concentration and purity were evaluated using a NanoDrop Spectrophotometer (Thermo Fisher Scientific), and samples with A260/A280 > 1.80 were prepared for subsequent cellular experiments. First, the total RNAs were subjected to reverse transcription following the instructions in the SYBR® PrimeScript™ RT-PCR Kit (Accurate Biology, China). The qRT-PCR experiments were conducted following the instructions of the SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology). U6 and GAPDH were used as housekeeping genes for standardization. The data are presented as the fold change of downregulation or upregulation [fold value = $2^{\Delta\Delta Ct}$, where ΔΔCt = (Ct of the gene of interest, treated - Ct of the housekeeping gene, treated) - (Ct of the gene of interest, control - Ct of the housekeeping gene, control); Ct = the number of threshold cycles]. The primer sequences are listed in Supplementary Table 1.

2.8. Western blot assay

After discarding the culture medium, the cells were washed with pre-chilled PBS. Lysate containing 10 % phenylmethylsulfonyl fluoride was added and the cells were lysed on ice for 30 min followed by manual scraping from the culture plates into 1.5 mL Eppendorf (EP) tubes. After centrifugation at 4 °C and 12,000 rpm for 15 min, the supernatant was transferred to a new EP tube. The cells were divided into two parts: one was frozen at −20 °C, and the other was used to determine the protein concentration, which was measured using a BCA protein concentration measurement kit (Bioground, China). To the other part, 5 × protein electrophoresis loading buffer mix was added at a 1:4 ratio and boiled in a water bath (5–10 min). Ten micrograms of samples per lane were loaded onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel for protein separation. Equal aliquots of total protein were separated by SDS-PAGE on an 8 % gel at a voltage of 70 V for 3 h, followed by transfer to a PVDF membrane at a current of 90 mA overnight at 4 °C, and blocked with TBST buffer containing 5 % fat-free milk for 2 h. The membranes were then incubated with primary antibodies at the recommended dilution overnight at 4 °C. Subsequently, the membranes were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) for 2 h. An anti-rabbit HRP-conjugated secondary antibody (Zen Bioscience, China) was used. Antigen–antibody complexes were detected using the ECL reagent (Millipore Corporation, USA). The antibodies used in this study are listed in Supplementary Table 2.

2.9. Bioinformatics analysis

The potential targets of miR-195-5p were predicted using TargetScanHuman (https://www.targetscan.org/vert_80/), ENCORI (https://rnasysu.com/ENCORI/), and ONCOMIR databases (https://oncomir.org). We used the intersection of genes targeted by miR-

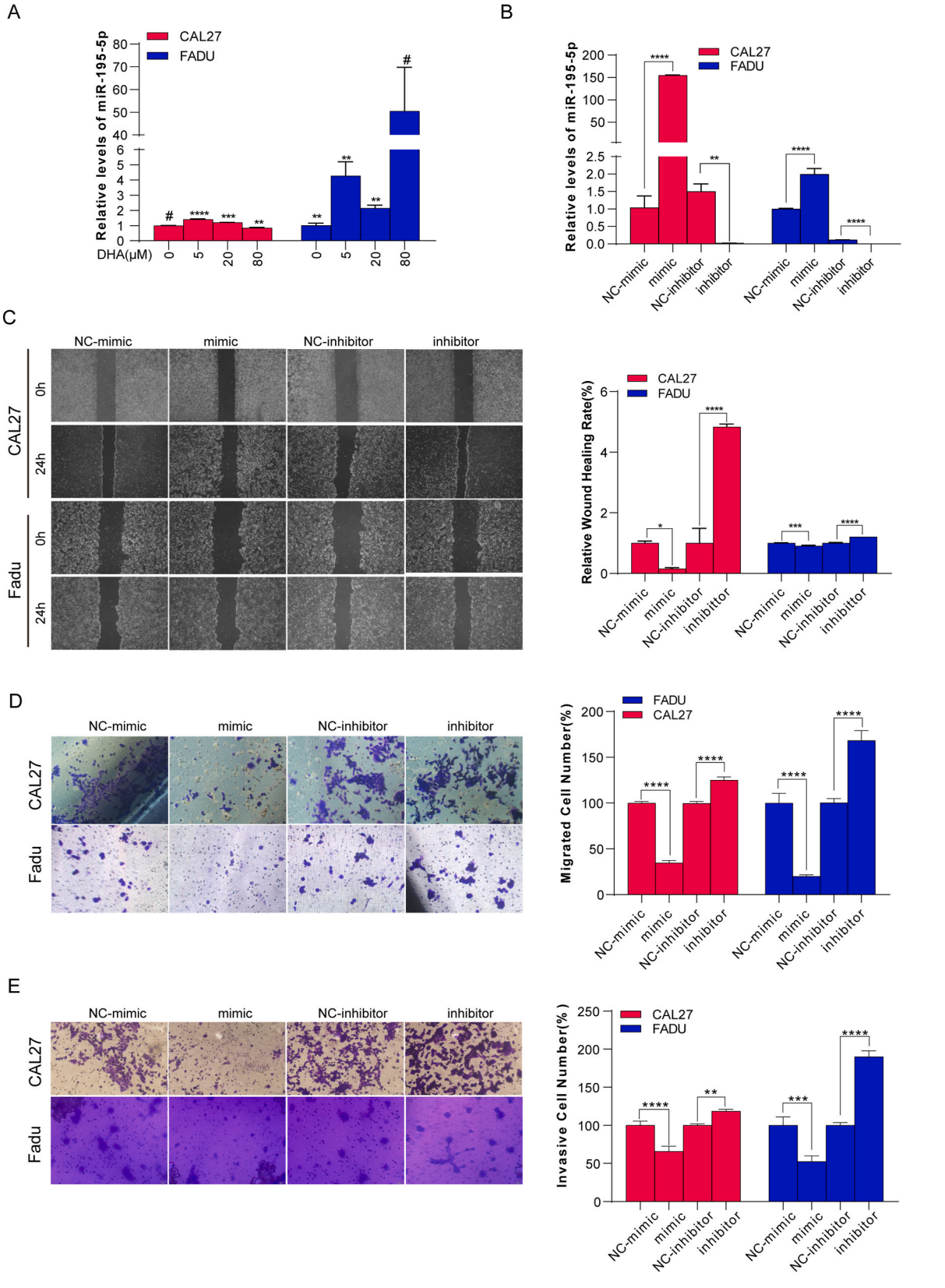

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Fig. 2. DHA-mediated inhibition of HNSCC cell invasion and migration via miR-195-5p regulation. A, Wound-healing rates in CAL-27 and FaDu cells after 0 and 24 h of transfection with miR-195-5p mimic or inhibitor. B–C, Transwell assay performed in CAL-27 and FaDu cells transfected with miR-195-5p mimic or inhibitor, and the analysis of tendencies of invasive cells. D, qRT-PCR analyzed miR-195-5p levels in DHA-treated CAL-27 and FaDu cells. E, qRT-PCR analyzed miR-195-5p expression in CAL-27 and FaDu cells transfected with miR-195-5p mimic or inhibitor. *P < 0.05, **P < 0.01, ***P < 0.001. miR/miRNA, microRNA; NC, negative control.

Table 1
Putative target gene of miR-195-5p.

Target gene	Representative transcript	Gene name	TargetScan	ENCORI	ONCOMIR
TENM2	ENST00000519204.1	teneurin transmembrane protein 2	✓	✓	✓
HPSE2	ENST00000370546.1	heparanase 2	✓	×	×
EIF2B5	ENST00000273783.3	eukaryotic translation initiation factor 2B, subunit 5 epsilon, 82 kDa	✓	×	×
PAX7	ENST00000420770.2	paired box 7	✓	×	×
DCAF5	ENST00000341516.5	DDB1 and CUL4 associated factor 5	✓	×	×
HCN1	ENST00000303230.4	hyperpolarization activated cyclic nucleotide-gated potassium channel 1	✓	×	×
SLC4A8	ENST00000453097.2	solute carrier family 4, sodium bicarbonate cotransporter, member 8	✓	×	×
EPT1	ENST00000260585.7	ethanolaminephosphotransferase 1 (CDP-ethanolamine-specific)	✓	×	×
ATP1B4	ENST00000218008.3	ATPase, Na+/K+ transporting, beta 4 polypeptide	✓	×	×
CACNB1	ENST00000394303.3	calcium channel, voltage-dependent, beta 1 subunit	✓	×	×
FSTL1	ENST00000295633.8	Follistatin Like 1	×	✓	×
ELMO2	ENST00000290246.11	engulfment and cell motility 2	×	✓	×
CREB3L1	ENST00000621158.5	cAMP responsive element binding protein 3-like 1	×	✓	×
CD44	ENST00000428726.8	CD44 molecule	×	✓	×
FAM219A	ENST00000651358.1	Family With Sequence Similarity 219 Member A	×	✓	×
SAMD4A	ENST00000554335.6	Sterile Alpha Motif Domain Containing 4A	×	✓	×
CIPC	ENST00000361786.7	CLOCK Interacting Pacemaker	×	✓	×
DLG1	ENST00000667157.1	Discs Large MAGUK Scaffold Protein 1	×	✓	×
BTG2	ENST00000290551.5	BTG Anti-Proliferation Factor 2	×	✓	×
CNTNAP1	ENST00000264638.9	Contactin Associated Protein 1	×	✓	×

195-5p in three public databases and represented them using a Venn diagram.

2.10. Dual luciferase reporter gene assay

Vectors pTENM2 UTR (1 μg/mL) and pTENM2 UTR MUT (1 μg/mL) were transfected into HNSCC cells by Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific). The effect of transfection was assessed using the Dual Luciferase Reporter Assay System (Promega, USA) in a Glo Max® -Multi Detection System Photometer (Promega). The miR-195-5p mimic, miR-195-5p inhibitor, mimic control, and inhibitor control were obtained from Gene RIB Bio (Guangzhou, China). Cells were seeded in a 24-well plate at a density of 1×10^5 cells/well, 24 h before transfection.

MiR-195-5p mimic (10 μM) or miR-195-5p inhibitor (10 μM) was transfected into the cells at 500 ng, together with 50 ng/well of pRL-TK (Promega). After 24 h of transfection, luciferase activity was measured using Varioskan Flash (Thermo Scientific). The experiment was performed thrice independently and the enzyme activity levels were presented as mean ± standard error.

2.11. Immunofluorescence

The tissue sections were deparaffinized in xylene and rehydrated using a series of ethanol solutions of increasing concentrations. The expression levels of TENM2 and tubulin were probed with primary antibodies, following the manufacturer's instructions. The antibodies used in this study are listed in Supplementary Table 2. Secondary antibodies Alexa Fluor 594 anti-sheep and Alexa Fluor 488 anti-mouse were used to label TENM2 and tubulin, respectively. Protein expression was quantified based on the staining intensity.

2.12. Statistical analysis

All experiments in vitro were done independently with at least three biological replicates. GraphPad Prism software version 9 was used to conduct statistical analysis. The student's t-test or one-way analysis of variance (ANOVA) was conducted to assess the comparative statistical significance among two or three groups. Values with $P < 0.05$ were considered as statistically significant. The results are expressed as mean ± standard deviation (SD).

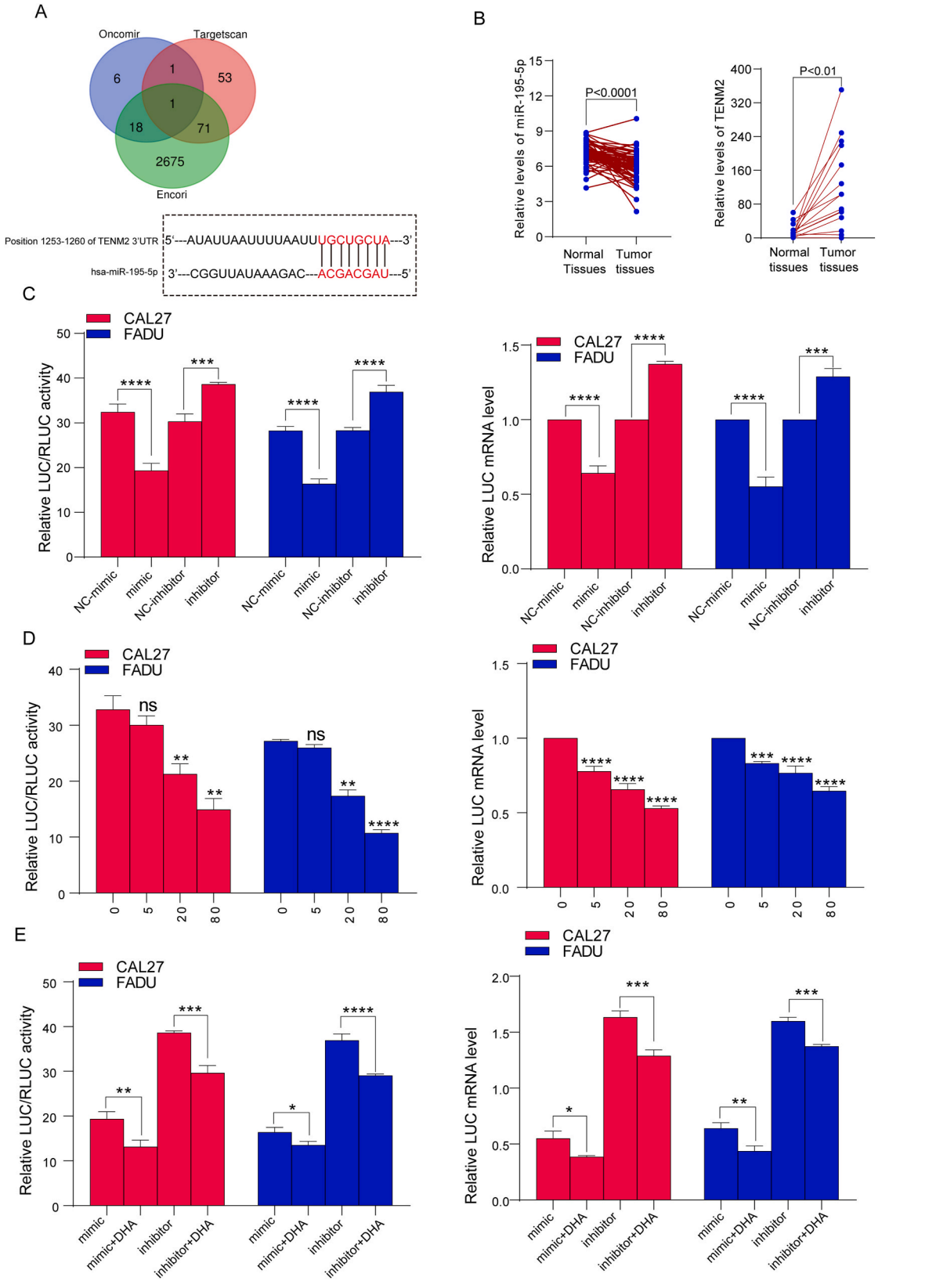

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Fig. 3. TENM2 is the direct target of miR-195-5p. A, Screening of differentially expressed miRNAs in HNSCC cells using the Gene Expression Omnibus and public miRNA databases. B, Expression of miR-195-5p and TENM2 in normal and HNSCC tissue samples in two GEO datasets. C, Luc/RLuc activity and Luc mRNA in CAL-27 and FaDu cells after 0 and 24 h of transfection with miR-195-5p mimic or inhibitor. D, Luc/RLuc activity and Luc mRNA in DHA-treated CAL-27 and FaDu cells. E, Luc/RLuc activity and Luc mRNA in CAL-27 and FaDu cells after stimulation with miR-195-5p mimic or DHA and miR-195-5p mimic, miR-195-5p inhibitor or DHA and miR-195-5p inhibitor. *P < 0.05, **P < 0.01, ***P < 0.001.

3. Results

3.1. DHA inhibits proliferation, migration, and invasion of HNSCC cells

Multiple assays were performed to explore the possible inhibitory effects of DHA on the proliferation, migration, and invasion of HNSCC cells. First, we performed CCK-8, wound-healing, and transwell assays after treating HNSCC cells with different concentrations of DHA for 24 h. The results indicated that DHA effectively inhibited the growth of FaDu and CAL-27 cells (Fig. 1A). Furthermore, we examined the effects of DHA on cell migration by treating HNSCC cells with varying doses of DHA for 24 h. The findings revealed significant inhibition of wound healing in both the cell lines when exposed to 20 and 80 μM DHA (Fig. 1B). Subsequently, we evaluated the potential of DHA to suppress the invasion of HNSCC cells. The transwell assay demonstrated reduced invasion of both CAL-27 and FaDu cells (Fig. 1C). The invasive cell count from the transwell assay confirmed that DHA effectively restrained the invasion of CAL-27 and FaDu cells (Fig. 1D). Overall, these results indicate that DHA suppresses the proliferation, migration, and invasion of CAL-27 and FaDu cells.

3.2. DHA inhibits HNSCC cell invasion and migration via miR-195-5p regulation

Previous studies suggest that miR-195-5p inhibits cell invasion and migration of various cancers and is strongly associated with poor HNSCC prognosis. We hypothesized that DHA inhibited cell invasion and migration of HNSCC cells via miR-195-5p regulation. Therefore, to explore the mechanism underlying DHA-mediated suppression of cell invasion and migration by regulating miR-195-5p expression, HNSCC cells were treated with various concentrations of DHA for 24 h qRT-PCR was performed to detect the expression of miR-195-5p. The results showed significant increase in the expression of miR-195-5p in HNSCC cells compared with that in the control group (Fig. 2A). HNSCC cells were transfected with miR-195-5p mimic or inhibitor after 0 and 24 h. We analyzed the expression of miR-195-5p by qRT-PCR. The results showed that miR-195-5p mimic increased the expression levels of miR-195-5p, whereas the inhibitor decreased its levels (Fig. 2B).

To estimate the effect of miR-195-5p on HNSCC cell migration and invasion, we analyzed the cell invasion and migration during miR-195-5p overexpression or sufficient inhibition. Compared with the untreated cells, 0 and 24 h of transfection with miR-195-5p mimic or inhibitor decreased and increased cell migration, respectively (Fig. 2C). For further exploration of the effect of miR-195-5p on cell invasion, a transwell assay was performed using CAL-27 and FaDu cells. Compared with the untreated cells, 24 h of transfection with miR-195-5p mimic or inhibitor decreased and increased the cell invasion, respectively (Fig. 2D and E). These data suggested that DHA inhibited cell invasion and migration by upregulating miR-195-5p expression in CAL-27 and FaDu cells.

3.3. TENM2 as the direct target of miR-195-5p

First, we determined the mechanisms underlying the effects of miR-195-5p on HNSCC cells. We used three public databases (ONCOMIR, TargetScan, and ENCORI) to identify the candidate targets of miR-195-5p. The target genes of miR-195-5p in three public databases are listed in Table 1. All three databases predicted TENM2 as a target candidate of miR-195-5p (Fig. 3A). Moreover, we observed significant differences in the expression of miR-195-5p and TENM2 between normal and HNSCC tissues, as identified in two GEO datasets (GSE216630 and GSE178537) (Fig. 3B).

Next, we constructed the luciferase reporter containing the complimentary seed sequence of miR-195-5p at the 3′-UTR region of TENM2 mRNA to confirm TENM2 as a target of miR-195-5p (Fig. 3C). Luciferase activity assay verified that the expression of Luc/RLuc was significantly reduced on co-transfection with hsa-miR-195-5p mimic, whereas it increased significantly on co-transfection with hsa-miR-195-5p inhibitor (Fig. 3C). To verify this phenomenon, qRT-PCR analysis was performed, and the results showed significant reduction in the levels of Luc mRNA on co-transfection with hsa-miR-195-5p mimic, whereas significant increase with the inhibitor (Fig. 3C).

Furthermore, we analyzed the luciferase activity in CAL-27 and FaDu cells treated with DHA (5, 20, and 80 μM). The activity of Luc/RLuc reduced in a dose-dependent manner, as confirmed by qRT-PCR results (Fig. 3D). Meanwhile, CAL-27 and FaDu cells co-stimulated with miR-195-5p mimic and DHA had significantly reduced Luc/RLuc activity compared with those stimulated with miR-195-5p alone (Fig. 3E). Luc/RLuc activity in DHA and miR-195-5p inhibitor co-treated cells was markedly upregulated compared with that in cells treated with miR-195-5p inhibitor alone. The qRT-PCR analysis confirmed these results (Fig. 3E). Overall, our data showed that miR-195-5p negatively regulated the expression of TENM2.

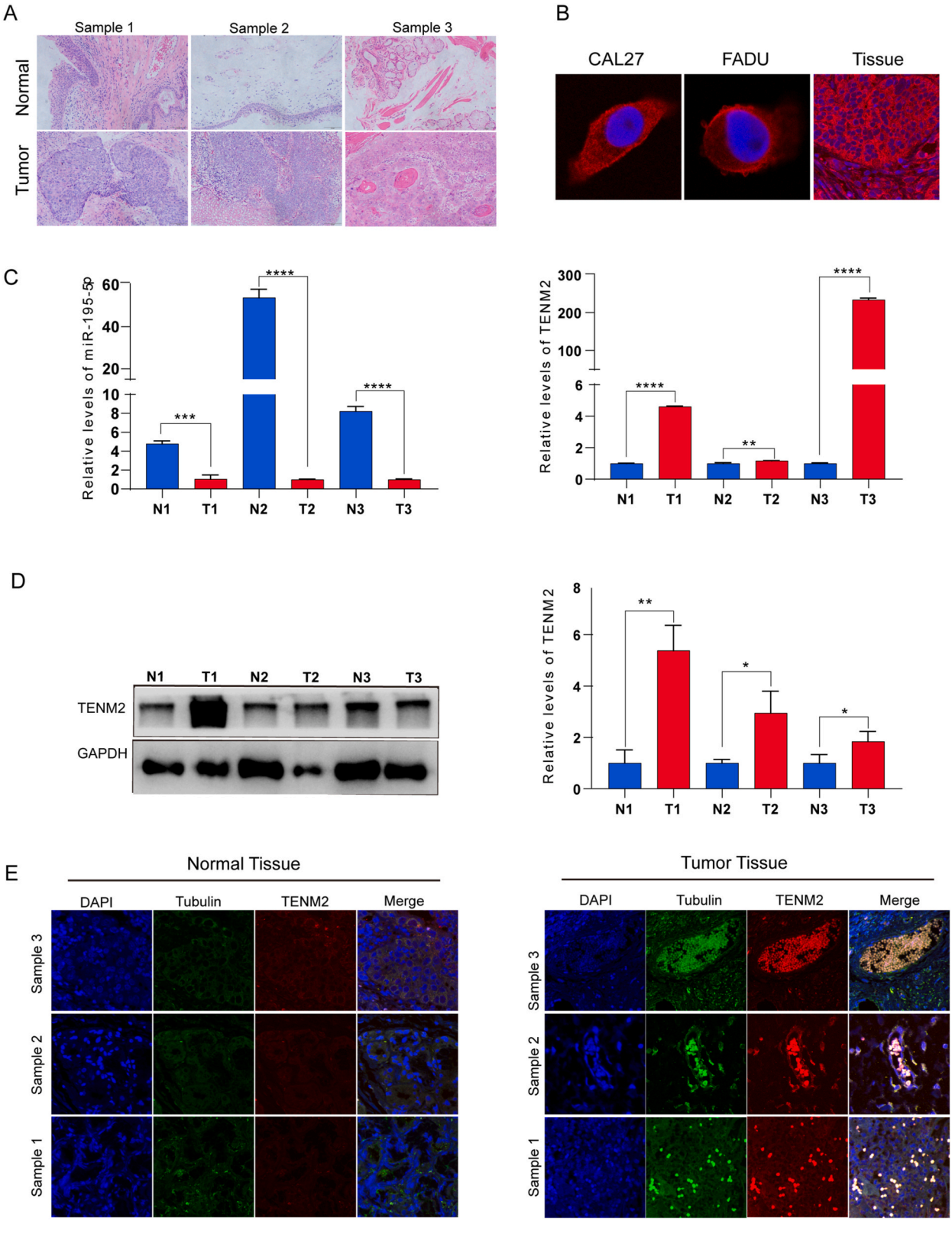

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Fig. 4. Different expression of miR-195-5p and TENM2 between normal and HNSCC tissue samples. A, HE staining of normal and tumor head and neck tissues. B, The location of TENM2 expression in CAL-27 cells by immunofluorescence. C, qRT-PCR analyzed the expression levels of miR-195-5p and TENM2 in normal and tumor head and neck tissues. D, Western blot analyzed TENM2 protein expression in tumor head and neck tissues. E, Immunofluorescence analyzed TENM2 expression in tumor head and neck tissues. Scale bars indicate 10 μm in all panels. *P < 0.05, **P < 0.01, ***P < 0.001.

3.4. Contrasting expressions of miR-195-5p and TENM2 between normal and HNSCC tissues

First, to explore the mechanism of action of miR-195-5p and TENM2, we analyzed the expression of TENM2 in normal and HNSCC tissue samples by performing HE staining and immunofluorescence (Fig. 4A and B). TENM2 was observed to be strongly expressed in the cell membrane (Fig. 4B).

Next, we analyzed the expression of miR-195-5p and TENM2 by qRT-PCR and western blotting. The results showed that decreased expression of miR-195-5p and increased expression of TENM2 in HNSCC tissue samples, whereas normal tissue samples showed the opposite trend (Fig. 4C and D). The immunofluorescence results further confirmed this phenotype (Fig. 4E). These results showed that the expression of miR-195-5p and TENM2 was significantly contrasting between normal and HNSCC tissues.

3.5. Both DHA and miR-195-5p mimic suppress the expression of TENM2

To elucidate the role of miR-195-5p/TENM2 regulation in DHA-induced inhibition of cell migration and invasion in HNSCC cells, we compared the effect of DHA and miR-195-5p mimic on TENM2 expression. The results of qRT-PCR and western blotting showed a notable decrease in TENM2 expression in DHA-treated (5, 20, and 80 μM) CAL-27 and FaDu cells compared with that in the untreated cells (Fig. 5A and B). The immunofluorescence results further confirmed this phenotype (Fig. 5C).

To assess the effect of miR-195-5p on TENM2 expression, qRT-PCR and western blotting were performed on CAL-27 and FaDu cells. The results showed decreased TENM2 expression with miR-195-5p mimic, whereas increased expression with the inhibitor (Fig. 5D and E). Additionally, immunofluorescence data indicated that DHA decreased TENM2 levels in CAL-27 and FaDu cells in a dose-dependent manner (Fig. 5F). These results suggested that both DHA and miR-195-5p mimic reduced the expression of TENM2 in CAL-27 and FaDu cells.

3.6. DHA inhibits HNSCC invasion and migration by miR-195-5p/TENM2 regulation

To explore the mechanism by which DHA inhibits cell migration and invasion by promoting TENM2 targeting by miR-195-5p, qRT-PCR and western blotting were performed to detect the expression of miR-195-5p and TENM2 in CAL-27 and FaDu cells. The results showed significant downregulation of TENM2 expression in the cells co-stimulated with miR-195-5p mimic and DHA compared with that in cells stimulated with miR-195-5p mimic alone. Moreover, treatment with DHA and miR-195-5p inhibitor significantly inhibited the expression of TENM2 compared with treatment with miR-195-5p inhibitor alone (Fig. 6A and B). The immunofluorescence results further confirmed this phenotype (Fig. 6C).

To further investigate the mechanism by which DHA inhibits cell migration by promoting miR-195-5p expression, we used wound-healing rates to detect cell migration. Compared with cells treated with miR-195-5p alone, cells co-stimulated with miR-195-5p mimic and DHA had low cell migration ability. The migration ability of cells co-treated with DHA and miR-195-5p inhibitor was markedly downregulated compared with that of the cells treated with miR-195-5p inhibitor alone (Fig. 6D). The transwell assay showed that DHA and miR-195-5p mimic co-treatment significantly inhibited cell invasion compared with miR-195-5p mimic treatment alone (Fig. 6E and F). These results suggested that DHA inhibited cell invasion and migration by regulating the miR-195-5p target, TENM2.

4. Discussion

DHA, one of the main derivatives of artemisinin, significantly inhibits malaria [5,6]. Recently, the anticancer activity of DHA has gained attention. Various molecular mechanisms are involved in the anti-tumor effects of DHA, including inhibition of proliferation, induction of apoptosis, inhibition of tumor angiogenesis and metastasis, promotion of immune function, and induction of autophagy and endoplasmic reticulum stress [18]. However, the specific mechanisms through which DHA suppresses cell invasion and migration in the context of its anticancer effects remain unclear. DHA has been demonstrated to inhibit cell invasion and migration by down-regulating TCTP in gallbladder cancer cells [7], by mediating angiogenesis in human melanoma cells [19], and by mediating RECK in human ovarian cancer cells [20]. Furthermore, DHA inhibits cell invasion and migration in liver cancer cells by suppressing the CaMKK2/NCLX signaling pathway, which is related to the production of ATP synthase [21]. Despite numerous reports on DHA-mediated inhibition of cell invasion and migration of various tumor cells, its effects on HNSCC cancer cells are still unknown. In this study, we found that DHA inhibited cell invasion and migration by regulating miR-195-5p expression in HNSCC cells in vitro. Our findings provide new evidence for the anti-invasive and anti-migratory activities of DHA on HNSCC cells, which provides a theoretical basis for the clinical application of DHA to HNSCC in the future.

MiR-195-5p inhibits cell invasion and migration in various cancers, including glioblastoma, non-small cell lung cancer, and prostate cancer, by targeting genes such as CCND3, MYB, and polypeptide 1 (RPS6KB1) [16,22,23]. In this study, we observed that the expression of TENM2 decreased, whereas that of miR-195-5p increased with DHA treatment. Upregulation of miR-195-5p resulted in

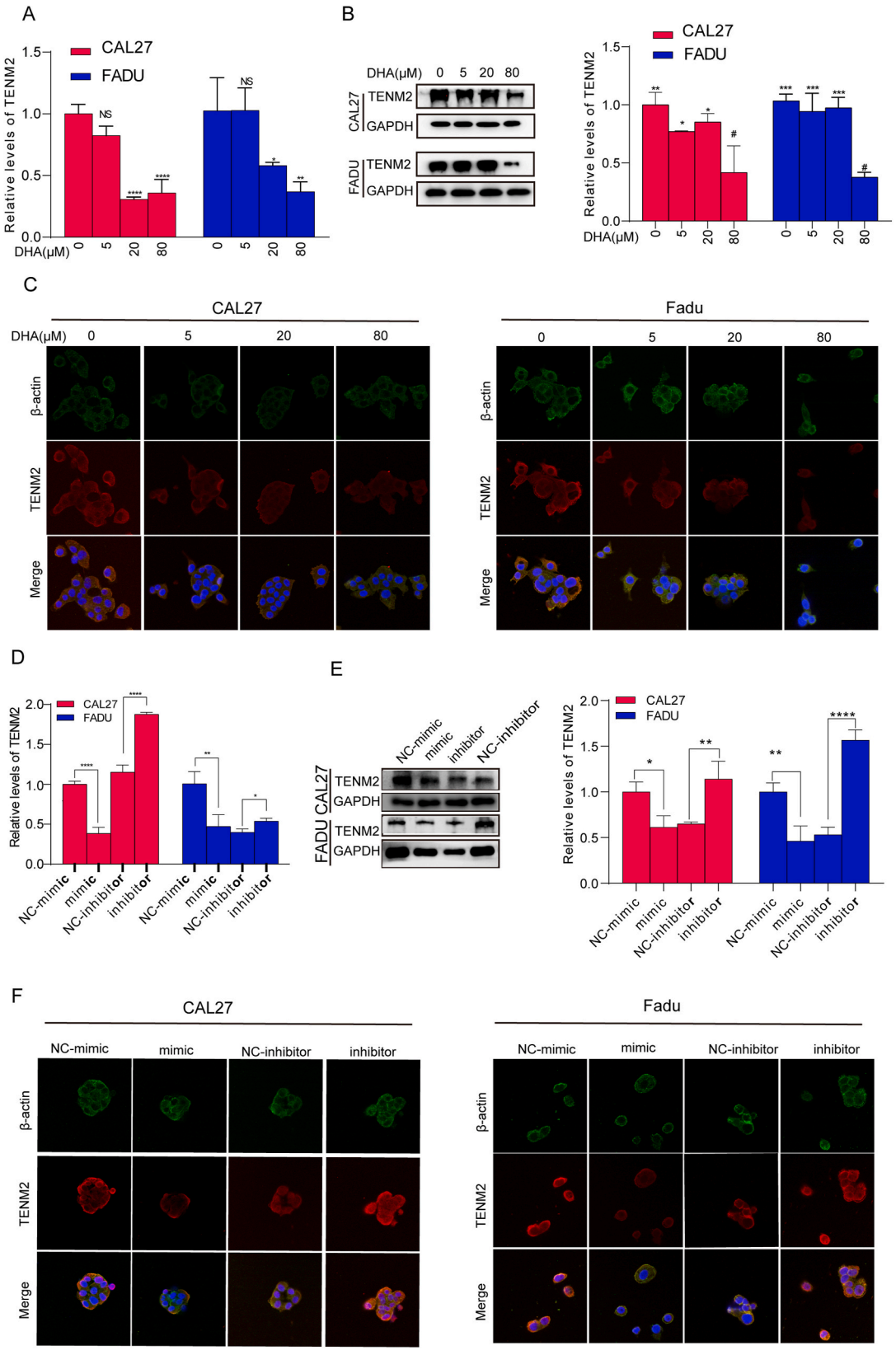

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Fig. 5. Both DHA and miR-195-5p downregulate TENM2 expression. A, qRT-PCR analyzed TENM2 transcript in DHA-treated CAL-27 and FaDu cells. B, TENM2 protein expression in DHA-treated CAL-27 and FaDu cells using Western blot. C, TENM2 expression in DHA-treated CAL-27 and FaDu cells was determined by immunofluorescence. Scale bars indicate 10 μm in all panels. D, qRT-PCR analyzed TENM2 and miR-195-5p mRNA levels in CAL-27 and FaDu cells transfected with miR-195-5p mimic or inhibitor. E, TENM2 protein expression in CAL-27 and FaDu cells transfected with miR-195-5p mimic or inhibitor using western blotting. F, TENM2 expression in CAL-27 and FaDu cells transfected with miR-195-5p mimic or inhibitor using immunofluorescence. Scale bars indicate 10 μm in all panels. *P < 0.05, **P < 0.01, ***P < 0.001.

downregulation of TENM2, whereas complete inhibition of miR-195-5p led to upregulation of TENM2. Meanwhile, we also found the expression of miR-195-5p and TENM2 was significantly contrasting between normal and HNSCC tissues. These findings suggested that miR-195-5p acted as a tumor suppressor in HNSCC by targeting TENM2.

However, our results indicated that TENM2 was not the only target gene of miR-195-5p in HNSCC cells. Bioinformatics analysis predicted that miR-195-5p had 21 target genes in HNSCC, some of which were associated with tumor invasion and migration. For example, the long non-coding RNA TRPM2 antisense RNA (TRPM2-AS) promotes invasion and migration of gastric cancer cells by suppressing miR-195/HMGA1 signaling axis [25]. Additionally, miR195-5p targets KIF23, inhibiting proliferation and migration in triple-negative breast cancer, as well as LITAF, which is related to colorectal cancer onset and progression [26,27]. These findings suggest that miR-195-5p has multiple target genes involved in the regulation of cell invasion and migration in different types of cancer. In the present study, miR-195-5p inhibited the invasion and migration of HNSCC cells by targeting TENM2. However, several questions require further investigation. First, it is necessary to determine whether DHA can inhibit cell invasion and migration by regulating other miRNAs in addition to miR-195-5p. Second, it is important to explore whether miR-195-5p contributes to cell invasion and migration by targeting other genes in HNSCC cells, in addition to TENM2. Furthermore, the interactions between the genes targeted by miR-195-5p remain unclear. Finally, the effect of DHA on cell invasion and migration through the regulation of these genes in HNSCC cells remains unknown. These unresolved issues are of great significance to this study and warrant further exploration, highlighting its limitations. In the future, we will focus on exploring other mechanisms of action of DHA in HNSCC, and animal experiments will be performed to further verify these potential mechanisms.

In conclusion, the present study provides credible evidence supporting the anti-invasive and anti-migratory effects of DHA on HNSCC cells. Experimental evidence strongly supports the role of DHA in regulating miR-195-5p and targeting TENM2, suggesting that DHA is a potential anti-tumor agent in HNSCC.

Consent for publication

All participants gave written informed consent, and the collection of clinical information was approved by the Institutional Review Board.

Availability of data and material

The data generated in the present study may be requested from the corresponding author.

Competing interests

The author reports no conflicts of interest in this work.

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Ethics statement

The research protocol received approval from the Ethics Committee of Chongqing General Hospital (S2020-110-01). Ethics approval date was 2021.2.8.

We understand and comply with the Ethics and Editorial Policies in the Elsevier policies (https://www.cell.com/heliyon/ethics).

Data availability statement

Data included in article/supp. material/referenced in article. The data supporting this study's findings are available on request from the corresponding author, [W.Y.]. The data are not publicly available due to their containing information that could compromise the privacy of research participants. Furthermore, for the use of data, the regulation requires the permission of this institution's Ethics Committee.

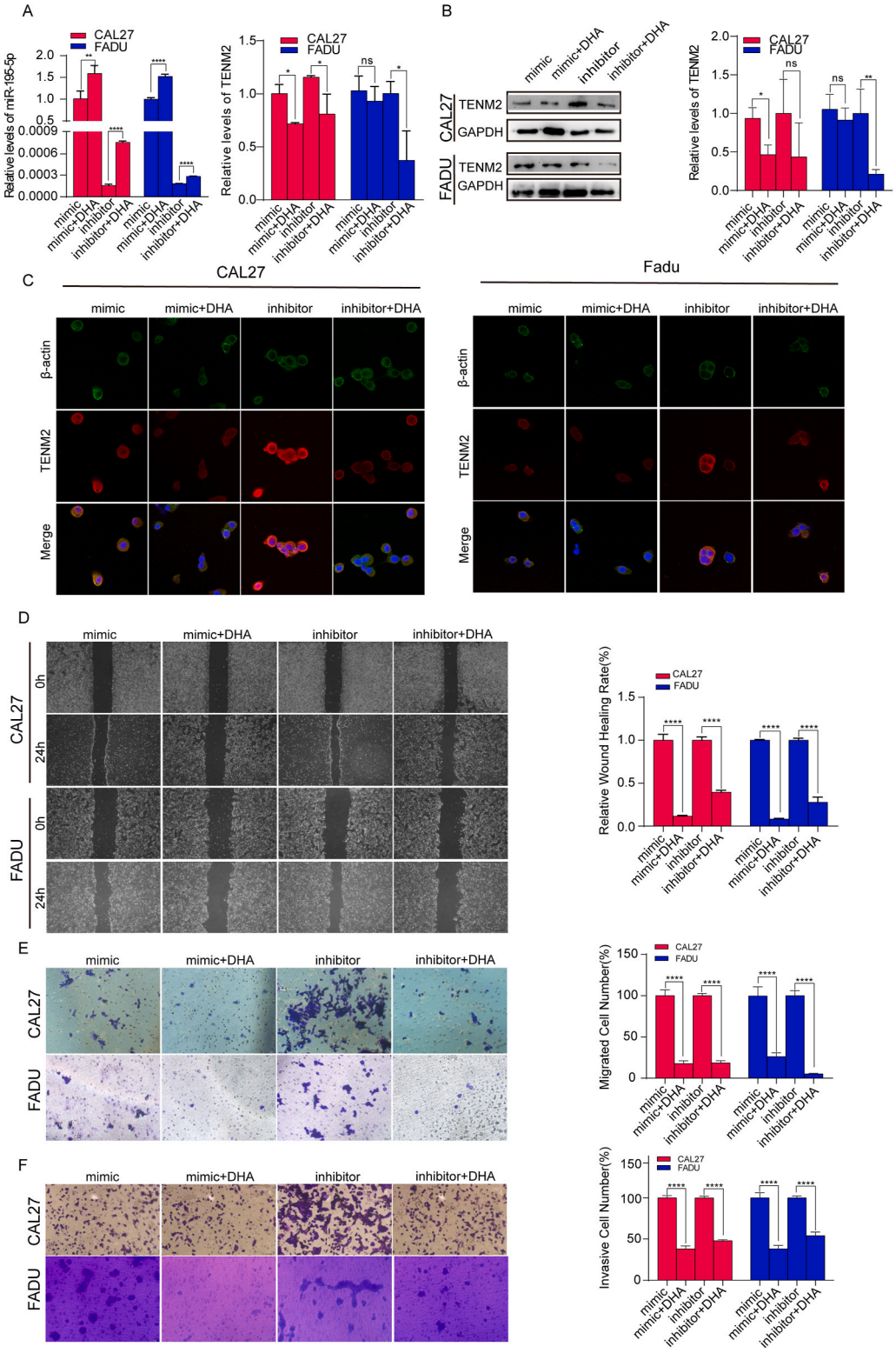

Fig. 6. DHA inhibits HNSCC invasion and migration by regulating miR-195-5p/TENM2. A, qRT-PCR analyzed TENM2 and miR-195-5p mRNA levels in CAL-27 and FaDu cells after stimulation with miR-195-5p mimic or DHA and miR-195-5p mimic, miR-195-5p inhibitor or DHA and miR-195-5p inhibitor. B, TENM2 protein expression in CAL-27 and FaDu cells after stimulation with miR-195-5p mimic or DHA and miR-195-5p mimic, miR-195-5p inhibitor or DHA and miR-195-5p inhibitor for 24 h using western blotting. C, TENM2 expression in CAL-27 and FaDu cells after stimulation

with miR-195-5p mimic or DHA and miR-195-5p mimic, miR-195-5p inhibitor or DHA and miR-195-5p inhibitor for 24 h using immunofluorescence. Scale bars indicate 10 μm in all panels. D, Wound-healing rates in CAL-27 and FaDu cells after stimulation with miR-195-5p mimic or DHA and miR-195-5p mimic, miR-195-5p inhibitor or DHA and miR-195-5p inhibitor for 24 h using western blotting. E-F, Transwell assay performed in CAL-27 and FaDu cells after stimulation with miR-195-5p mimic or DHA and miR-195-5p mimic, miR-195-5p inhibitor or DHA and miR-195-5p inhibitor for 24 h were exhibited, and the tendency of invasive cells was analyzed. *P < 0.05, **P < 0.01, ***P < 0.001.

CRediT authorship contribution statement

Xiaolu Wu: Writing – original draft, Software, Methodology, Conceptualization. **Congwen Yang:** Writing – review & editing, Visualization, Data curation, Conceptualization. **Zhongwan Li:** Validation. **Ping Lv:** Validation. **Xiang An:** Formal analysis. **Xiaohe Peng:** Methodology. **You Li:** Methodology. **Xiaojun Jiang:** Investigation. **Xuemei Mao:** Investigation. **Donghong Chen:** Investigation. **Lifeng Jia:** Writing – review & editing, Project administration, Funding acquisition. **Wei Yuan:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32522.

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