Dihydroartemisinin suppresses proliferation, migration, the Wnt/β-catenin pathway and EMT via TNKS in gastric cancer

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Received December 19, 2020; Accepted May 11, 2021

DOI: 10.3892/ol.2021.12949

Abstract. Gastric cancer is a common malignancy worldwide. However, the molecular mechanisms underlying this malignancy remain unclear and there are a lack of effective drugs. The present study aimed to investigate the antitumor effect of Dihydroartemisinin (DHA) or inhibition of Tankyrases (TNKS), and determine the underlying molecular mechanisms of gastric cancer. Immunohistochemistry and immunofluorescence analyses were performed to detect the expression levels of TNKS, epithelial-to-mesenchymal transition (EMT) and Wnt/β-catenin pathway-related proteins in gastric cancer tissues and adjacent normal tissues. The Cell Counting Kit-8 assay was performed to assess the viability of HGC-27 and AGS cells following treatment with different concentrations of HLY78 (a Wnt activator) or DHA. Following treatment with HLY78, DHA or small interfering (si)-TNKS1/si-TNKS2, colony formation and migratory abilities were assessed via the colony formation, wound healing and Transwell assays. Furthermore, western blot and immunofluorescence analyses were performed to detect the expression levels of TNKS, EMT- and Wnt/β-catenin-pathway-related proteins. The results demonstrated that the expression levels of TNKS, AXI2, β-catenin, N-cadherin and Vimentin were upregulated, whereas E-cadherin expression was downregulated in gastric cancer tissues compared with normal tissues. Furthermore, HLY78 and DHA suppressed the viability of HGC-27 and AGS cells, in a concentration-independent manner. Notably, TNKS knockdown or treatment with DHA suppressed colony formation, migration, TNKS expression, EMT and the Wnt/β-catenin pathway. Opposing effects were observed following treatment with HLY78, which were ameliorated following co-treatment with DHA. Taken together, these results suggest that DHA or inhibition of TNKS can suppress the proliferation and migration of gastric cancer cells, which is partly associated with inactivation of the Wnt/β-catenin pathway and EMT process.

Introduction

Gastric cancer is one of the most common malignancies worldwide, as the fifth most frequently diagnosed cancer and the third-leading cause of cancer-associated mortality, particularly in East Asian countries (1). Its 5-year overall survival rate is <30% (2). Most patients are diagnosed at an advanced stage (3). Some clinical trials have tested new targeted drugs for advanced gastric cancer (4-6); however, the results are disappointing due to notable toxic effects and low response rate (7). Thus, it is important to investigate the molecular pathogenesis and develop novel drugs for patients with gastric cancer.

The occurrence of gastric cancer is an intricate process, involving the abnormal expression of several genes (8,9). Tankyrases (TNKS), as member of the poly (ADP-ribose) polymerase (PARP) family, has two subtypes, TNKS1 and TNKS2 (10). Both subtypes have 85% overlap in the amino acid sequence (10). TNKS participate in various biological processes. For example, TNKS hyperactivates the Wnt/β-catenin pathway by destabilizing AXIN (11). Furthermore, TNKS regulates PARylation of BLZF1, as well as CASC3, thereby recruiting RNF146 and subsequent ubiquitination (12). In addition, it participates in centrosome maturation during prometaphase by regulating PARylation of HEPACAM2/MIKI (13). It may regulate vesicle trafficking, as well as subcellular distribution of SLC2A4/GLUT4-vesicles (14). By mediating PARylation of TERF1, TNKS is involved in telomere length (15). It has been reported that TNKS expression is upregulated in different types of cancer, including gastric cancer (16). In addition, TNKS1 expression is significantly associated with stage and differentiation of gastric cancer (17). Recently, it was

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Abbreviations: DHA, Dihydroartemisinin; TNKS, Tankyrases; EMT, epithelial-to-mesenchymal transition; MMPs, matrix metalloproteinases; siRNA, small interfering RNA

Key words: gastric cancer, DHA, TNKS, migration, Wnt/β-catenin, EMT
demonstrated that TNKS knockdown can inhibit the proliferation, invasion and epithelial-to-mesenchymal transition (EMT) process in hepatocellular carcinoma cells (11). However, the underlying molecular mechanisms of TNKS in gastric cancer remain unclear.

Several signaling pathways, such as EMT, contribute to tumor invasion and metastasis (18). EMT has a profound influence on the early events of metastatic spread of gastric cancer cells (18). In the EMT process, the expression of adhesion proteins, such as E-cadherin, decrease, followed by loss of polarity of epithelial tumor cells and loosening of cell connections (19). The interaction between epithelial cells gradually disappears (19). Subsequently, the expression of mesenchymal cell characteristics (N-cadherin and Vimentin) and matrix metalloproteinases (MMPs) increases, and the expression of signal transduction proteins, such as Twist, is activated (20). Tumor cells have acquired the ability to resist apoptosis and degrade the extracellular matrix, and their migratory ability increases, resulting in invasion and metastasis (21). Clinically, EMT is associated with poor prognosis of patients with gastric cancer (22). Activation of the Wnt/β-catenin pathway accelerates the EMT process, thereby promoting invasion and metastasis of gastric cancer (23,24). HLY78 can bind to the DAX domain of Axin, which has been widely used as the Wnt/β-catenin pathway agonist (25).

Dihydroartemisinin (DHA) is the main component of artemisinin extracted from the traditional Chinese medicine, Artemisia annua (26). Several studies have reported that DHA exerts broad biological characteristics, including anti-tumor effects (26-28). As reported in a recent study, DHA can restrain proliferative, migrative and invasive abilities of gastric cancer cells (27). Furthermore, DHA prevents Helicobacter pylori-induced gastric cancer by inhibiting NF-xB activity (28). However, the exact molecular mechanism of DHA remains unclear. Thus, the present study aimed to investigate the effects of DHA on proliferation, migration, the Wnt/β-catenin pathway, as well as the EMT process in gastric cancer cells.

Materials and methods

Patients and tissue specimens. A total of 87 pairs of gastric cancer tissues and adjacent normal tissues were collected from patients (42 men and 45 women; mean age, 60.1 years; age range, 43-72 years) following surgical resection at The First Hospital of Yulin between February 2018 and February 2020. Normal tissues were at least 5 cm away from tumor tissues. The inclusion criteria were as follows: i) Patients did not receive chemotherapy or radiotherapy prior to surgery; ii) patients were diagnosed as primary gastric cancer; iii) patients did not have other types of cancer; iv) patients did not have any history of other types of cancer; iv) patients did not have any history of chemotherapy or radiotherapy prior to surgery; ii) patients were diagnosed as primary gastric cancer tissues and adjacent normal tissues were collected from patients (42 men and 45 women; mean age, 60.1 years; age range, 43-72 years) following surgical resection at The First Hospital of Yulin between February 2018 and February 2020. Normal tissues were at least 5 cm away from tumor tissues. The inclusion criteria were as follows: i) Patients did not receive chemotherapy or radiotherapy prior to surgery; ii) patients were diagnosed as primary gastric cancer; iii) patients did not have other types of cancer; iv) patients did not have any history of surgery and v) patients did not have any concomitant diseases. Patients without complete clinical information were excluded from the present study. Tissue samples were transferred into liquid nitrogen following surgery and stored at -80°C. All specimens were fixed in 10% formalin, followed by gradual alcohol dehydration, transparent xylene and paraffin embedding within 24 h. Patients were diagnosed by two pathologists, in line with the guidelines of the Union for International Cancer Control (29). The present study was approved by the Ethics Committee of The First Hospital of Yulin (Yulin, China; approval no. 2018031) and written informed consent was provided by all patients prior to the study start.

Immunohistochemistry. Gastric cancer tissues were fixed overnight with 10% formalin solution at 4°C. Paraffin-embedded gastric cancer tissues were cut into 4-µm-thick sections. Following dewaxing and rehydrating, the sections were incubated with 3% H2O2 for 20 min to inhibit endogenous peroxidase activity at room temperature. Following antigen retrieval, the sections were blocked with 5% BSA blocking solution at room temperature for 1 h. The sections were incubated with primary antibodies against TNKS (1:200; cat. no. 18030-1-AP), AXIN2 (1:150; cat. no. 20540-1-AP), Vimentin (1:200; cat. no. 10366-1-AP), β-catenin (1:100; cat. no. 17565-1-AP), E-cadherin (1:100; cat. no. 20874-1-AP) and N-cadherin (1:100; cat. no. 22018-1-AP) overnight at 4°C (all purchased from ProteinTech Group, Inc.). Following the primary incubation, membranes were incubated with HRP-conjugated secondary antibodies (1:1,000; cat. no. ab6721; Abcam) for 2 h at room temperature. DAB reagent (Sigma-Aldrich; Merck KGaA) was used for color development. The sections were stained with hematoxylin for 3 min at room temperature, and differentially with 1% hydrochloric acid in ethanol for 15 sec, and 1% ammonia water for 1 min. Following a series of ethanol dehydration, the sections were made transparent with xylene and sealed with neutral resin. Images were observed under a light microscope (Olympus Corporation) at magnification of x200. The optical density values were determined using ImageJ software (version 1.48; National Institutes of Health). Furthermore, the semi-quantitative values of TNKS, AXIN2 and β-catenin expression were assessed via the percentage of positive cells (<5%, 0 point; 5-25%, 1 point; 26-50%, 2 points; 51-75%, 3 points and 76-100%, 4 points) and staining intensity (no staining, 0 point; light yellow, 1 point; brown, 2 points and tan, 3 points), as previously described (30). The product of the two was defined as follows: 0, negative (-) and >1 positive (+).

Cell culture. The human gastric cancer cell lines, AGS and HGC-27, were purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), at 37°C with 5% CO2, and 95% saturated humidity environment (all purchased from Gibco; Thermo Fisher Scientific, Inc.). When the cells reached 80% confluence, they were digested with 0.25% trypsin. After passaging three times, cells were harvested and seeded into a 6-well plate at a density of 3x10^3 cells/well.

Transient transfection. Small interfering RNAs (siRNAs) targeting TNKS1 or TNKS2 (5 nM, Sangon Biotech, Co., Ltd.) and the corresponding siRNA negative control (si-NC; Sangon Biotech, Co., Ltd.) were separately transfected into AGS and HGC-27 cells using Lipofectamine® 2000 transfection reagent (cat. no. 11660-019; Thermo Fisher Scientific, Inc.). The siRNA sequences were as follows: hTNKS1 forward, 5′-GCAUUG AGCUUUGUUGUAU-3′ and reverse, 5′-AUUAACACACAGCUUCAUCGU-3′; hTNKS2 forward, 5′-GAGGGAUC...
UCAUUAGGUAAU-3' and reverse, 5'-UACCUAUGAGA UACCUCUU-3'; and si-NC forward, 5'-CGUACUUUU UGUAGUACAA-3' and reverse, 5'-UGUAGUACACA AAAGUACCG-3'. After 48 h, transfection efficiency was verified via western blotting.

Cell Counting Kit-8 (CCK-8). AGS and HGC-27 cells were respectively seeded into 96-well plates at a density of 5x10^3 cells/well. After the cells fully adhered, the original culture medium was discarded. Subsequently, each group was treated with different concentrations of HLY78 (cat. no. HY-122816, MCU; 0, 5, 10, 20, 30, 40, 50 and 100 µM) or DHA (cat. no. HY-N0176; MCE; 0, 5, 10, 20, 30, 50 and 100 µM) in RPMI-1640 complete medium (100 µl/well). Following incubation for 48 h at 37°C, cells were cultured in medium containing CCK-8 (100 µl, RPMI-1640 complete medium + 10 µl CCK-8; Beyotime Institute of Biotechnology) for 1 h. Cell viability was analyzed at a wavelength of 450 nm, using a microplate reader (Bio-Rad Laboratories, Inc.).

Western blotting. AGS and HGC-27 cells were seeded into 6-well plates at a density of 5x10^5 cells/well and lysed on ice using RIPA lysis buffer (Beyotime Institute of Biotechnology). Following centrifugation at 12,000 x g for 20 min at 4°C, the supernatant was harvested. Protein concentration was determined using the BCA protein concentration determination kit (Thermo Fisher Scientific, Inc). The proteins samples were diluted by an equal volume of 2X loading buffer and heated for 10 min to denature the protein. A total of 40 µl protein sample was added to each well, separated via 12% SDS-PAGE, transferred onto PVDF membranes (MilliporeSigma) and blocked with 5% skimmed milk powder for 1 h at 4°C. The membranes were incubated with primary antibodies against TNKS (1:1,000; cat. no. 18030-1-AP), AXIN2 (1:1,000; cat. no. 20540-1-AP), TWIST (1:1,000; cat. no. 20540-1-AP), MMP2 (1:1,000; cat. no. 10366-1-AP), Vimentin (1:1,000; cat. no. 10366-1-AP), β-catenin (1:200; cat. no. 17565-1-AP), E-cadherin (1:1,000; cat. no. 20874-1-AP), N-cadherin (1:500; cat. no. 22018-1-AP) and β-actin (1:1,000; cat. no. 20536-1-AP) overnight at 4°C (all purchased from ProteinTech Group, Inc.). Following the primary incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. nos. ab205719 and ab6721; Abcam) for 1 h at room temperature. ECL color luminescent liquid (GE Healthcare) was evenly added to the PVDF membranes and allowed to react for 1 min. The gray values of the target proteins were quantified using ImageJ software (version 1.48; National Institutes of Health).

Colonies formation assay. AGS and HGC-27 cells were seeded into a 6-well plate at a density of 5x10^3 cells/well. Following transfection or treatment for 24 h, cells were digested using trypsin, and 2 ml cell suspension was added to each well. Cell colony formation was observed every 2 days and the medium was changed every 3-4 days. The medium was discarded after 1 week and cells were fixed with 600 µl methanol for 30 min at 4°C. Cells were subsequently stained with 600 µl 0.1% crystal violet for 20 min at room temperature. Stained cells were observed under a light microscope (Olympus Corporation) at magnification of x200.

Wound healing assay. AGS and HGC-27 cells were seeded into a 6-well plate at a density of 5x10^3 cells/well. Once the bottom of the plate was covered by cells and the confluence was up to 95% under the microscope field of view, a 10 µl pipette tip was used to scratch the cell monolayers. The original medium was discarded and the plates were washed three times with PBS to remove cell debris. The cells were serum-starved during the wound healing assay. Following treatment with 5 nM si-TNKSI, 5 nM si-TNKSII, 20 µM HLY78 or 30 µM DHA, cells were observed at 0 and 48 h. Images were observed under a light microscope (Olympus Corporation) at magnification of x200.

Migration assay. The Transwell chamber (Corning, Inc.) was placed into a 24-well plate. Serum-free medium was used to routinely prepare the cells into a single cell suspension. AGS and HGC-27 cells were plated in the upper chambers of Transwell plates (5x10^5 cells/well), while 500 µl medium supplemented with 10% FBS was plated in the lower chambers. Following incubation for 12 h at room temperature, the 24-well plate was washed 2-3 times with PBS and the migratory cells were fixed with 4% paraformaldehyde for 15 min at 4°C. Cells were subsequently stained with Giemsa for 30 min at room temperature and counted in three randomly selected fields using a light microscope (Olympus Corporation).

Immunofluorescence. AGS and HGC-27 cells were seeded into a 6-well plate at a density of 5x10^5 cells/well and covered with a cover glass. Once the cells adhered to the wall, the glass slide was fixed with 4% paraformaldehyde for 15 min at 4°C. The sections were incubated with penetrating agent for 20 min at room temperature and subsequently blocked with blocking solution for 30 min at room temperature. Sections were incubated with primary antibodies against TNKS (1:200; cat. no. 18030-1-AP), AXIN2 (1:150; cat. no. 20540-1-AP) and β-catenin (1:100; cat. no. 17565-1-AP) overnight at 4°C (all purchased from ProteinTech Group, Inc.). Following the primary incubation, the sections were incubated with Alexa Fluor® 488-conjugated secondary antibody (1:500; cat. nos. ab150077 and ab150113; Abcam) for 90 min at room temperature and counted in three randomly selected fields using a light microscope (Olympus Corporation).

Statistical analysis. Statistical analysis was performed using SPSS 22.0 software (IBM Corp.). Unpaired Student's t-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's post hoc test was used to compare differences between multiple groups. The χ² test was used to assess the association between TNKS expression and the clinicopathological characteristics of patients with gastric cancer. P<0.05 was considered to indicate a statistically significant difference.

Results

Activation of TNKS, Wnt/β-catenin and EMT in gastric cancer. The expression levels of TNKS, Wnt/β-catenin and EMT proteins were detected in 87 pairs of gastric cancer tissues and
adjacent normal tissues. The results demonstrated that TNKS expression was 1.95 times higher in gastric cancer tissues compared with normal tissues \((P<0.01; \text{Fig. 1A and B})\). In the Wnt/β-catenin pathway, AXIN2 \((P<0.001; \text{Fig. 1A and C})\) and β-catenin \((P<0.001; \text{Fig. 1A and D})\) both exhibited higher expression in gastric cancer tissues compared with normal tissues. The positive rates of TNKS, AXIN2 and β-catenin in gastric cancer, as well as adjacent normal tissues were calculated in a cohort of 87 patients with gastric cancer, as listed in Table I. Furthermore, the expression of EMT-related proteins was detected. Among them, E-cadherin \((P<0.001; \text{Fig. 1A and E})\) was downregulated, while N-cadherin \((P<0.001; \text{Fig. 1A and F})\) and Vimentin \((P<0.001; \text{Fig. 1A and G})\) were upregulated in gastric cancer tissues compared with normal tissues. Consistent with immunohistochemistry, immunofluorescence analysis demonstrated that TNKS \((P<0.001; \text{Fig. 2A and B})\), AXIN2 \((P<0.001; \text{Fig. 2A and C})\) and β-catenin \((P<0.001; \text{Fig. 2A and D})\) expression levels were distinctly higher in gastric cancer tissues compared with normal tissues. Furthermore, E-cadherin expression was downregulated by 0.47-fold in gastric cancer tissues compared with normal tissues \((P<0.01; \text{Fig. 2A and E})\). Collectively, the TNKS, Wnt/β-catenin and EMT pathways are activated in gastric cancer \((16,18,23)\). The association between TNKS expression and the clinicopathological characteristics of patients with gastric cancer was also evaluated. As presented in Table II, TNKS expression was significantly associated with depth of invasion \((P=0.012)\), lymph metastasis \((P=0.001)\), TNM stage \((P=0.018)\) and survival status \((P<0.001)\).

**Figure 1. Immunohistochemistry analysis of TNKS, Wnt/β-catenin and EMT-related proteins between gastric cancer tissues and adjacent normal tissues.** (A) Representative images of immunohistochemistry analysis. Scale bar, 20 µm. (B) TNKS, (C) AXIN2, (D) β-catenin, (E) E-cadherin, (F) N-cadherin and (G) Vimentin expression levels were quantified in gastric cancer tissues and normal tissues. **\(P<0.01\)** and ***\(P<0.001\) vs. normal tissues. TNKS, Tankyrases.

DHA treatment significantly suppresses proliferation of gastric cancer cells partly by TNKS2. Gastric cancer cells were treated with different concentrations of HLY78, a Wnt activator. The results of the CCK-8 assay demonstrated that the viability of HGC-27 and AGS cells decreased as the concentration of HLY78 increased (both \(P<0.0001\); Fig. 3A and B). HLY78 \((20 \mu M)\) was selected as the optimal concentration for subsequent analyses. The viability of HGC-27 and AGS cells treated different concentrations of DHA was assessed. The results demonstrated that DHA significantly suppressed viability of the gastric cancer cells in a concentration-independent manner (both \(P<0.0001\); Fig. 3C and D). DHA \((30 \mu M)\) was determined the optimal concentration. Western blot analysis confirmed that TNKS expression decreased...
following transfection with si-TNKS1 and si-TNKS2 (P<0.0001; Fig. 3E and F). Cell colony formation was assessed for the transfected or treated gastric cancer cells. The results demonstrated that HLY78 significantly increased the colony formation ability by 2.78-fold and 2.88-fold for HGC-27 and AGS cells compared with the controls (both P<0.0001; Fig. 3G-I). Furthermore, the colony formation ability decreased following transfection with si-TNKS1 or si-TNKS2. Treatment with DHA notably decreased the number of cell colonies by 0.31-fold and 0.13-fold in HGC-27 and AGS cells compared with the controls. In addition, treatment with DHA significantly attenuated the enhancement of colony formation induced by HLY78. Taken together, these results suggest that DHA significantly suppresses proliferation partly by silencing TNKS2 expression in gastric cancer cells.

**DHA treatment suppresses migration of gastric cancer cells partly by silencing TNKS.** The wound healing and Transwell assays were performed to assess the migratory ability of treated gastric cancer cells. The results demonstrated that HLY78 elevated the migration ability of HGC-27 and AGS cells (P<0.001 and P<0.01; Fig. 4A-C). The wound distance was significantly shorter following transfection with si-TNKS1 by 1.55-fold and 1.56-fold or transfection with

| Protein  | Gastric cancer tissues (n=87) | Adjacent normal tissues (87) |
|----------|-----------------------------|-----------------------------|
|          | Positive rate, n (%)       | Negative rate, n (%)       | Positive rate, n (%) | Negative rate, n (%) | P-value |
| TNKS     | 61 (70.1)                  | 26 (29.9)                  | 21 (24.1)          | 66 (75.9)          | <0.0001 |
| AXIN2    | 57 (65.5)                  | 30 (34.5)                  | 23 (26.4)          | 64 (73.6)          | <0.0001 |
| β-catenin| 68 (78.2)                  | 19 (21.8)                  | 23 (26.4)          | 64 (73.6)          | <0.0001 |

**Table I.** Expression levels of TNKS, AXIN2 and β-catenin in 87 gastric cancer tissues and 87 adjacent normal tissues from a cohort of patients with gastric cancer.

**Figure 2.** Immunofluorescence analysis of TNKS, AXIN2, β-catenin and E-cadherin between gastric cancer tissues and adjacent normal tissues. (A) Representative images of immunofluorescence analysis. Scale bar, 20 µm. (B) TNKS, (C) AXIN2 and (D) β-catenin expression levels were upregulated in gastric cancer tissues compared with normal tissues. (E) E-cadherin expression was downregulated in gastric cancer tissues compared with normal tissues. **P<0.01 and ***P<0.001 vs. normal tissues. TNKS, Tankyrases.
**Table II. Association between TNKS expression and the clinicopathological characteristics of patients with gastric cancer (n=87).**

| Characteristic                        | Total (n=87) | Positive, % (n=61) | Negative, % (n=26) | χ²  | P-value |
|--------------------------------------|--------------|--------------------|--------------------|-----|---------|
| Sex                                  |              |                    |                    |     |         |
| Male                                 | 42           | 28 (66.7)          | 14 (33.3)          | 0.461 | 0.497  |
| Female                               | 45           | 33 (73.3)          | 12 (26.7)          |     |         |
| Age, years                           |              |                    |                    |     |         |
| <60                                  | 43           | 32 (74.4)          | 11 (25.6)          | 0.752 | 0.386  |
| ≥60                                  | 44           | 29 (65.9)          | 15 (34.1)          |     |         |
| BMI                                  |              |                    |                    |     |         |
| <24                                  | 27           | 17 (63.0)          | 10 (37.0)          | 0.978 | 0.613  |
| 24-27.9                              | 31           | 23 (74.2)          | 8 (25.8)           |     |         |
| ≥28                                  | 29           | 21 (72.4)          | 8 (27.6)           |     |         |
| Smoking                              |              |                    |                    |     |         |
| No                                   | 46           | 33 (71.7)          | 13 (28.3)          | 0.123 | 0.726  |
| Yes                                  | 41           | 28 (68.3)          | 13 (31.7)          |     |         |
| Drinking                             |              |                    |                    |     |         |
| No                                   | 39           | 24 (61.5)          | 15 (38.5)          | 2.481 | 0.115  |
| Yes                                  | 48           | 37 (77.1)          | 11 (22.9)          |     |         |
| Helicobacter pylori infection        |              |                    |                    |     |         |
| No                                   | 54           | 37 (68.5)          | 17 (31.5)          | 0.173 | 0.677  |
| Yes                                  | 33           | 24 (72.7)          | 9 (27.3)           |     |         |
| Depth of invasion                    |              |                    |                    |     |         |
| T1/T2                                | 36           | 19 (52.8)          | 17 (47.2)          | 6.363 | 0.012  |
| T3/T4                                | 51           | 40 (78.4)          | 11 (21.6)          |     |         |
| Lymph metastasis                     |              |                    |                    |     |         |
| N0                                   | 27           | 9 (33.3)           | 18 (66.7)          | 11.379 | 0.001b |
| N1/N2/N3/N3                         | 60           | 43 (71.7)          | 17 (28.3)          |     |         |
| TNM stage                            |              |                    |                    |     |         |
| I-II                                 | 40           | 22 (55.0)          | 18 (45.0)          | 5.572 | 0.018a |
| III-IV                               | 47           | 37 (78.7)          | 10 (21.3)          |     |         |
| Survival status                      |              |                    |                    |     |         |
| Dead                                 | 33           | 24 (72.7)          | 9 (27.3)           | 23.351 | <0.001c |
| Alive                                | 54           | 11 (20.4)          | 43 (79.6)          |     |         |

*P<0.05; *P<0.01; **P<0.001. TNKS, Tankyrases; TNM, tumor-node-metastasis.

DHA suppresses activation of the EMT process and the Wnt/β-catenin pathway in gastric cancer cells. The present study investigated whether DHA can affect activation of EMT and the Wnt/β-catenin pathway in gastric cancer cells via western blot analysis. The results demonstrated that DHA significantly decreased TNKS expression by 0.42-fold (P<0.0001; Fig. 5A and B). Furthermore, HLY78 elevated TNKS expression by 1.84-fold in gastric cancer cells, which was ameliorated following DHA co-treatment (both P<0.0001). HLY78 treatment increased AXIN2 expression by 0.55-fold, the effects of which were reversed following treatment with DHA (both P<0.0001; Fig. 5A and C). MMP2 expression is used to assess the invasive ability of tumor cells (20). The results demonstrated that DHA significantly inhibited MMP2 expression.
expression by 0.47-fold and ameliorated the increase in MMP2 expression induced by HLY78 (both P<0.0001; Fig. 5A and D). In Fig. 5A and E, TWIST expression was increased by HLY78 and decreased by DHA in gastric cancer cells both (P<0.0001). DHA treatment significantly decreased Vimentin expression by 0.69-fold (P<0.001), and improved HLY78-induced increase in Vimentin expression (P<0.0001; Fig. 5A and F). Furthermore, β-catenin (Fig. 5A and G) and N-cadherin (Fig. 5A and H) expression levels significantly decreased following treatment with DHA (both P<0.01), the effects of which were ameliorated following treatment with HLY78 (both P<0.0001). In Fig. 5A and I, DHA significantly increased E-cadherin expression by 2.25-fold (P<0.0001), while HLY78 suppressed E-cadherin expression by 0.50-fold in gastric cancer cells. Collectively (P<0.0001), these results suggest that DHA inactivates EMT and the Wnt/β-catenin pathway in gastric cancer cells.

**DHA suppresses activation of the EMT process and the Wnt/β-catenin pathway in gastric cancer partly by silencing TNKS.** The effects of TNKS knockdown and DHA on EMT and the Wnt/β-catenin pathway in gastric cancer cells were investigated. As expected, TNKS expression significantly decreased by 0.47-fold and 0.22-fold in gastric cancer cells treated with si-TNKS1/si-TNKS2 or DHA (all P<0.0001; Fig. 6A and B). Furthermore, TNKS1 or TNKS2 knockdown significantly decreased AXIN2 expression by 0.39-fold and 0.38-fold (both P<0.0001; Fig. 6A and C), MMP2 by 0.37-fold and 0.35-fold (both P<0.0001; Fig. 6A and D), TWIST by 0.32-fold and 0.30-fold (both P<0.0001; Fig. 6A and E), Vimentin by 0.55-fold and 0.51-fold (both P<0.0001; Fig. 6A and F), β-catenin by 0.32-fold and 0.30-fold (both P<0.0001; Fig. 6A and G) and N-cadherin by 0.28-fold and 0.53-fold (both P<0.0001; Fig. 6A and H). In Fig. 6A and I,
E-cadherin expression significantly increased by 1.95-fold and 2.00-fold following transfection with si-TNKS1/si-TNKS2 (all P<0.0001). Similar results were observed following treatment with DHA (all P<0.0001). Taken together, these results suggest that DHA inhibits activation of EMT and the Wnt/β-catenin pathway in gastric cancer cells partly by silencing TNKS.
DHA inactivates the Wnt/β-catenin pathway in gastric cancer partly via silencing TNKS. Immunofluorescence analysis was performed to detect the expression levels of TNKS, AXIN2 and β-catenin in HGC-27 and AGS cells. Consistent with western blotting, TNKS expression significantly decreased following transfection with si-TNKS1/si-TNKS2 or treatment with DHA in HGC-27 cells (all P<0.0001; Fig. 7A and B). Conversely, HLY78 treatment significantly increased TNKS expression, which was reversed following DHA co-treatment. Similar results were observed in AGS cells. Both TNKS knockdown and DHA decreased TNKS expression (P<0.05, P<0.01 or P<0.001; Fig. 7A and E), AXIN2 (all P<0.01; 0.001 Fig. 7A and F) and β-catenin (P<0.01, P<0.001 or P<0.0001; Fig. 7A and G) expression levels in gastric cancer cells. Collectively, these results suggest that DHA inactivates the Wnt/β-catenin pathway in gastric cancer partly by silencing TNKS.

Discussion
The results of the present study demonstrated that DHA and inhibition of TKNS suppressed proliferation, migration,
EMT process, as well as the Wnt/β-catenin pathway in gastric cancer. Following co-treatment with Wnt activator, HLY78, the inhibitory effect of DHA was not affected. Inhibition of TNKS is considered a therapeutic strategy for different types of cancer, such as hepatocellular carcinoma (11) and gastric cancer (17). The results of the present study demonstrate that DHA inhibited TNKS expression. Thus, DHA may be a promising drug for the treatment of gastric cancer.

The EMT process and Wnt/β-catenin pathway contribute to migration and invasion in gastric cancer (19,24,31). In the present study, activation of the EMT process and Wnt/β-catenin pathway were detected in gastric cancer tissues. The results demonstrated that DHA inhibited activation of the Wnt/β-catenin pathway and EMT process in gastric cancer. HLY78, a Wnt activator, activated Wnt/β-catenin, as well as the EMT process, thereby promoting proliferation and migration of gastric cancer cells (32). Notably, DHA ameliorated the HLY78-induced malignant transformation in gastric cancer cells, suggesting that DHA may be a promising novel drug for the treatment of gastric cancer. Vimentin, as a mesenchymal marker, promotes gastric cancer cell migration and adhesion (19). AXIN2 protein functions in the canonical Wnt pathway (33). TWIST is associated with EMT process in gastric cancer (34). The expression levels of Vimentin, AXIN2 and TWIST were suppressed following treatment of gastric cancer cells with DHA. For gastric cancer, the loss of E-cadherin expression may stimulate cells to transform into a more invasive characteristic via the EMT process (19). The results of the present study suggest that DHA can recover E-cadherin expression in gastric cancer cells. MMPs are involved in tumor cell invasion and migration, as well as degradation of the extracellular matrix (35). MMP-2, an important member of the MMPs family, is overexpressed in gastric cancer (36-38). Its high expression can induce migration, as well as invasion in gastric cancer cells (35). In the present study, DHA distinctly decreased MMP2 expression in gastric cancer cells.
cancer cells, suggesting that DHA can ameliorate the invasion and metastasis in gastric cancer. It has been reported that DHA can suppress EMT formation in breast cancer cells (39) and esophageal cancer cells (40). Furthermore, treatment with...
DHA can inhibit the proliferative ability of squamous cancer cells via the Wnt/β-catenin pathway (41). Thus, DHA inactivates EMT and the Wnt/β-catenin pathway in gastric cancer cells.

TNKS inhibitor is considered a candidate drug for inactivation of the Wnt/β-catenin process in cancers (42). It has been reported that TNKS induces aerobic glycolysis and proliferation by activating the Wnt/β-catenin pathway in ovarian cancer (43). Furthermore, its overexpression is closely associated with poor clinical outcomes of patients with colorectal cancer (44). However, the role of TNKS in gastric cancer remains unclear. The results of the present study demonstrated that TNKS inhibition may be used as a potential treatment strategy for gastric cancer. Collectively, these results suggest that treatment with DHA significantly inhibits TNKS expression in gastric cancer, suggesting that DHA may be an inhibitor of TNKS. However, further studies are required to validate the results presented here.

In conclusion, the results of the present study demonstrated that DHA or TNKS inhibition suppressed proliferation and migration of gastric cancer cells. Furthermore, the Wnt/β-catenin pathway and EMT process were inactivated following treatment with DHA or TNKS knockdown. DHA distinctly decreased TNKS expression, suggesting that TNKS may be a potential target of DHA. However, the present study is not without limitations. First, the therapeutic effects of DHA on gastric cancer need to be further investigated in vivo. Secondly, whether TNKS is a direct target of DHA requires further investigation. Thus, prospective studies will aim to investigate the therapeutic effects of DHA on gastric cancer and determine its underlying molecular mechanisms.

Acknowledgements

Not applicable.

Funding

The present study was funded by the Key R & D plan of Shaanxi Province (grant no. 2020SF-236), the Project of Hebei Medical Science Research Project (grant no. 20191127) and the Science and Technology Research and Development Project of Yulin City in 2020 (grant no. YF-2020-039).

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

HM conceived and designed the present study. YM, PZ and QZ performed most of the experiments, analyzed the data and drafted the initial manuscript. XW, QM, XL and BC performed some experiments and analyzed the data, and drafted and revised the manuscript. HM and YM confirmed the authenticity of all the raw data. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Hospital of Yulin (Yulin, China; approval no. 2018031) and written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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