Telomerase inhibition decreases esophageal squamous carcinoma cell migration and invasion

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Abstract. Telomerase has been shown to be associated with a variety of cancer types. To elucidate the role of telomerase in esophageal squamous carcinoma (ESCC), tissue samples from 100 patients with ESCC, and paired paracancerous tissues from 75 of these patients, were collected for use in the present study. Using immunohistochemical analysis, the expression of telomerase reverse transcriptase (hTERT) in the cytoplasm of ESCC cells was revealed to be significantly higher compared with that in paracancerous tissues, and no significant difference was observed between hTERT expression in the nucleus of ESCC and paracancerous tissue cells. Combined analysis revealed that the cytoplasmic hTERT-positive rate of patients with ESCC was significantly associated with pathological grade, N stage and Tumor-Node-Metastasis (TNM) stage; these data support the association between hTERT expression and poor patient prognosis. *In vitro* experiments demonstrated that hTERT knockdown does not inhibit the proliferation of ESCC Kyse410 or Kyse520 cells, but inhibits their migration and invasion abilities. These findings indicate that hTERT expression is associated with ESCC metastasis. Interestingly, decreased colony-formation ability was observed in Kyse410 cells, but not in Kyse520 cells. Collectively, the results of the present study suggest that hTERT may serve as a potential therapeutic target for ESCC.

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Abbreviations: ESCC, esophageal squamous cell carcinoma; hTERT, telomerase reverse transcriptase; EADC, esophageal adenocarcinoma; hTR, telomerase RNA; TMAs, tissue microarrays; FBS, fetal bovine serum; NC, negative control

Key words: ESCC, telomerase, migration, invasion, prognosis

Introduction

Esophageal cancer is considered to be one of the most malignant tumors, with a high mortality rate that ranks sixth worldwide. Esophageal cancer is categorized into two subtypes: i) Esophageal squamous cell carcinoma (ESCC), which accounts for ~90% of all cases; and ii) adenocarcinoma (EADC), which accounts for the remaining ~10% of cases (1,2). ESCC is a common malignant gastrointestinal tumor with a high incidence rate in certain rural areas of China, for example in Linxian, Cixian, Shexian and Yanting the incidence rates of esophageal cancer (per 100,000 population) for 2011 were 83.8, 91.5, 64.2 and 99.0, respectively, and is characterized by a unique geographical distribution along the Taihang Mountains (3,4). The risk factors of ESCC include nutritional deficiency, and the consumption of betel quid, tobacco, pickled vegetables and hot food and drinks (2,5). With changes in socioeconomic status, living conditions and eating habits, the incidence of esophageal cancer has declined, but the situation remains severe (3,6).

Surgical resection is the first choice for the treatment of early ESCC. However, the majority of patients have already developed advanced ESCC before symptoms become apparent, which is usually indicated by difficulties in swallowing. At this point, radiotherapy is considered to be the primary treatment option, although the outcome is often unsatisfactory; following radiotherapy alone, the 5-year survival rate is ~10% due to poor local control and distant metastasis (7), and as such, the development of novel anticancer strategies is highly warranted.

Telomerase maintains telomere length by adding TTAGGG hexamers and inhibiting cellular senescence (8,9). Telomerase has been reported to be closely associated with cancer, serving crucial roles in tumor growth and progression, in part through the maintenance of telomere structure (10). Telomerase reactivation is an essential step in malignant tumor progression, and almost 90% of cancer in humans exhibit telomerase activity (11). Therefore, the expression and activity of telomerase are indispensable for tumor formation (12).

Telomerase consists of telomerase RNA (hTR) and telomerase reverse transcriptase (hTERT). hTERT is the core catalytic subunit of telomerase, and has historically been considered to play an important role in telomere length maintenance (13,14). hTERT is distinguishable from other reverse

transcriptases by its unique mode of action, which promotes template realignment to enable continued synthesis of multiple DNA sequence repeats (15). hTERT transcription induces telomerase activity, which is critical for cellular proliferation, differentiation and senescence (16,17). Moreover, hTERT is expressed in proliferative cells such as germ and stem cells, as well as in tumors, but is not found in the majority of somatic cells (16,17). The hTERT promoter is located within a dense CG-rich CpG-island, indicating a role for methylation in the regulation of hTERT expression (18). hTERT promoter hypermethylation has been observed in numerous cancer cell types (compared with normal, noncancerous cells), which correlates with hTERT expression, especially in epithelial tumors (19). Furthermore, hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity (20). hTERT has also been noted as enzymatically active in different cancer types, and serves a crucial role in tumorigenesis and progression. As well as maintaining telomere length, hTERT was found to be associated with migration and invasion in various cancer cell types (21). hTERT expression is also the rate-limiting factor for telomerase activity, and increased telomerase activity can be blocked by inhibiting hTERT (22). Genome-wide studies of clinical samples have also highlighted the importance of hTERT in numerous malignancies (23). However, previous studies have largely focused on the enzymatic activity of hTERT, rather than its other biological functions (24). Studies in which telomerase was inhibited by genetic, antisense and pharmacological strategies indicate that telomerase, especially hTERT, is an ideal target for cancer therapy (25).

Metastasis is the leading cause of death in patients with esophageal cancer, and the liver, lung and bones are the most common sites of metastasis (26). Tumor metastasis involves a series of complex processes, including cellular migration. Abnormal regulation of cellular migration results in tumor cell invasion and metastasis, which enables tumor cell escape from the primary site, invasion into lymphatic and blood vessels, and ultimately, colonization at distant sites (27). Cellular migration is a dynamic, multi-step process (28). Key molecules in cancer cell migration are of great importance for tumor metastasis, and may therefore serve as potential targets for cancer treatment. To date, a large number of studies have reported the role of hTERT in malignancies such as urological tumors (29-32), melanomas (30,33,34), gastric cancer (35-37), gliomas (38-41) and hepatocellular tumors (42). However, few studies have reported the association between hTERT expression and the migration and invasion of ESCC cells (43,44).

In the present study, hTERT expression was analyzed in ESCC tissues, and its association with specific clinicopathological characteristics was determined. Moreover, hTERT knockdown using RNA interference (RNAi) methods resulted in decreased cellular migration and invasiveness. It was therefore concluded that targeting hTERT may highlight novel approaches for the treatment of ESCC.

Materials and methods

Patients and samples. The present study included 100 patients with ESCC, of which 75 also donated paired paracancerous tissues (located <3 cm away from the cancer tissue). The mean age of the patients was 65.29 years (range, 48-82 years), including 74 males and 26 females. All the samples in the tissue microarrays (TMAs) were purchased (Shanghai Outdo Biotech Co., Ltd.) and the use of the TMAs for research purposes was approved by the Institutional Research Ethics Committee, The Second Hospital of Nanjing (approval no. 2018-LY-KY068). Specimens from patients with incomplete clinical data were not included in the statistical analysis.

Immunohistochemistry analysis. The expression of Ki67 (proliferation cell-associated nuclear antigen in tumor tissues), p53 (tumor suppressor) and hTERT was assessed by immunohistochemistry. TMAs were embedded with paraffin at 63˚C for 1 h, deparaffinized in xylene at room temperature for 30 min and then rehydrated in absolute ethanol for 14 min (solvent refreshed at 7 min), then rehydrated in 90, 80 and 70 ethanol (7 min each), and finally placed in distilled water for 9 min (solvent refreshed every 3 min). The TMAs were then immersed in boiling sodium citrate buffer for 5 min and left to cool at room temperature. Following incubation in 10% BSA (Sangon Biotech Co., Ltd.) for 1 h at room temperature, the TMAs were incubated with primary antibodies specific to hTERT (rabbit anti-human TERT, polyclonal; 1:100; cat. no. ab183105; Abcam), p53 (rabbit anti-human, polyclonal; 1:100; cat. no. 9282; Cell Signaling Technology, Inc.) and Ki-67 (mouse anti-human, monoclonal; 1:100; cat. no. sc-23900; Santa Cruz Biotechnology, Inc.) at 4˚C overnight; this was followed by a further incubation with goat anti-mouse IgG (H+L) (1:1,000; cat. no. SA00001-1; ProteinTech Group, Inc.) or goat anti-rabbit IgG (H+L) (1:1,000; cat. no. SA00001-2; ProteinTech Group, Inc.) secondary antibodies at 37˚C for 20 min. The sections were stained at room temperature with DAB for 5 min and counterstained with hematoxylin and eosin (H&E) for 2 min at room temperature. The TMAs were then dehydrated and dried, and subsequently mounted using neutral gum. The TMAs were scanned using an Aperio ScanScope system (Leica Microsystems, Inc.), and staining was analyzed using Aperio Imagescope version 12.4.0.5043 (Aperio Technologies, Inc.). The results were verified by a senior pathologist who was blinded to the clinicopathological data of the patients. Three fields with different staining intensities were analyzed at x20 magnification. Approximately 100 cells in each field of view were analyzed and the percentage of positive cells in nucleus and cytoplasm were calculated manually. The final staining positive rate of the tissue point was the average number of three fields.

Cell culture and transfection. Kyse410 and Kyse520 ESCC cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, and cultured in RPMI-1640 with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin (all Gibco; Thermo Fisher Scientific, Inc.) at 37˚C (5% CO_2). The sequences of the negative control (NC) and hTERT siRNAs (Guangzhou RiboBio Co., Ltd.) were as follows: NC forward, 5'-UUCUCCGAA CGUGUCACGUdTdT-3'; NC reverse, 5'-ACGUGACACGUU CGGAGAAdTdT-3'; anti-hTERT forward, 5'-GCGACGACG UGCUGGUUCAdTdT-3'; and anti-hTERT reverse, 5'-dTd TCGCUGCUGCACGACCAAGU-3'. On the day before transfection, $5x10^5$ cells were seeded into 6-well plates without

antibiotics and cultured until 70% confluence was achieved. The NC or hTERT siRNA (25 nmol/l) and Lipofectamine® RNAiMAX Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) were diluted in Opti-MEM® Reduced Serum Medium (Gibco; Thermo Fisher Scientific, Inc.) and gently mixed; both reagents were then combined, mixed gently and incubated for 10-20 min at room temperature. The complexes were added to each well of the 6-well plates, and incubated at 37˚C for the times indicated in each subsequent experimental section.

Western blot analysis. Following transfection for 48 h, Kyse410 and Kyse520 cells were harvested and lysed with RIPA buffer (KeyGEN BioTECH); protein concentration was quantified using a bicinchoninic acid protein assay kit (Nanjing KeyGen Biotech Co., Ltd.). The samples were boiled with 5X loading buffer, and 20-μg aliquots of total protein were separated using 8% SDS-PAGE gels, prior to transfer onto PVDF membranes (EMD Millipore). The membranes were blocked at room temperature in 5% nonfat milk for 2 h and incubated with GAPDH (1:500; cat.no. GB11002; Wuhan Servicebio Technology Co., Ltd.) or hTERT (rabbit anti-human TERT, polyclonal; 1:1,000; cat. no. ab183105; Abcam) primary antibodies overnight at 4˚C. The membranes were than washed, and subsequently incubated with goat anti-rabbit IgG (H+L) secondary antibody (1:10,000; cat. no. SA00001-2; ProteinTech Group, Inc.) for 1 h at room temperature. Proteins were visualized using the FluorChem M System (ProteinSimple).

MTT assays. The effects of hTERT knockdown on cell proliferation were detected using an MTT Cell Proliferation and Cytotoxicity Assay Kit (Beijing Leagene Biotechnology Co., Ltd.). The cells were separated into three groups: i) Untreated; ii) NC; and iii) hTERT siRNA. Kyse410 and Kyse520 cells were seeded into 96-well plates ($2x10^3$ cells/well) 12 h post-transfection, and left to adhere for a further 6 h. Following 0-, 24-, 48- and 72-h incubation periods at 37˚C, 10 μl MTT solution was added to each well and the cells were incubated for an additional 4 h. The culture supernatants were carefully removed and 110 μl formazan solvent was added per well to dissolve the blue–purple formazan crystals. Absorbance was detected at 570 nm using a spectrophotometer (SN.1510-05687; Thermo Fisher scientific, Inc.).

Cell Counting Kit-8 (CCK-8) assay. The CCK-8 assay was performed to analyze the effect of hTERT knockdown on cellular proliferation, according the manufacturer's protocol (Dojindo Molecular Technologies, Inc.). Cells were seeded into 96-well plates at a density of $2x10^3$ cells/well (n=5). After incubation for 24, 48 and 72 h, 10 μl CCK-8 reagent was added to each well and incubated at 37˚C (5% CO_2) for 3 h. Absorbance was detected at 450 nm using the aforementioned spectrophotometer.

Clonogenic assay. To assess clonogenic cell survival, Kyse410 and Kyse520 cells were harvested with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) and seeded into 3.5-cm tissue culture dishes at various cell densities. The cells were incubated at 37˚C for 7-12 days, fixed with 95% ethanol and stained using hematoxylin. Clonogenic cells were defined as those able to form a colony of ≥50 cells. Colony images were captured using a commercial digital camera (PowerShot S110; Canon, Inc), and the colonies from four independent replicates were counted using Adobe Photoshop CS5 software version 12.01 (Adobe Systems, Inc.).

Table I. Clinicopathological characteristics of patients with esophageal squamous carcinoma.

Clinicopathological features	Patients, n	Censored patients, n	P-value
Sex			0.024
Male	74	9	
Female	26	10	
Total	100	19	
Age, years			0.723
≤65	51	8	
>65	49	11	
Total	100	19	
Tumor size, cm			0.021
≤5	56	14	
>5	29	4	
Total	85	18	
Pathological grade			0.631
I	6	2	
II	66	12	
III	28	5	
Total	94	19	
T-stage			0.026
T0	4	3	
T1	11	4	
T2	79	11	
T3	3	0	
Total	97	18	
N-stage			0.001
N0	45	13	
N1	31	5	
N2	17	0	
N3	5	0	
Total	98	18	
TNM-stage			<0.001
TNM1	4	3	
TNM2	42	12	
TNM3	50	2	
Total	96	17	

N, node; T, tumor; TNM, Tumor-Node-Metastasis.

Invasion assays. For the invasion assays (12 h post-transfection), $1x10^5$ cells were resuspended in 100 μl RPMI-1640 medium (without serum) and seeded onto the upper surfaces of 24-well Transwell filter inserts (pore diameter, 8 μm; Corning Inc.); the inserts were pre-coated with Matrigel (BD Biosciences). The lower chamber was loaded with 600 μl RPMI-1640 medium supplemented with 10% FBS as a chemoattractant; each sample was assessed in triplicate. After 48 h, cells in the upper chamber were removed with wet cotton swab. The cells

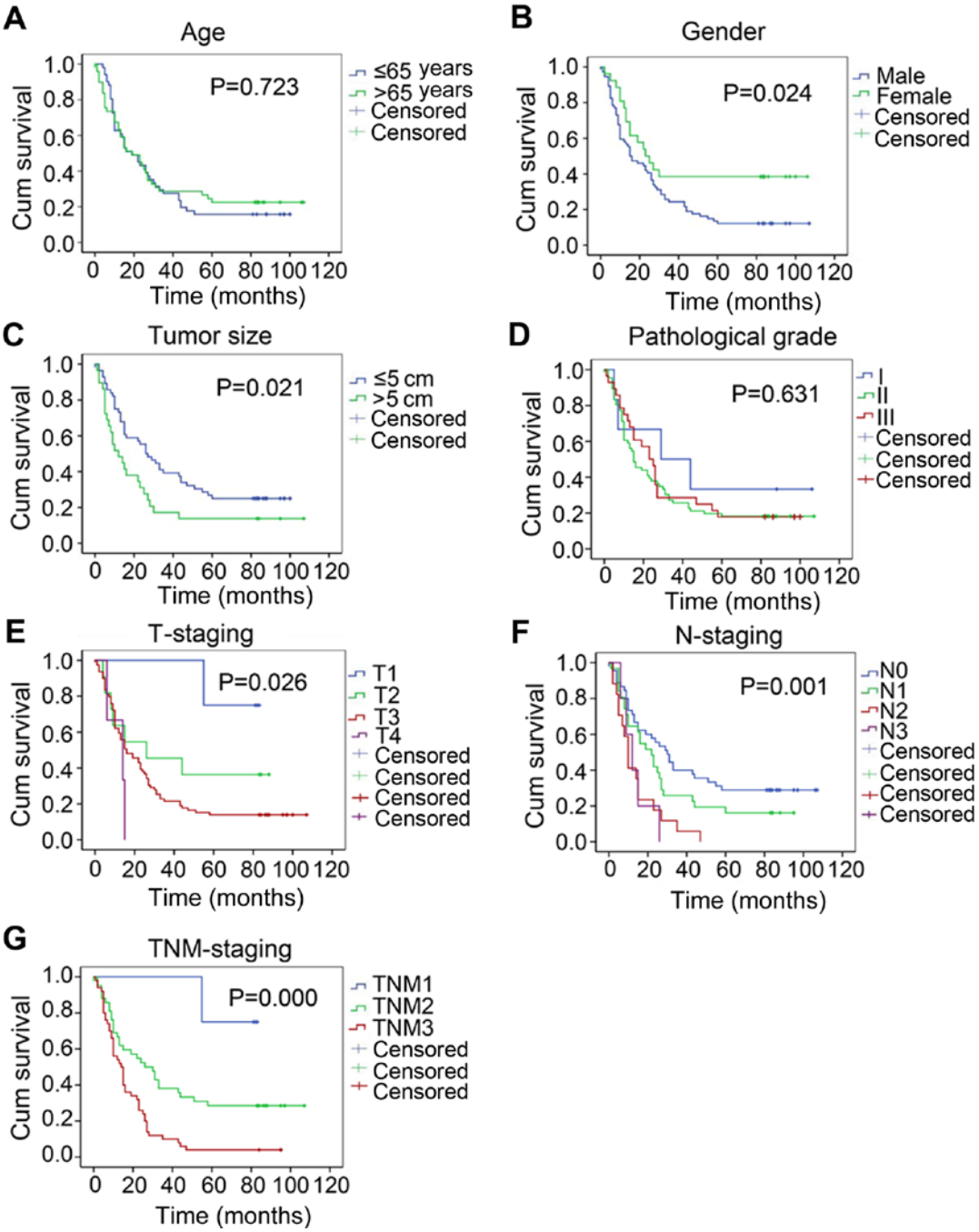

Figure 1. Survival times of patients with esophageal squamous cell carcinoma according to different clinicopathological characteristics. Survival curves according to (A) age, (B) sex, (C) tumor size, (D) pathological grade, (E) T-staging, (F) N-staging and (G) TNM-staging. TNM, Tumor-Node-Metastasis; T, primary tumor; N, regional lymph nodes; M, distant metastasis; Cum, cumulative.

in the lower chamber were fixed with 99.99% methanol at 4˚C for 30 min, stained with 0.1% crystal violet at 37˚C for 15 min (Invitrogen; Thermo Fisher Scientific, Inc.), and observed and images were captured under an inverted phase contrast microscope (magnification, x100) (Olympus Corporation).

Wound healing assay. Following 24 h of transfection, a straight-line wound was made in each cell monolayer using a 100-μl pipette tip. Detached cells were gently removed with PBS. Each well was replenished with RPMI-1640, and then observed using a phase contrast microscope (magnification, x40) and images were captured at 0 and 24-h time intervals.

Statistical analysis. Data analysis was performed using SPSS version 25 (IBM Corp.). The χ^2 test was used to determine significant differences between the frequency of different categories. Fisher's exact test was used to assess if high hTERT-expression rate in ESCC tissues and paracancerous tissues different. ANOVA followed by Fisher's LSD post hoc test was used to assess whether the mean difference between groups was significant. The Kaplan-Meier method and log-rank test were used to calculate survival rate, and Spearman's rank correlation was used to assess the degree of association between two variables. $P<0.05$ was considered to indicate a statistically significantly difference.

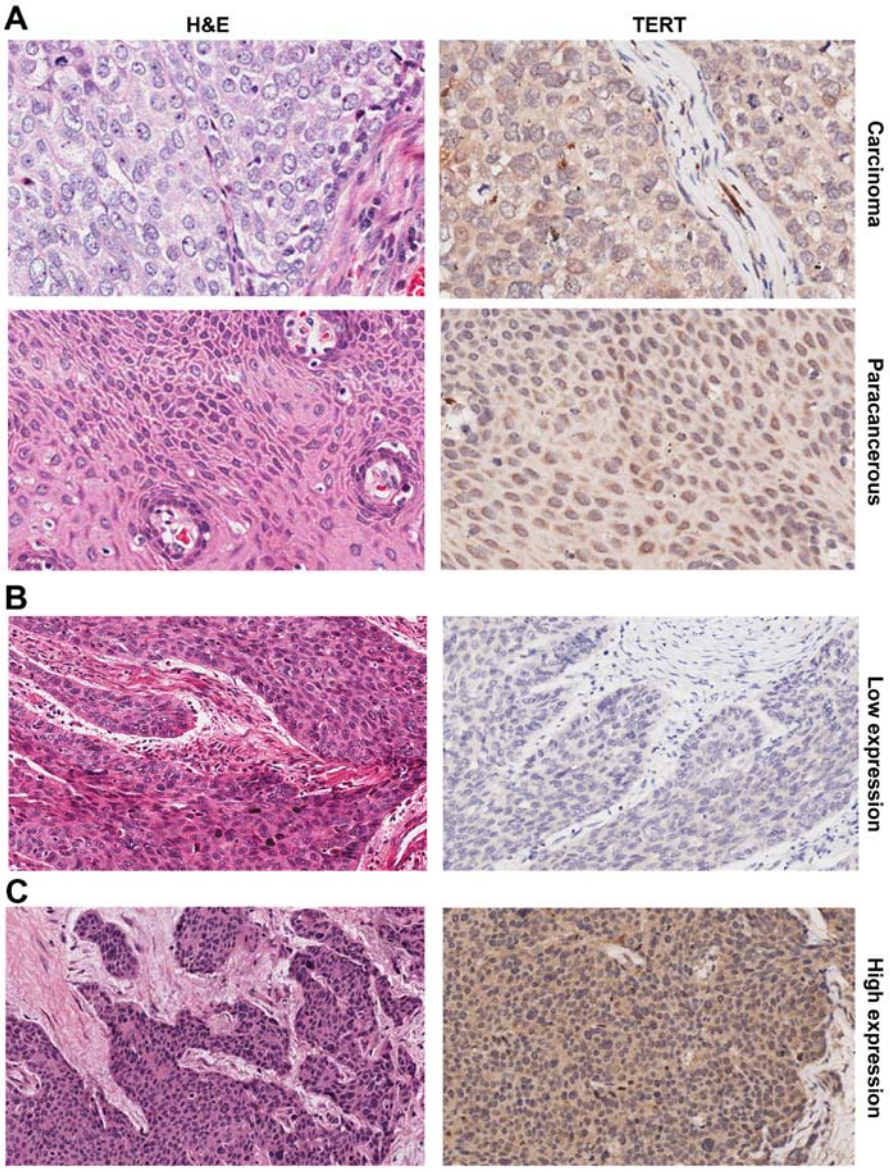

Figure 2. hTERT expression is higher in the cytoplasm of esophageal cancer cells compared with those of paired paracancerous tissues. Pathological changes in esophageal squamous cell carcinoma tissues were evaluated by H&E staining. hTERT expression in tissues is indicated by brown coloration. (A) hTERT expression in cancer cell cytoplasm and paired paracancerous tissues. (B-C). Representative photos show differential expression of hTERT in cancer cell cytoplasm within tissues. hTERT, telomerase reverse transcriptase; H&E, hematoxylin and eosin. Magnification, x20.

Results

HTERT is highly expressed in tumors and is associated with patient survival. The present study included 100 patients with ESCC (74 males and 26 females). The assessed patient clinicopathological characteristics included sex, age, tumor size, pathological grade and tumor stage (Table I). The incidence of ESCC among males (74%) was higher compared with that of females (26%), which corresponds with the findings of a previous study (69 and 31%, respectively) (2). Patient follow-up revealed that following surgery, the survival time of females was significantly higher compared with that of males (P=0.024; Fig. 1B), and that the combined 5-year survival rate was 20%, consistent with data the National Cancer Institute (NIH) (5-30%; https://www.cancer.gov/types/esophageal/hp/esophageal-treatment-pdq). There was no significant difference in the survival time of patients with ESCC among different age groups and pathological grades (Fig. 1A and D). However, significant differences were observed in survival times according to different tumor sizes, T stages, N stages and TNM stages of patients with ESCC (Fig. 1C and E-G).

Pathological changes in esophageal carcinoma were evaluated by H&E staining. hTERT staining is exhibited as a brown color (Fig. 2A-C). Ki67 and p53 were also assessed using immunohistochemistry. hTERT expression level of ≤90% was defined as low expression, and >90% was defined as high expression. No significant differences were observed in the proportions of high nuclear hTERT expression between esophageal carcinoma and paracancerous tissues (85 and 84% respectively; Fig. 2A). However, a significant difference was observed in the proportions of high hTERT expression in the cytoplasm between these tissue types (66 and 6.7% respectively; P<0.05; Table II, Fig. 2A). The follow-up revealed no

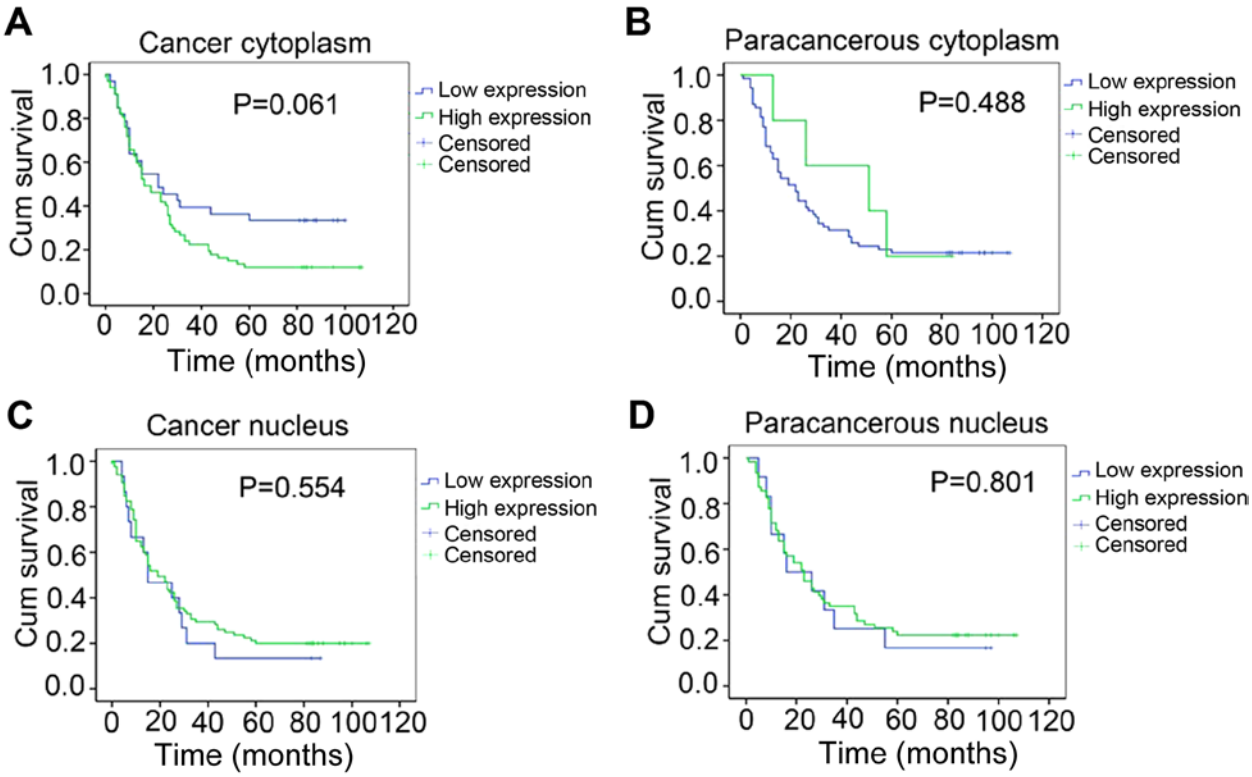

Figure 3. Association between high hTERT expression in cancer cell cytoplasm and poor prognosis in patients with ESCC. (A) Survival curves according to hTERT expression in the cytoplasm of (A) ESCC and (B) paracancerous cells. (C) Survival curves according to hTERT expression in the nucleus of (C) ESCC and (D) paracancerous cells. Exp, expression; hTERT, telomerase reverse transcriptase; ESCC, esophageal squamous carcinoma; Cum, cumulative.

Table II. hTERT expression in 100 esophageal squamous carcinoma tissues and 75 paracancerous tissues.

	Expression, n (%)		
Tissue type	High	Low	P-value
Cancer tissues	67 (67)	33 (33)	<0.0001
Paracancerous tissues	5 (6.7)	70 (93.3)	

hTERT expression level of ≤90% was defined as low expression, and >90% was defined as high expression. hTERT, telomerase reverse transcriptase.

Table III. Correlation of the hTERT-positive rate in the cytoplasm of esophageal squamous carcinoma cells with clinical parameters.

Clinical parameter	N	ρ	P-value
Age	100	0.051	0.612
Sex	100	-0.056	0.577
Tumor size	85	0.014	0.896
Pathological grade	100	0.243[a]	0.015
N stage	98	0.290[b]	0.004
TNM stage	96	0.298[b]	0.003

[a]P<0.05, [b]P<0.01.

significant differences between the survival times of patients in different hTERT expression groups (Fig. 3B-D). However, the P-value between the survival time of patients with different cytoplasmic hTERT expression levels was 0.061, which is close to 0.05, indicting a potential association between hTERT expression and poor prognosis in patients with ESCC (Fig. 3A).

Considering the increased hTERT expression in the cytoplasm of cancer tissue cells, the association between positive cytoplasmic hTERT expression and clinical data are presented in Table III. The hTERT-positive rate in the cytoplasm of esophageal carcinoma cells was significantly correlated with pathological grade (r=0.243, P=0.015), N stage (r=0.290, P=0.004) and TNM stage (r=0.298, P=0.003), but not with age, sex or tumor size.

hTERT knockdown does not significantly inhibit Kyse410 or Kyse520 cell proliferation. To investigate the mechanism by which high hTERT expression correlates with the poor prognosis of patients with esophageal carcinoma, hTERT knockdown was performed in Kyse410 and Kyse520 esophageal carcinoma cell lines using RNAi methods. Western blotting was used to confirm successful knockdown in both cell lines (Fig. 4A and B). The effect of hTERT knockdown on proliferation was analyzed using MTT assays (Fig. 4C and D). At 48 and 72 h, the proliferation of hTERT-knockdown Kyse410 and Kyse520 cells was slightly lower compared with that of the control cells, however no significant differences were observed. The results of the CCK-8 assays (Fig. 4E and F) were consistent with those of the MTT assays.

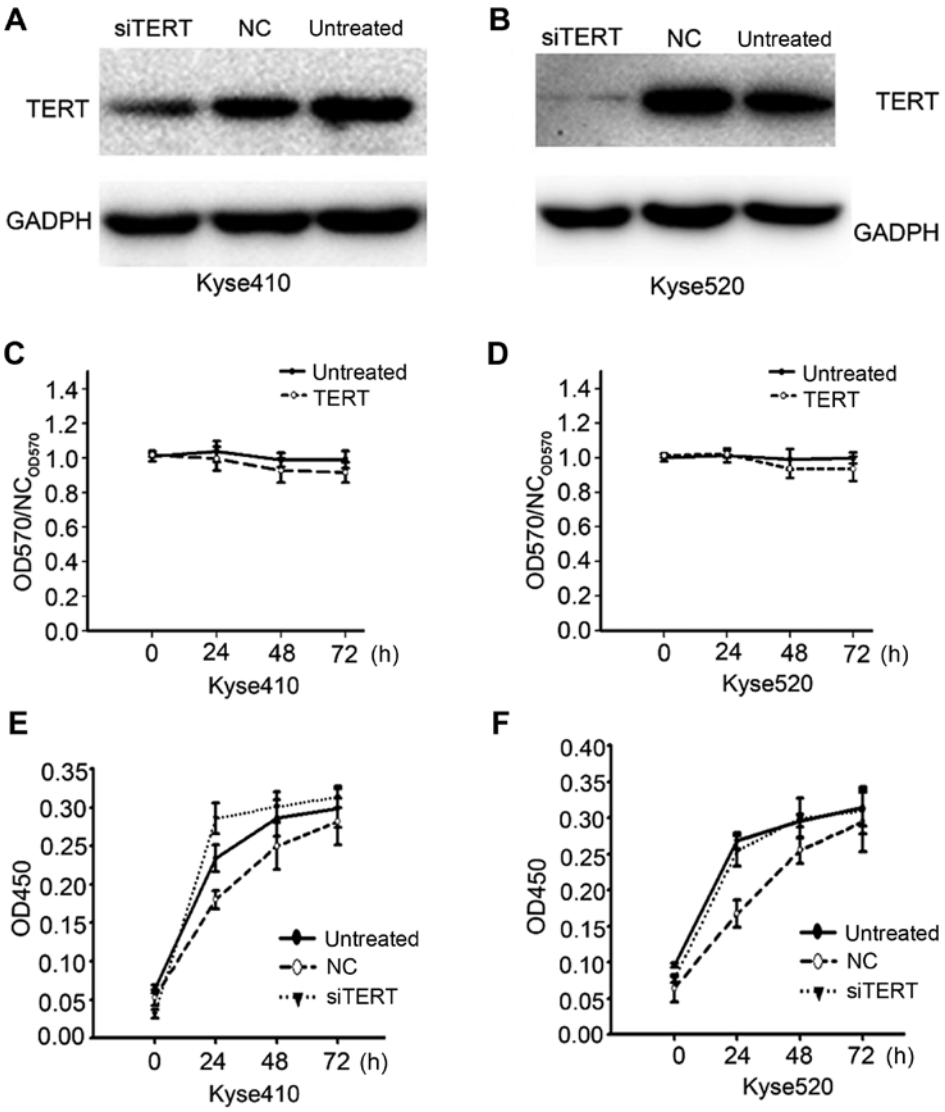

Figure 4. hTERT knockdown does not inhibit Kyse410 or Kyse520 cell proliferation. (A) Kyse410 and (B) Kyse520 cells were transfected with hTERT-specific siRNAs and the expression levels were assessed by western blotting. Proliferative ability of (C) Kyse410 and (D) Kyse520 cells was evaluated using an MTT assay (n=6 each). Results were characterized as a change in absorbance at 570 nm. Proliferative ability of (E) Kyse410 and (F) Kyse520 cells was also evaluated using the Cell Counting Kit-8 assay (n=5 each), and the results were characterized as a change in absorbance at 450 nm. hTERT, telomerase reverse transcriptase; si, small interfering (RNA); NC, negative control; OD, optical density.

hTERT knockdown inhibits Kyse410 cell colony formation. To investigate whether hTERT expression influences the colony-formation ability of esophageal carcinoma cells, hTERT expression was knocked down in Kyse410 and Kyse520 cells using RNAi. Colony-formation ability was decreased by ~80% following hTERT knockdown in Kyse410 cells (Fig. 5A, upper panel), but no significant decrease was observed in Kyse520 cells (Fig. 5A, lower panel).

hTERT knockdown inhibits Kyse410 and Kyse520 cell invasion and migration. To confirm the effect of hTERT knockdown on the migration and invasion abilities of Kyse410 and Kyse520 cells, Transwell assays were used to assess invasion, and wound-healing assays were used to assess migration. Cellular invasion was measured after transfection for 48 h. The number of invading cells from three random fields was determined using light microscopy. As shown in Fig. 5B, the invasion ability of Kyse410 cells (upper panel) was higher compared with that of Kyse520 cells (lower panel), and hTERT knockdown significantly decreased the invasiveness of both cell types ($^{*}P<0.05$). Similar results were observed for the wound healing assay. The data revealed that the migration ability of Kyse410 cells was higher than that of Kyse520 cells (Fig. 6A and C, left panel), and that hTERT knockdown significantly inhibited the migration of both cell types (Fig. 6B and D; $P<0.05$).

Discussion

Abnormalities in hTERT are considered to be associated with the tumorigenesis of 85% of all cancer types tested (45), and hTERT has been found to be overexpressed in a number of different cancer types, such as cervical cancer and gastric cancer (35,46,47). hTERT plays critical roles in tumorigenesis by preventing apoptosis, and enhancing motility and invasiveness (48,49). RNAi is a gene-silencing technology that was developed by Fire *et al* (50). Previous studies have

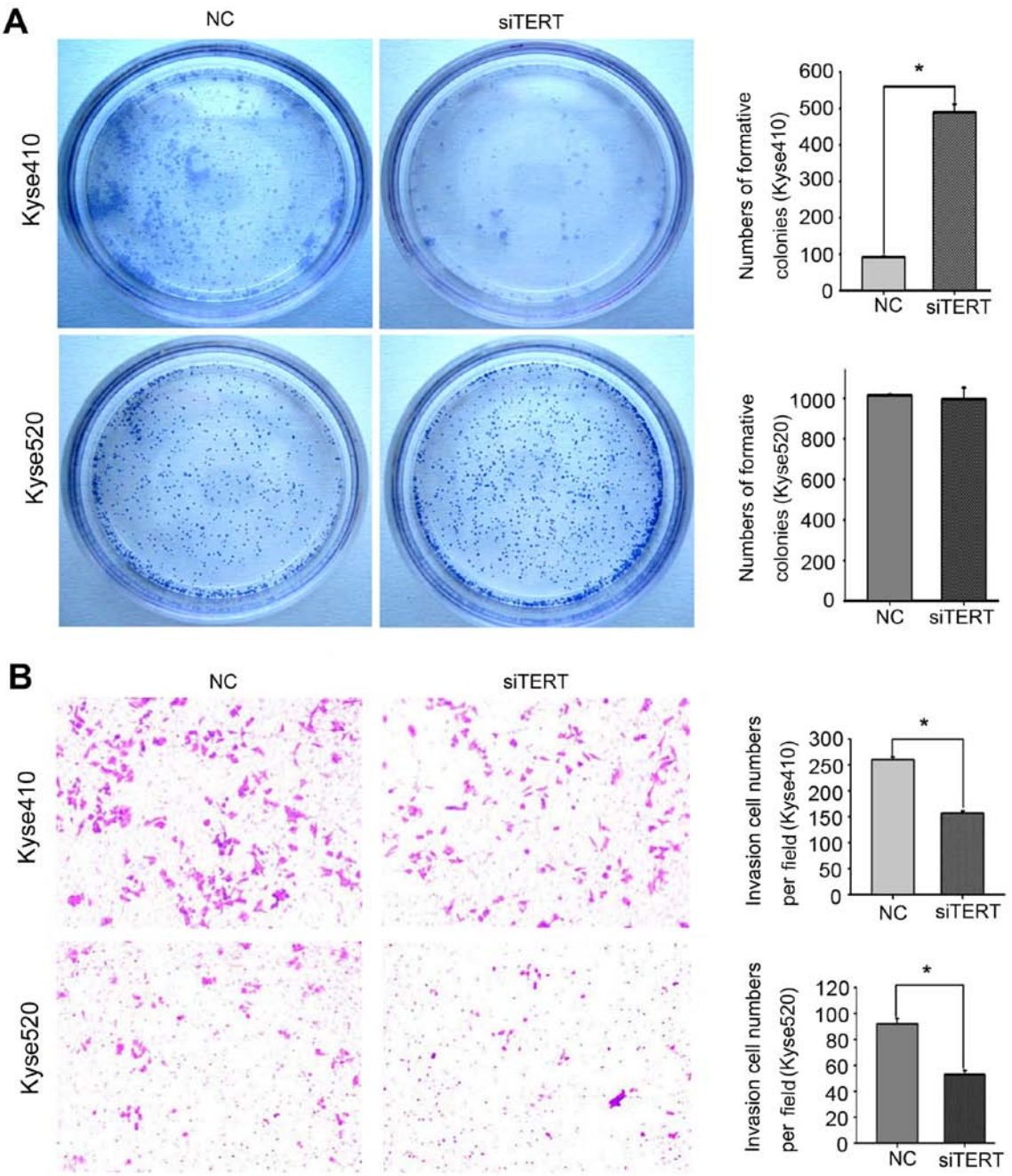

Figure 5. hTERT knockdown suppresses Kyse410 cell colony formation and inhibits Kyse410 and Kyse520 cell invasiveness. (A) Colony formation ability of Kyse410 (upper panel) and Kyse520 cells (lower panel) was analyzed using clonogenic assays (n=4 each). (B) Invasive ability of Kyse410 (upper panel) and Kyse520 cells (lower panel) was evaluated using Transwell assays 48 h post-transfection, and the number of cells from three random fields of the membrane was counted using light microscopy (n=3 each) *P<0.05. hTERT, telomerase reverse transcriptase; si, small interfering (RNA); NC, negative control.

reported that hTERT knockdown inhibits cellular proliferation and induces apoptosis in numerous types of cancer cells, for example anaplastic thyroid cancer and osteosarcoma cells (51,52). RNAi-induced silencing of hTERT is considered to be a promising strategy for cancer gene therapy by inhibiting tumorigenesis and progression, and the results of the present study provide insights into the development of novel therapeutic approaches for esophageal cancer.

In the present study, H&E staining was used to evaluate alterations in ESCC tissues compared with adjacent normal tissues. p53 is expressed at low levels in most normal fetal and adult tissues, and also has a short half-life. Since p53 and ki67 are reportedly highly expressed in esophageal cancer tissues (53,54), their expression was evaluated in pathological sections in the present study to further confirm this observation. High hTERT expression was observed in the cytoplasm of ESCC tissues, indicating that esophageal cell carcinogenesis is accompanied by an increase in hTERT synthesis in the cytoplasm. Following synthesis, hTERT is trafficked to the nucleus, where it is then assembled and activated. The biogenesis, trafficking, recruitment and activation of hTERT affects the development of ESCC in a complex manner (55). Therefore, further studies are required to assess the progress of nuclear-cytoplasmic hTERT trafficking for

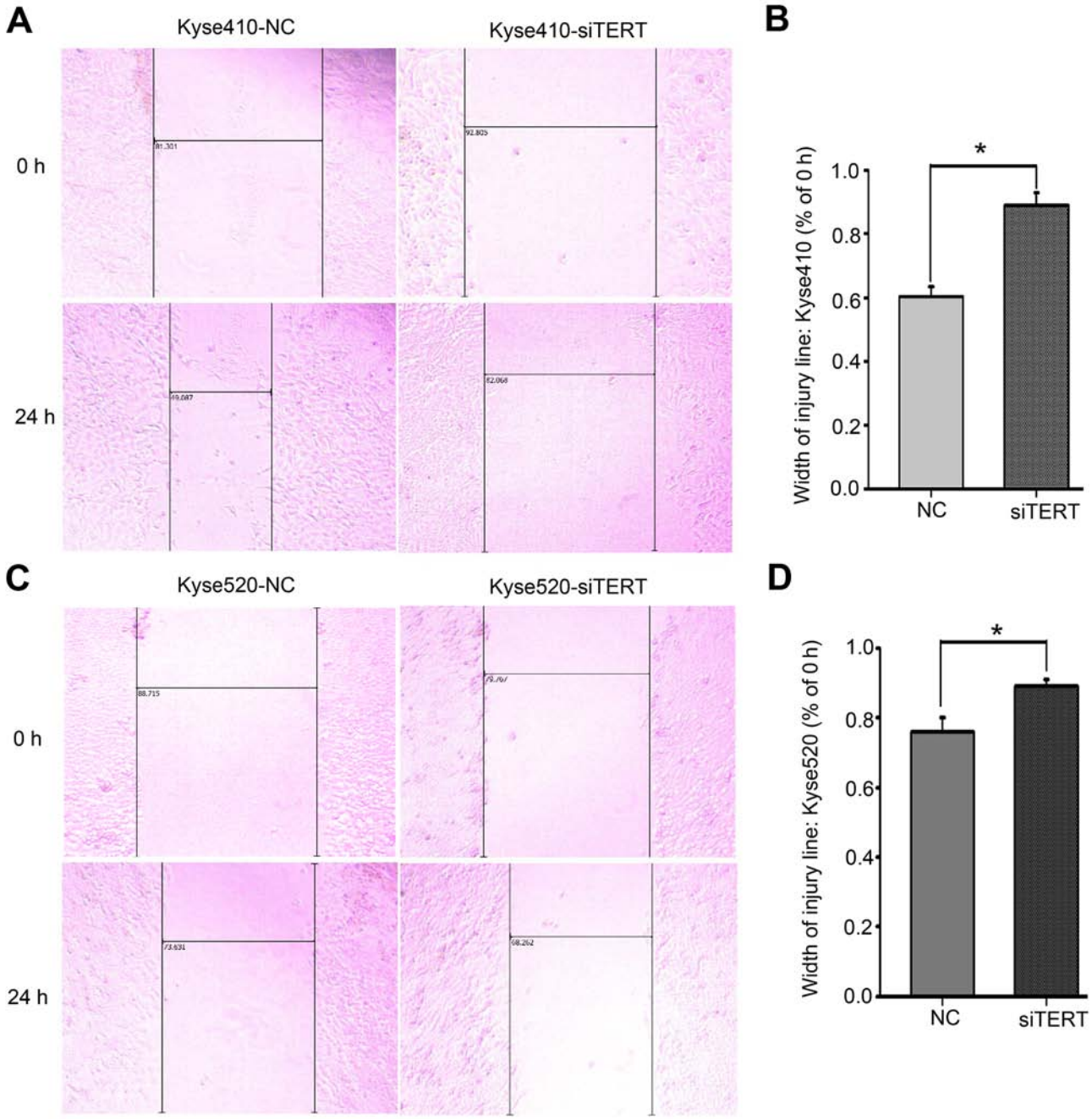

Figure 6. hTERT knockdown inhibits Kyse410 and Kyse520 cell migration. (A and B) Migration abilities of Kyse410 and (C and D) Kyse520 cells were evaluated using wound healing assays 24 h post-transfection, and images were captured at 0- and 24-h time points using a phase contrast microscope (magnification, x40). (n=3 each). *P<0.05. hTERT, telomerase reverse transcriptase; si, small interfering (RNA); NC, negative control.

telomerase maturation and activity in esophageal cell carcinogenesis (55).

Next, the correlation between hTERT expression and the clinicopathological data of patients with ESCC was analyzed; hTERT expression was found to be significantly correlated with pathological grade, T stage, N stage and TNM stage. Survival curve analysis also revealed significant differences in the survival rates of postoperative patients at different T, N and TNM stages. It was therefore speculated that a high expression level of hTERT in the cytoplasm of ESCC cells is associated with poor patient prognosis.

A preliminary study on the effects of hTERT on the occurrence and development of ESCC was subsequently conducted. hTERT was knocked down in Kyse410 and Kyse520 cells using RNAi methods, which was found to inhibit the proliferation of both cell types at the 72-h timepoint, although no significant difference was observed; this was consistent with a previous study in which imetelstat was found to block hTERT activity and consequently inhibit Kyse410 and Kyse520 cell proliferation after 3-4 weeks (1). A possible reason for this increased onset period is that cellular proliferation within a few days does not cause a great degree of telomere shortening. It was speculated that cell proliferation would not be affected until the telomeres were shortened below a specific threshold, thus hTERT knockdown did not immediately cause a significant inhibition in proliferation. The effects of hTERT knockdown

on migration and invasion were then evaluated using cell lines with the longest telomere length (Kyse410,6.32Kb) and the shortest telomere length (Kyse520, 3.50Kb) among several esophageal cancer cell lines (56). Notably, the results demonstrated that the migration and invasion abilities of Kyse410 cells were stronger compared with those of Kyse520 cells, thus Kyse410 appears to be the more aggressive ESCC cell line.

Matrix metalloproteinases (MMPs) represent a major group of extracellular matrix regulatory proteins, which serve an important role in tumor invasion and metastasis (57). A previous study demonstrated that hTERT regulates MMP expression in U2OS cells independently of telomerase activity (24). hTERT knockdown also significantly inhibits the colony-formation ability of Kyse410 cells, confirming its effects on the tumor-formation ability of these cells. In addition, hTERT promotor hypermethylation was observed in esophageal adenocarcinoma and its precancerous lesions in a number of different studies (58,59). According to the aforementioned observations, hTERT may regulate esophageal cancer by targeting numerous different molecular pathways. In the present study, hTERT was found to be associated with ESCC cell migration, invasion and colony formation, but the associated mechanisms remain to be elucidated.

In conclusion, the present study determined the association between high hTERT expression in the cytoplasm and the poor prognosis of patients with ESCC. *In vitro* experiments confirmed that hTERT knockdown suppresses the migration and invasion abilities of ESCC cells, and suppresses the colony-formation ability of Kyse410 cells. The previous study reported that Wnt5a promotes ESCC cell invasion via retinoic acid-related orphan receptors (ROR)1 and ROR2 and dishevelled-associated activator of morphogenesis 1/RhoA signaling pathway (60), thus the potential association between hTERT, Wnt5a and ROR needs to be further studied.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW designed the study. JL, JS and GT performed the experiments. JL and GD analyzed and interpreted the data. JL, XW and GD drafted and edited the manuscript. JL, GD and XW revised the manuscript critically and approved the final of version of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of the clinical specimens for research purposes was approved by the Institutional Research Ethics Committee, The Second Hospital of Nanjing (approval no. 2018-LY-KY068).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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