Undifferentiated embryonic cell transcription factor-1 (UTF1) inhibits the growth of cervical cancer cells by transactivating p27^Kip1

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Undifferentiated embryonic cell transcription factor-1 (UTF1) is an important transcription factor during development, which plays critical roles in cell fate determination. However, its expression and function in somatic tissues remain unclear. Here, we investigated the expression pattern of UTF1 in the human normal and cancerous lesions of cervix and found that UTF1 was downregulated in cervical carcinogenesis, which was related to the hypermethylation of UTF1 promoter. Exogenous expression of UTF1 resulted in the significant inhibition of cell proliferation in vitro and tumorigenesis in vivo through attenuating cell cycle arrest via increasing the level of p27^Kip1. Luciferase reporter assay indicated that the region containing an intact activating transcription factor site between nucleotides −517 and −388 of the p27^Kip1 promoter was indispensable for its activation by UTF1. Chromatin immunoprecipitation analysis confirmed the physical interaction between UTF1 and the p27^Kip1 promoter. Taken together, our findings reveal that UTF1 attenuates cell proliferation and is inactivated in cervical carcinogenesis through epigenetic modification, which strongly supports that UTF1 is a potential tumor suppressor.

Introduction

Worldwide, cervical cancer (CC) is the fourth most common cancer in females (1). Persistent infection with high-risk human papillomavirus (HPV) types (16 or 18) is regarded as the primary risk factor involved in CC (2), followed by other risk factors such as smoking, immunosuppression (3) and many sexual partners (4). However, other genetic and molecular factors that contribute to the initiation and progression of CC have been poorly elucidated (5). Identification of crucial gene alterations in CC will provide a conceptual framework to guide future analyses in the early detection and effective treatment. In recent years, the relationship between carcinogenesis and abnormal expression of stem cell–related transcription factors, such as Sox2, Stat3, NANOG and GATA-3, has been revealed in CCs (6–9).

Undifferentiated embryonic cell transcription factor-1 (UTF1), a transcriptional co-activator, was first isolated and identified from mouse F9 embryonic carcinoma cells (10). The human UTF1 gene is located on chromosome 10q26 and consists of two exons and one short intron (11). Both the 5′ promoter and 3′ enhancer elements are essential for UTF1 expression. The 5′ TATA-less promoter consists of four GC boxes, whereas the 3′ enhancer element harbors two octamer sequences (ACTAGCATAACATG), which bind the Oct4/Sox2 complex, and an M1 octamer sequence (GTCTGGGT), which is conserved in the NANOG promoter (12,13). In addition, UTF1 is a stably chromatin-associated transcriptional repressor with histone-like properties and potentially plays a role in the maintenance of a specific epigenetic profile that allows for lineage-specific differentiation of embryonic stem, but it is not involved in stem cell renewal (14,15).

UTF1 functions as a co-activator of activating transcription factor-2 (ATF-2) and boosts its transcription by transmitting the signaling to basal transcriptional machinery (11,16). It is highly expressed in the cells of the inner cell mass and epiblast during early embryonic development and is maintained in the primordial germ cells of the developing embryo and in the gonads of the adults while rapidly downregulated upon the initiation of differentiation (17,18). The genetic selection system based on a human UTF1 promoter/enhancer-driven neomycin resistance transgene led to virtually absolute pluripotent culture (19). Zhao et al. (18) reported that the expression of UTF1 and p53 siRNAs with c-Myc, KLF4, Oct4 and Sox2 significantly increased the efficiency of induced pluripotent stem cells generation by 200-fold. Therefore, it indicates that UTF1 has important roles in development.

Increasing evidence has revealed that UTF1 is highly expressed in the human epididymis, prostate epithelia (20), endometriosis samples (21) and germ cell tumor, and is barely detectable in non-germ cell tumors (22,23). Guenin et al. (24) recently reported the expression of UTF1 during cervical carcinogenesis. Hence, this study aimed to explore the possible role and potential molecular mechanisms of human UTF1 as a stem cell–related transcription factor in the development and progression of cervical carcinomas.

Materials and methods

Tissue samples

The human CC tissue microarray (n = 50) was purchased from Chaoying Biotechnology Company (Xi’an, China). The specimens (n = 67) were obtained from patients at the First Affiliated Hospital of Xi’an Jiaotong University Medical College from 2004 to 2012. Of the 67 patients, 30 were normal cervical epithelium (NC), 7 were low-grade squamous intraepithelial lesions (LSIL) and 30 were high-grade squamous intraepithelial lesions (HSIL). None of the patients had received chemotherapy, immunotherapy or radiotherapy prior to specimen collection for tissue microarray and the primary specimens. Histopathologic classification was based on the two-tier Bethesda system. The procedures followed medical ethics approval practices, and the patients had provided their informed consents prior to specimen collection. All specimens obtained for routine pathological studies were fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin.

Cell culture and 5-aza-2′-deoxycytidine treatment

Human CC cell lines, HeLa, SiHa, CaSiKi and C33-A, and the human embryonic stem cell, Tera-1 cells were purchased from American Type Culture Collection and cultured according to their specifications at 37°C in a humidified 5% CO2 incubator. CC cells were treated with 5-aza-2′-deoxycytidine (5-azaC; 5 μM; Sigma-Aldrich, St. Louis, MO) for up to 72 h. Fresh medium containing 5-azaC was added every 24 h.

Plasmid construction and transfection

The full-length human UTF1 complementary DNA was amplified from Tera-1 cells via PCR using the following primers: UTF1-F (GTTGAATTCGGAAGATCCTGTCGGCCCGC) and UTF1-R (GTGGATCCATCTACGGACGGGTTCCCTGAG). The product was cleaved with EcoRI and BamHI (TaKaRa, Tokyo, Japan) and ligated into pIRE2- AcGFP1 to generate the pIRE2-AcGFP-UTF1 vector. All constructs were verified by sequencing. The p27^Kip1 promoter reporter constructs (both the full-length construct and a series of 5′ deletion mutants) were originally obtained from Dr Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan) (25).

Transfection was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, and stable clones from HeLa
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and SiHa cells were selected with 0.8 and 1 mg/ml G418 (Calbiochem, La Jolla, CA), respectively.

Immunohistochemistry and immunocytochemistry

Formalin-fixed, paraffin-embedded tissue sections were mounted on positively-charged microscope slides. Tissue sections were then placed in a 60°C incubator overnight. After being dewaxed and rehydrated, the sections were incubated in 10 mM sodium citrate buffer (pH 6.0) in a pressure cooker for 2 min. Endogenous peroxidase was blocked with 3% H2O2 in methanol for 10 min at room temperature. The sections were incubated in a humid chamber with the primary antibodies UTF1 (1:200, MAB4337; Chemicon International, Temecula, CA), Ki-67-MIB-1 (1:100, sc-23900; Santa Cruz Biotechnology, Santa Cruz, CA) and p27Kip1 with the secondary antibodies (Vector Laboratories, Burlingam, CA) and development of 3,3'-diaminobenzidine. For negative controls, the primary antibody was replaced with phosphate-buffered saline (PBS).

Immunocytochemistry was performed as described above. Briefly, cells were cultured on autoclaved cover slips, fixed in 4% paraformaldehyde, washed, blocked and incubated with the primary antibodies listed above.

All slides were examined under Olympus-CX31 microscope (Olympus, Tokyo, Japan), and two investigators scored the results in five randomly selected fields at ×100 magnification. The expression of immunohistochromical parameters was represented by a semi-quantitative immunoreactivity score (IRS) according to the method of Remmele and Stegner (26). Briefly, the IRS (negative 1–4, weak 5–6, strong 7–12) was evaluated by multiplying the values for staining intensity (scored as 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) and the values for the percentage of positive cells (scored as 1, 0–25%; 2, 26–50%; 3, 51–75%; 4, 76–100%) in each sample. Stromal cells were used as negative controls.

Western blot analysis

Cells were lysed for 30 min on ice in lysis buffer. Lysates were collected by centrifugation. Protein concentrations were measured using the Bradford assay, and equal amounts of protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto activated polyvinylidene difluoride membranes (Millipore, Billerica, MA). Then, blots were incubated with primary antibodies UTF1 (1:500, MAB4337; Chemicon International) and p27Kip1 (1:150, sc-528; Santa Cruz Biotechnology), respectively, followed with incubation of biotinylated secondary antibodies (Vector Laboratories, Burchingam, CA) and development of 3,3'-diaminobenzidine. For negative controls, the primary antibody was replaced with phosphate-buffered saline (PBS).

Bisulfite sequencing

Genomic DNA was extracted using the Universal Genomic DNA Extraction Kit ver.3.0 (TaKaRa) as recommended by the manufacturer. For validation of shore regions, 1 μg of genomic DNA of each sample was bisulfite-treated using an EpiTect kit (Qiagen, Tokyo, Japan) according to the manufacturer’s specifications. For bisulfite sequencing, converted DNA was amplified using primers: forward 5′-GGGGGTAGTATTGGGTTG-3′ and reverse 5′-ATCTCTAAAAATTTACTTTTAAACC-3′. Amplified products were subcloned using the pEASY-T1 Cloning Kit (TransGen Biotech, Beijing, China). At least 10 positive inserted clones were selected for sequencing (Invitrogen). Methylation density was quantified by BiQ Analyzer software.

Reverse transcriptional PCR and quantitative real-time PCR

Total RNA was extracted from cultured cells with TRIzol reagent (Invitrogen) and reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit (#K1621; Fermentas, Burlington, Ontario, Canada). PCR was performed using Taq PCR MasterMix (TianGen, Beijing, China) and the following primers: UTF1 (ATGGGCGTGTCGGCCGAACG and GGGGGGCGTCGCAACCTGC); HPV16E6 (AAATGTTTCAGGACCCTACGG and TCAGGACAATGCCGCTCCTCTG); p57 (GCCGCGTGTACAAAGCATGCT and ATGCCGCGCTGATTCTCA); Cdk2 (GCTAGCAGCTTTGGACTGACCG and AGTCTGGTACAGGTCGA); Cdk4 (ATGTTTCCGCCGTAGAAG and CACCAAGGTATACCTGACCTTGC) and cyclin D1 (AAACAGATCAGCGAACAAC and GTTGGGGCTCCTGACCTGTC). The primers for β-actin and ubiquitin C were adapted from a previous study (27).

Cell proliferation assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and colony formation assay

Cells were plated in six-well plates at a density of 2 × 104 cells per well in media supplemented with 10% fetal bovine serum. Cells were dissociated with 0.25% trypsin to a single cell suspension and manually counted with a hemocytometer on days 0, 3 and 7. Cell numbers were measured from three independent experiments for multiple cell lines.

As to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cells (1000 cells per well) were seeded in 96-well plates (each condition had six parallel samples). Cells were assessed every other day (7 days total) using the MTT (Sigma–Aldrich) dye according to standard protocols. Briefly, 0.5 ml of MTT (5 mg/ml) was added to each well and incubated for 4 h at 37°C followed by 150 μl of dimethyl sulfoxide solubilizing the amount of live cells was determined by the absorbance at 490 nm (Bio-Rad). Each experiment was performed in triplicate.

Cells (200 cells per well) were seeded in 10 cm dishes and cultured with Dulbecco’s modified Eagle’s medium (containing 10% fetal bovine serum) for 3–4 weeks. When most colonies were large enough to be visualized, the plates were washed twice with PBS, fixed in methanol, stained with Giemsa (Sigma–Aldrich) and counted. Clones with more than 50 cells were counted as positive colonies. Each experiment was performed in triplicate.

Flow cytometry analysis

For cell cycle analysis, 1 × 106 cells were collected and fixed in 70% ice-cold ethanol at 4°C overnight. Before staining, all samples were washed twice with PBS, treated with 1 mg/ml ribonuclease A and stained with 20 μg/ml propidium iodide (Sigma–Aldrich), then analyzed by flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ). For apoptosis analysis, cells were harvested and stained with a fluorescent Annexin V (Sigma–Aldrich) according to the manufacturer’s instructions. The percent of apoptotic cells was referrred as the apoptosis index. Each experiment was performed in triplicate.

Luciferase reporter assay

Briefly, plasmids containing firefly luciferase reporters were co-transfected into tumor cells in triplicate using Lipofectamine 2000 (Invitrogen). pMiniTK-Rluc was used as an internal control. After 48 h, the cell monolayers were washed with PBS, harvested by scraping and resuspended in passive lysis buffer. The luciferase activity was measured in a luminometer (Promega, Madison, WI), and transduction efficiency was normalized to the paired Renilla luciferase activity using the Dual Luciferase Reporter Assay system (Promega) according to the manufacturer’s instructions. The specific promoter activity was expressed as the fold change of the experimental group versus the control group.

Quantitative chromatin immunoprecipitation assay

HeLa and SiHa cells were subjected to chromatin immunoprecipitation (ChIP) using the EZ-ChIP™ Assay Kit (Cat#178–371; Millipore). Briefly, cells were treated with 0.8% formaldehyde, which was terminated with 0.125M glycine. Cells were then lysed and sonicated to produce 0.2–1 kb DNA fragments. Chromatin protein complexes were immunoprecipitated with 5 g of anti-UTF1 antibody (sc-130911; Santa Cruz Biotechnology).

The negative control was immunoprecipitated with normal mouse IgG, and input DNA was precipitated without antibody. Real-time PCR was performed to amplify the region between nucleotides −517 and −388 of the p27Kip1 promoter, containing the ATF site, with the following primer pair (designated as P1): P1F, 5′-GGATCTCCCTCTGTTTTA-3′ and P1R, 5′-GAGGCGGAGAAAACAACCCCGA-3′. The 3′ untranslated region of the p27Kip1 gene was amplified as a control for the ChIP assay using the following primer set (designated as P2): P2F, 5′-AAGCTTGGATGATGACATTGC-3′ and P2R, 5′-AGATCACCGATGACTCCACAAATTGATG-3′. The reaction aliquots were amplified using SYBR Premix Ex Taq™ II (TaKaRa) and Bio-Rad IQ5 software v.2.0 analyses according to the manufacturer’s instructions. All experiments had at least biological duplicates and assay triplicates, and results were analyzed via the ΔΔCt method using GAPDH, β-actin and ubiquitin C as the housekeeping genes. Primers used were as follows: GAPDH (GCACGGTGCAAGGCTGAGAC and TGGTGAGAGCGCATGGGA); p27 (CCTGCCCTCCACGTCCTTCCT and CAGAACCTGGTGATTITT); p16 (CATAATGTCGGCGCAGAAGTT and CCCAGGAGTTTTTCAGAGGCCT); p21 (GCAGACCAGATGACAGATTCT and CGGATTAAGGCTTCTCTTG); p57 (GCCGCGTGTACAAAGCATGCT and ATGCCGCGCTGATTCTCA); Cdk2 (GCTAGCAGCTTTGGACTGACCG and AGTCTGGTACAGGTCGA); Cdk4 (ATGTTTCCGCCGTAGAAG and CACCAAGGTATACCTGACCTTGC) and cyclin D1 (AAACAGATCAGCGAACAAC and GTTGGGGCTCCTGACCTGTC). The primers for β-actin and ubiquitin C were adapted from a previous study (27).

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Tumor xenograft assay
The animal experiments were performed in accordance with the institutional guidelines for use of laboratory animals. About 4- to 6-week-old BALB/c athymic nude mice (Jackson Laboratories, Inc., Bar Harbor, ME) were supplied by the Medical College Experimental Animal Center of Xi’an Jiaotong University. To assess the tumorigenicity in vivo, cells were collected and inoculated into nude mice dorsa (5 mice per group). About 1 × 10⁶ cells mixed with 30% Matrigel (BD) in 300 µl total volume were used for inoculation. Mice were monitored weekly for body weight and tumor size for up to 11 weeks after injection. Xenograft tumor volume was calculated using the following formula: (length × width²)/2. The mice were killed and tumors were dissected out and weighed, fixed in 4% paraformaldehyde buffer (pH 7.0) and paraffin-embedded for histological analysis.

Statistical analysis
Statistical analyses were performed using SPSS v13.0 software (SPSS, Chicago, IL). All data were expressed as the group means ± standard deviation of the mean (SD). To test associations between categorical variables, we used the χ² or Fisher’s exact test. Univariate analysis was analyzed by the Student’s t test (two-tailed) and the Mann–Whitney U-test. The correlation of the expression of UTF1 and p27kip1 in CC was analyzed by Pearson’s correlation test. Differences were considered statistically significant at values of P < 0.05.

Results
The expression of UTF1 in NC and cervical cancerous lesions
To investigate the differential expression of UTF1 in NC and different cervical cancerous lesions, we performed immunohistochemistry in the clinic samples. Representative images of UTF1 immunostaining were shown in Figure 1A. The expression of UTF1 was located in nuclear of the parabasal cell layers in NC (Figure 1A, a2) and LSIL (Figure 1A, b2), but not basal layer, which suggested that UTF1 was negatively associated with cell proliferation. Additionally, UTF1 was weakly expressed in tumor parenchyma in HSIL (Figure 1A, c2) and CC (Figure 1A, d2). The average IRS of UTF1 staining was 8.734 ± 0.987 in NC (n = 30), 8.000 ± 1.890 in LSIL (n = 7), 5.267 ± 0.823 in HSIL (n = 30) and 3.220 ± 0.535 in CC (n = 50), respectively (Figure 1B). Each positive percentage of UTF1 staining was 73.3% (22/30) of NC, 71.43% (5/7) of LSIL, 46.7% (14/30) of HSIL and 24% (12/50) of CC (Figure 1C). Further analysis of the relationship of UTF1 and clinicopathologic parameters showed that the expression of UTF1 was significantly correlated with the grade of squamous carcinoma, but none with the age of the patients, FIGO stage or pelvic lymph node invasion (Supplementary Table 1, available at...
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Fig. 2. The methylation status of the UTF1 promoter in cultured and primary CC. (A) Schematic representation of the CpG islands found in the promoter region of the UTF1 genomic locus. Numbers indicated the position relative to the transcriptional start site. The regions interrogated by bisulfite sequencing below were marked in red. (B) UTF1 promoter methylation was measured in human CC cell lines, HeLa, C33-A, SiHa and CaSki. Results were shown by 10 independent clones for each cell line. The methylation included all 18 CpG dinucleotides within the 181 bp amplicon. (C) Western blot analysis of CC cell lines before and after 5-azadC treatment was visualized with anti-UTF1 (top) and β-actin (bottom). (D) Graph of the methylation of UTF1 in normal cervical tissue and CCs were analyzed. The UTF1 promoter was aberrantly hypermethylated in primary tumors.

Fig. 3. Exogenous UTF1 expression inhibits the cell proliferation in vitro. (A) The stable transfected cells were identified by western blot analysis. (B) The growth curves and (C) MTT assay were used to assess proliferation of the indicated cell lines. Data show the mean (SD) from three independent experiments. (D) Colony formation assay and (E) quantification of E demonstrates exogenous UTF1 expression markedly inhibited colony-forming ability (t-test, *P < 0.05). CFU: colony-forming units.
The immunohistochemistry analysis revealed that UTF1 expression was gradually decreased from normal cervical epithelia and LSIL to HSIL and carcinomas. The densitometry analysis of UTF1 expression in clinic samples showed that the average level of UTF1 in NC (n = 8) was higher than that in CC (n = 20; Figure 1D and 1E; \( P < 0.01 \)). These results indicated that UTF1 was downregulated in cervical carcinogenesis and strongly implied that UTF1 acts as a tumor suppressor in the progression of cervical carcinomas.

The methylation status of UTF1 promoter in CC cell lines and primary tumors

We firstly examined the messenger RNA (mRNA) and protein levels of UTF1 in CC cell lines. As shown in Supplementary Figure S1A and B, available at Carcinogenesis Online, with teratoma cell line hTera-1 as positive control, the expression of UTF1 at mRNA and protein levels was detectable in C33-A and SiHa cells, but not in HeLa and CaSki cells, which fit in with GEO DataSet results regarding the UTF1 level in CC cell lines (28). To explain the dramatically reduced level of UTF1 in cervical carcinomas, we supposed that the expression of UTF1 was interfered by promoter epigenetic modification. Based on methyl primer express analysis, we noticed that there were high densities of CpG islands in the proximal promoter of UTF1. For confirmation, we designed a pair of primers to amplify a 181 bp region of the UTF1 promoter that encompassed 18 CpG sites upstream of the transcription start site (Figure 2A) and analyzed their methylation status by bisulfite sequencing from four cell lines (HeLa, C33-A, SiHa and CaSki). As shown in Figure 2B, dense methylation of the UTF1 promoter were detected in HeLa, SiHa and CaSki cell lines, respectively (92.8, 92.2 and 98.9%), whereas only C33-A cell exhibited rather low frequency (2.23%), which have retained UTF1 expression in western blotting. After 5-aza-dC treatment, we found that the abundance of UTF1 protein was pronounced reversed in HeLa, SiHa and CaSki cells, but not in C33-A cells, which implied the expression of UTF1 in cervical cell lines was indeed regulated by methylation modification (Figure 2C). Hence, we concluded that methylation status would be one of the reasons affecting differential expression level of UTF1 in CC cell lines. Moreover, UTF1 methylation status was also detected in five normal cervixes and nine CCs (Figure 2D). UTF1 was found to be highly hypermethylated in most of the analyzed primary tumors (mean 63.21%) compared with that in the normal tissues (mean 4.35%). These findings reinforced that the hypermethylation of the UTF1 promoter and low expression of UTF1 were related to the progression of cervical cancer and UTF1 worked as a tumor suppressor in CC.

**Fig. 4.** Exogenous UTF1 expression inhibits tumorigenesis in vivo. Tumor volume (a1 and c1), weight (a2 and c2) and growth curves (a3 and c3) in BALB/c nude mice were shown. (White arrowhead, control group; black arrowhead, UTF1-expression cells; (A) HeLa group; (C) SiHa group.) Tumor growth was monitored weekly. UTF1-overexpressing group dramatically suppressed tumor growth compared with the controls. The error bar represents the mean tumor volume ± SD from three independent experiments (***P < 0.001). (B and D) Immunohistochemistry of HeLa and SiHa xenografts or cells stained with for UTF1 and Ki-67 (black arrowhead, positive staining of UTF1; red arrowhead, positive of Ki-67). Magnification ×100; scale bars, 50 µm.
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To functionally evaluate the role of UTF1 as a tumor suppressor in CC, we reconstituted UTF1 expression in HeLa and SiHa cell lines (Figure 3A). Cell proliferation and MTT assays demonstrated the significant suppression of cell growth in HeLa-UTF1 and SiHa-UTF1 cell lines compared with controls on days 5 and 7 (Figure 3B and 3C; P < 0.001). Colony formation assay showed that UTF1-overexpressing cell lines had dramatically reduced efficiency of foci formation (HeLa-UTF1, 28% colonies; SiHa-UTF1, 14% colonies) compared with the corresponding clones (HeLa-GFP, 48%; SiHa-GFP, 26% colonies) (Figure 3D and Supplementary Figure S3, available at Carcinogenesis Online, P < 0.001). These data demonstrated that enforced ectopic expression of UTF1 led to the suppression of cancer cell growth in vitro.

To evaluate the effect of UTF1 on tumorigenicity in vivo, we implanted the exogenous UTF1-expressing cells in athymic nude mice (Figure 4A, a1, and C, c1). Tumors with UTF1-expressing groups (HeLa-UTF1 and SiHa-UTF1) formed much slower than those with the controls (HeLa-GFP and SiHa-GFP). The tumor volume from HeLa-UTF1 cells (0.5 ± 0.1 cm³) was much smaller than that from the control HeLa-GFP cells (1.6 ± 0.13 cm³) 10 weeks after transplantation (Figure 4A, a3; P < 0.001). Similarly, after 11 weeks, SiHa-UTF1 cells did not form any tumors, but the control SiHa-GFP cells did (1.3 ± 0.12 cm³) (Figure 4C, c3; P < 0.001). The average weight of xenografts of UTF1-overexpressing groups was lighter than the controls (HeLa-GFP and SiHa-GFP) (Figure 4A, a2, and C, c2; P < 0.001). These data suggested that UTF1 inhibited the tumorigenicity in vivo. In addition, we stained the tumor xenografts with anti-Ki67 and anti-UTF1 antibodies. As shown in Figure 4B and 4D, HeLa-UTF1 xenografts and SiHa-UTF1 cells showed strong positive UTF1 staining but weak Ki-67 staining. In contrast, the HeLa-GFP- and SiHa-GFP-xenografted tumors were negative for UTF1, but positive for Ki-67. Taken together, these results suggested that exogenous expression of UTF1 attenuated the tumor proliferation in vitro and tumorigenesis in vivo.

UTF1 inhibits cell proliferation through attenuating cell cycle

To explore the mechanism by which UTF1 inhibits cell proliferation, we performed cell cycle analysis. As shown in Figure 5A, the proportion of G0/G1 phase in HeLa-UTF1 cells was 58.32% compared with the control group 45.68%, and the proportion of S phase was 27.43% compared with 45.95% correspondingly (Figure 5A; P < 0.05). The ratio of HeLa-UTF1 cells in the G1/S phase (55.32%/27.43%, 2.13) was much higher than that of HeLa-GFP cells (45.68%/45.95%, 0.99), suggesting that UTF1 overexpression induced G1/S phase transition arrest in HeLa cells. The similar effect was observed in SiHa-UTF1 cells with a ratio (G1/S phase) of 2.22 compared with 1.17 in SiHa-GFP cells (Figure 5B; P < 0.05). Meanwhile, there were no obvious hypodiploid (sub-G1) population in the cell cycle analysis (Figure 5A and 5B) as well as statistical changes in the apoptotic analysis (Figure 5C and 5D; P > 0.05), suggesting that UTF1 inhibited cell proliferation through inducing G1/S phase transition arrest rather than inducing apoptosis.

UTF1 transactivates p27Kip1 through binding to the ATF site of the p27Kip1 promoter

To further investigate the potential molecular mechanism of UTF1-induced G1/S arrest, the cell cycle regulatory factors, including p16,
p21, p27, p57, CDK2, CDK4 and cyclin D1, were screened by real-time PCR. The elevated mRNA level of p27Kip1 was demonstrated in UTF1-overexpressing cells (Figure 6A; P < 0.001) and the increased protein level was confirmed by western blotting and xenografts immunostaining (Figure 6B and 6C), which demonstrated that UTF1-overexpression mediated p27Kip1 upregulation. In addition, from a panel of 21 CC samples, the correlation between the expression level of nuclear UTF1 and that of p27Kip1 was confirmed (Figure 6D and 6E; P < 0.001).

To investigate the transactivation mechanism of p27Kip1 by UTF1, luciferase reporter assay was performed with the p27Kip1 promoter constructs and its deletions. As shown in Figure 6F, the sequences between nucleotides −3568 and −462 in the p27Kip1 promoter showed much stronger transcriptional activity than the sequences without these nucleotides in HeLa-UTF1 cells (Figure 6F; f1; P < 0.05). Furthermore, the shortest sequence spanning nucleotides −511 to −462 demonstrated the strongest activity for the induction of p27Kip1 promoter activity, which implied that this region contains the regulatory elements for p27Kip1 transcriptional activation. The similar results were observed in SiHa-transfected cell lines (Figure 6F, f2; P < 0.05). Next, ChIP assay was carried out to identify whether UTF1 could directly interact with p27Kip1 promoter in UTF1-overexpression cells in vivo. Following immunoprecipitation, real-time PCR was performed to amplify the p27Kip1 promoter binding region (between nucleotides −517 and −388) with specific P1 primer for putative UTF1-binding sites (ATF) and 3′ untranslated region control P2 primers. The results showed that signals from HeLa-UTF1 cells were eight times stronger than HeLa-GFP cells with P1 primers, which strongly confirmed that UTF1 could physically bind to p27Kip1 promoter for its transactivation. Taken together, our findings clarified that UTF1 inhibited cell proliferation through directly binding to the promoter of cell cycle regulatory factor p27Kip1 to induce cell cycle arrest.

Discussion

UTF1 is well known as a stem cell–related transcription factor (10). In the past few years, several stem-cell transcription factors (such as Sox2 and KLF4) have been studied in various cancers. Expression of Sox2 was downregulated in gastric carcinogenesis and inhibited cell growth through cell cycle arrest and apoptosis (29, 30), whereas KLF4 acted as a tumor suppressor in bladder cancer (31), pancreatic carcinoma (32) and colorectal cancer (33). However, the study of UTF1 principally concentrated on embryonic stem cells, embryonic carcinoma cells (14, 34), induced pluripotent stem cells (18, 35) and germ cell tumors (23, 36, 37), whereas rarely considered its role in...
human somatic tumors. Forte (21) and Kristensen (20) demonstrated that UTF1 was expressed in human urogenital epithelia and endometriosis. In 2010, Liu et al. (23) reported that UTF1 staining was negative in thymomas and thymic carcinomas. Wang et al. (22) also demonstrated that 12 cases showed weak UTF1 staining among the 323 extra-testicular non-germ cell tumors, including 123 metastatic carcinomas, 11 melanomas, 13 mesotheliomas, 85 sarcomas and 91 hematolymphoid tumors. Our study found that UTF1 expression was downregulated during cervical carcinogenesis by immunohistochemistry and western blotting, suggesting that UTF1 may function as a tumor suppressor in CC. Conversely, Guenin et al. (24) reported that UTF1 expression was upregulated in cervical carcinogenesis. An alternate explanation to this discrepancy may be derived from the number of cases of clinic specimens tested.

As we all know, tumor suppressor genes are defined as genes, which encode proteins that inhibit the formation of tumors (38). Their mutations, deletions or epigenetic changes in the promoters would contribute to cancer development by preventing their inhibitory function (39,40). UTF1 is usually considered to contribute to development of embryonic cells. The mechanism for its silencing during development and carcinogenesis became an attractive direction. Previous studies have reported that promoter hypermethylation contributes to the diminished expression of several tumor suppressors, such as PTEN (41) and E-cadherin (42) in CC. Based on the gene analysis, we found UTF1 silence was subjected to hypermethylation in CC cell lines and primary tumors. Upon 5-azaC treatment, in UTF1 hypermethylation cell lines (HeLa, SiHa and CaSkI), the abundance of UTF1 protein was pronounced reversed, whereas cell proliferation capacity was significantly inhibited, but no change in C33-A (Figure 2 and Supplementary Figure S2B, available at Carcinogenesis Online).

Moreover, a significantly negative correlation between UTF1/b-actin protein density ratios and methylation ratios of UTF1 promoter in corresponding primary specimens was determined by Pearson’s correlation test \( r = -0.9448; \) \( P < 0.0001; \) Supplementary Figure 2A, available at Carcinogenesis Online. Therefore, all findings clearly showed that hypermethylation of UTF1 promoter contributed to the diminished expression. However, Guenin et al. (24) showed different UTF1 expression pattern in CC cell lines, and UTF1 expression was not related to its promoter hypermethylation. The features of human UTF1 gene with unique spliced transcripts and translation product make us exclude the possibility of the confusions of pseudogenes and isoforms. Thus, we speculated that these differences may be result from the different cell lines used and the different UTF1 mRNA versus protein levels. Consequently, our results reinforced the concept that hypermethylation of UTF1 promoter was related to low expression of UTF1 in CC and suggested that UTF1 was a potential tumor suppressor gene. Strikingly, we also noticed that the methylation status of the UTF1 promoter occurred in all HPV-positive cell lines including HeLa (HPV18), SiHa (HPV16) and CaSkI (HPV16), but not in C33-A (HPV-negative) (Supplementary Figure S1C, available at Carcinogenesis Online). It is known that repeatedly persistent HPV infection can induce chromatin genomic instability, and some evidence had revealed the association between the gene promoter methylation and HPV infection (43). Thus, we presumed that there might be a potential link between UTF1 gene silencing and HPV infection status, which need further investigation to confirm this assumption.

To explore how UTF1 functions in cervical carcinogenesis, functional study was performed in UTF1-expressing cell lines. We found that ectopic expression of UTF1 significantly inhibited cell proliferation through cell cycle (G1) arrest and attenuated tumor formation in nude mice. Generally speaking, growth is due to the deviation of canonical cell cycle progression and/or the absence of the apoptosis (44,45). Our results showed that UTF1-overexpressing cells did not undergo apoptosis but transacted p27\(^{kip}\) resulting in G1/S arrest. As the cell cycle inhibitor, p27\(^{kip}\) has been reported to be lost in CC (46,47). We also noticed nuclear UTF1 expression was positively correlated with nuclear and cytoplasmic p27\(^{kip}\) expression in CC (\( r = 0.6835 \)), but both of them were expressed weakly compared with normal tissues. In addition, the luciferase reporter and ChIP assays identified that UTF1 directly transactivated p27\(^{kip}\) through physical interaction with its proximal promoter region between nucleotides \(-511\) and \(-426\). According to present evidences, many tumor suppressors are involved in the regulation of p27\(^{kip}\) expression, such as KLF4 (32), BRCA1 (48) and E-cadherin (49). Therefore, we proposed that UTF1 functions as a tumor suppressor through activating p27\(^{kip}\) signal pathway.

In summary, our studies reveal that the expression of UTF1 is negatively correlated to the progression of CC, and the promoter hypermethylation results in UTF1 silencing in carcinogenesis. Furthermore, we provide the evidence that UTF1 functions as a tumor suppressor through activating p27\(^{kip}\) in CC, which suggests a potential novel strategy for CC therapy in the clinical study.

**Supplementary material**

Supplementary Table 1 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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