Human Telomerase Reverse Transcriptase Is a Promising Target for Cancer Inhibition in Squamous Cell Carcinomas

YOUNG-JIN PARK, EUN-KYOUNG KIM, SOOK MOON, DOO-PYO HONG, JUNG YOON BAE and JIN KIM

Department of Oral Pathology, Oral Cancer Research Institute and Brain Korea PLUS 21 Project, College of Dentistry, Yonsei University, Seoul, Republic of Korea

Abstract. *Background/Aim: The present study aimed to investigate whether the down-regulation of human telomerase reverse transcriptase (hTERT) may induce an anti-invasive effect in oral squamous cell cancer cell lines. Materials and Methods: A genetically-engineered squamous carcinoma cell line overexpressing hTERT in immortalized oral keratinocytes transfected by human papilloma virus (HPV)-16 E6/E7 (IHOK) was used. In vivo tumorigenicity was examined using an orthotopic xenograft model of nude mice. For evaluating anti-invasive activity by knockdown of hTERT expression, transwell invasion assay and real-time polymerase chain reaction (PCR) for matrix metalloproteinases (MMP) were employed. Results: The down-regulation of hTERT expression reduced the invasive activity and MMP expression. This result was re-confirmed in the HSC3 oral squamous carcinoma cell line. Conclusion: Targeting hTERT may lead to novel therapeutic approaches.*

The World Cancer Report published by the International Agency for Research on Cancer and the World Health Organization projects 20 million new cancer patients globally in 2025, compared to 14 million cases in 2012 (1). With cancer populations sharply increasing, the incidence of oral squamous cell carcinoma (OSCC) has been expected to increase as well. Given this trend, the paradigm shift from cancer treatment to cancer prevention is important for overcoming increased cancer death in the future.

The initial step of cancer progression is the immortalization of normal epithelial cells. For this step, telomerase activity is required, which maintains telomere length by adding TTAGGG hexamers and inhibiting cellular senescence, eventually resulting in persistent epithelial proliferation (2, 3). In addition, human telomerase reverse transcriptase (hTERT) has active roles in tumorigenesis by preventing apoptosis (4, 5) and by enhancing motility and invasiveness (6, 7). Thus, the expression and activity of telomerase are indispensable for cancer formation (4, 8). In light of the role of hTERT in carcinogenesis, targeting hTERT can be a promising tool to inhibit cancer initiation and progression.

Among innumerable causative factors of cancer development, about 2 million (16%) new cancer cases in 2008 are attributable to infections (1). The overall incidence of OSCC related to high-risk human papilloma virus (HPV) infection is gradually increasing. In India, the prevalence of HPV-16 infection in OSCC reaches the range of 20-50% (9). High-risk HPV infection has been acknowledged as the main cause of uterine cervical cancer (10-12). However, several key questions with regard to HPV-associated OSCC, such as unclear HPV-related oral precancerous lesions and unproven multi-step progression of infection to cancer, restrict opportunities for developing preventive and therapeutic modalities (13). Although numerous and progressive chromosomal abnormalities occur during the immortalization process of HPV-infected keratinocytes, high-risk HPV infection itself was found not to be tumorigenic in nude mice (14, 15). Hence, proving stepwise carcinogenesis by HPV-16/18 infection from immortalization to invasive carcinoma is required for prevention of and improved survival outcomes for OSCC.

Given the importance of high-risk HPV infection in OSCC, we attempted to investigate whether the down-regulation of hTERT can inhibit cancer progression in HPV-16-infected OSCC. For this study, we established a genetically-manipulated OSCC cell line by overexpression of CDK4 and hTERT in immortalized human oral keratinocytes (IHOK) transfected by HPV-16 E6/E7. This study showed that knockdown of hTERT reduced invasive activity of HPV-16-infected OSCC. In addition, knockdown of hTERT inhibited the invasive activity of HPV-non-related OSCC cells, suggesting that targeting hTERT can be a novel therapeutic approach for cancer treatment.

Correspondence to: Professor Jin Kim, DDS, Ph.D., Oral Cancer Research Institute, Department of Oral Pathology, College of Dentistry, Yonsei University, Seoul 120-749, South Korea. Tel: +82 222283031, Fax: +82 23922959, e-mail: jink@yuhs.ac

Key Words: Human telomerase reverse transcriptase (hTERT), immortal oral keratinocytes, multi-step process, squamous cell carcinoma.

Materials and Methods

Cell culture. IHOK cells were established by transfecting normal human gingival epithelial cells with the pLXSN vector containing the *E6*/*E7* open reading frame of HPV-16 as previously described (16). IHOK/CDK4/hTERT cells (Figure 1A) stably expressing CDK4 and hTERT have also been previously described (17). In brief, the coding region of human *CDK4* was amplified by polymerase chain reaction (PCR) using human umbilical vascular epithelial cells and ligated to plpc-*hTERT* vector (Clontech, Palo Alto, CA, USA). GP2-293 packaging cells were transiently transfected with the plpc-*CDK4-hTERT* and pVSV-G vectors (Clontech) to produce retrovirus particles, which were subsequently used to infect IHOK cells. All immortalized cell lines were maintained in keratinocyte growth media (KGM; Lonza, Walkersville, MD, USA) with supplementary bullet kit (Lonza).

HSC3 OSCC cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were maintained with a mixture of Dulbecco's Modified Eagles Medium (DMEM; Gibco BRL, Grand Island, NY, USA) and Ham's Nutrient Mixture F12 (Gibco BRL) at a 3:1 ratio supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 0.01 μg/ml cholera toxin, 0.04 μg/ml hydrocortisone, 0.5 μg/ml insulin, 0.5 μg/ml apo-transferrin and 0.2 μg/ml triiodotyronine (all purchased from Sigma, St. Louis, MO, USA).

Mouse orthotopic xenograft model. Animal studies were approved by the animal ethics committee at Yonsei University College of Dentistry. BALB/c nu/nu male mice (16±2 g, 4 weeks of age) were purchased from the Shizuoka Laboratory Center Inc, Shizuoka, Japan. To search for *in vivo* tumorigenicity of IHOK/CDK4/hTERT cells, cells (1×10^6) were injected into the dorsal tongue of 10 mice. The mice were then sacrificed after 8 weeks. The tongues of the mice were fixed in 10% neutral formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for morphologic analysis.

Telomerase repeat amplification protocol (TRAP) assay. The TRAP assay was analyzed using a TRAPeze kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. Telomerase activity was assayed from 0.5 μg and 1.0 μg of cell extract including Hela cell extract as a telomerase-positive control. One microgram of cell extracts derived from all samples were heat-treated at 85˚C for 10 min and used as negative controls for inactivation of telomerase. Template suppression reagent (TSR) DNA template was used as a PCR-positive control. Telomerase derived from all samples was allowed to add telomeric repeats onto a 3' end of a biotinylated substrate oligonucleotide. The products were amplified using PCR. The PCR products were immobilized onto streptavidin-coated 96-well plates *via* biotin-streptavidin interaction and then detected by anti-dinitrophenol antibody conjugated to horseradish peroxidase. The amount of colored products was measured at 450 and 690 nm and telomerase activity was measured using the equation A450 – A690.

PCR, Reverse Transcription-PCR (RT-PCR) and real-time PCR. Cellular DNA was extracted from each cell using the QIAamp DNA minikit (Qiagen, Hilden, Germany). Total cellular RNA was extracted from each cell using a RNeasy plus mini kit (Qiagen) and the complementary DNA product was synthesized by using the Transcriptor First strand cDNA Synthesis kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The following primers for PCR were used. HPV-16 *E6*; Forward (F): 5'-ATGTTTCAGGACCCGCAGGAGCGA-3', Reverse (R): 5'-TTACAGCTGGGTTTCTCTACGTG-3', HPV-16 *E7*; (F): 5'-TGTTAGATTTGCAACCAGAGACA-3', Reverse (R): 5'-TTATGGTTTCTGAGAACAGATGGG -3'. The following primers for RT-PCR and real-time PCR were used. *CDK4*; Forward (F): 5'-CCTGGCCAGAATCTACAGCTA-3', Reverse (R): 5'- ACATCTCGAGGCCAGTCATC-3'. *hTERT*; F: 5'-CGGAAGAGTG TCTGGAGCAA-3', R: 5'-GGATGAAGCGGAGTCTGGA-3'. Matrix-metalloproteinase (*MMP*)-2; F: 5'-GCGACAAGAAGTA TGGCTTC-3', R: 5'-TGCCAAGGTCAATGTCAGGA-3'. *MMP-9*; F: 5'-CGCGAGCATCGTCATCCAGT-3', R: 5'-GGATTGGCCT TGGAAGATGA-3'. *GAPDH*; F: 5'-GAAGGTGAAGGTCGG AGT-3', R: 5'- GAAGATGGTGATGGGATTTC-3'. DNA or cDNA was amplified by using the Accu Power Hot Start PCR Pre Mix (Bioneer, Daejeon, South Korea) with conditions of 30 cycles of 30 s at 94˚C, 30 s at 60˚C, and 40 s at 72˚C. The amplified products were then separated on 1.0% agarose gel stained with 0.1 μg/ml of ethidium bromide and photographed under UV light (Bio-Rad, Hercules, CA, USA). Real-time PCR was carried-out using the SYBR Green I Master (Roche Applied Science) and normalized to *GAPDH*. The LightCycler 480 Software (Roche Applied Science) was used for data analysis.

Western blotting. Cells were lysed using a cell lysis buffer (Cell Signaling, Beverly, MA, USA). Twenty-five micrograms of protein were separated to 10-15% SDS-polyacrylamide gel (PAGE) (Bio-Rad). The separated proteins were then transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were probed with antibodies specific for CDK4 (1:1000, Cell Signaling) and TERT (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The respective anti-mouse or anti-rabbit secondary antibodies (1:2000, Cell Signaling Technology) were conjugated with horseradish peroxidase and visualized by chemiluminescence (Santa Cruz Biotechnology).

siRNA transfection. CDK4 and hTERT-specific siRNA and control siRNA were purchased from Bioneer Corporation (Daejeon, South Korea). siRNA-mediated inhibition of gene expression was carried out using Lipofectamine RNAi Max (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. In brief, cells (3×10^5) were seeded in a 6-well plate 24 h before transfection. siRNA-transfected cells were employed to a transwell invasion assay 48 h post-transfection. Total cellular RNA was also extracted from each cell 48 h post-transfection for RT-PCR and real-time PCR.

Transwell invasion assay. A membrane invasion culture system (Corning incorporated – Life Sciences, Tewksbury, MA, USA) was used to evaluate invasive activity as previously described (18). In brief, the upper inserts containing 8 μm pore in 24-transwell plates were coated with type I collagen (45 μg/30 μl/well). Cells (2×10^4) were placed in the upper chambers of transwell. Medium contained with supplementary were added to the lower chambers. After incubating at 37˚C for 48 h, the upper well plates were fixed and stained with 0.25% crystal violet. The cells that penetrated the upper filter were counted manually under a light microscope (Olympus, Tokyo, Japan).

Statistical analysis. The results were analyzed using the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). All experiments were tested in three independent experiments and each experiment was carried out in triplicate. Comparison between two groups was analyzed using the student's *t*-test. *p*-Values <0.05 were considered statistically significant.

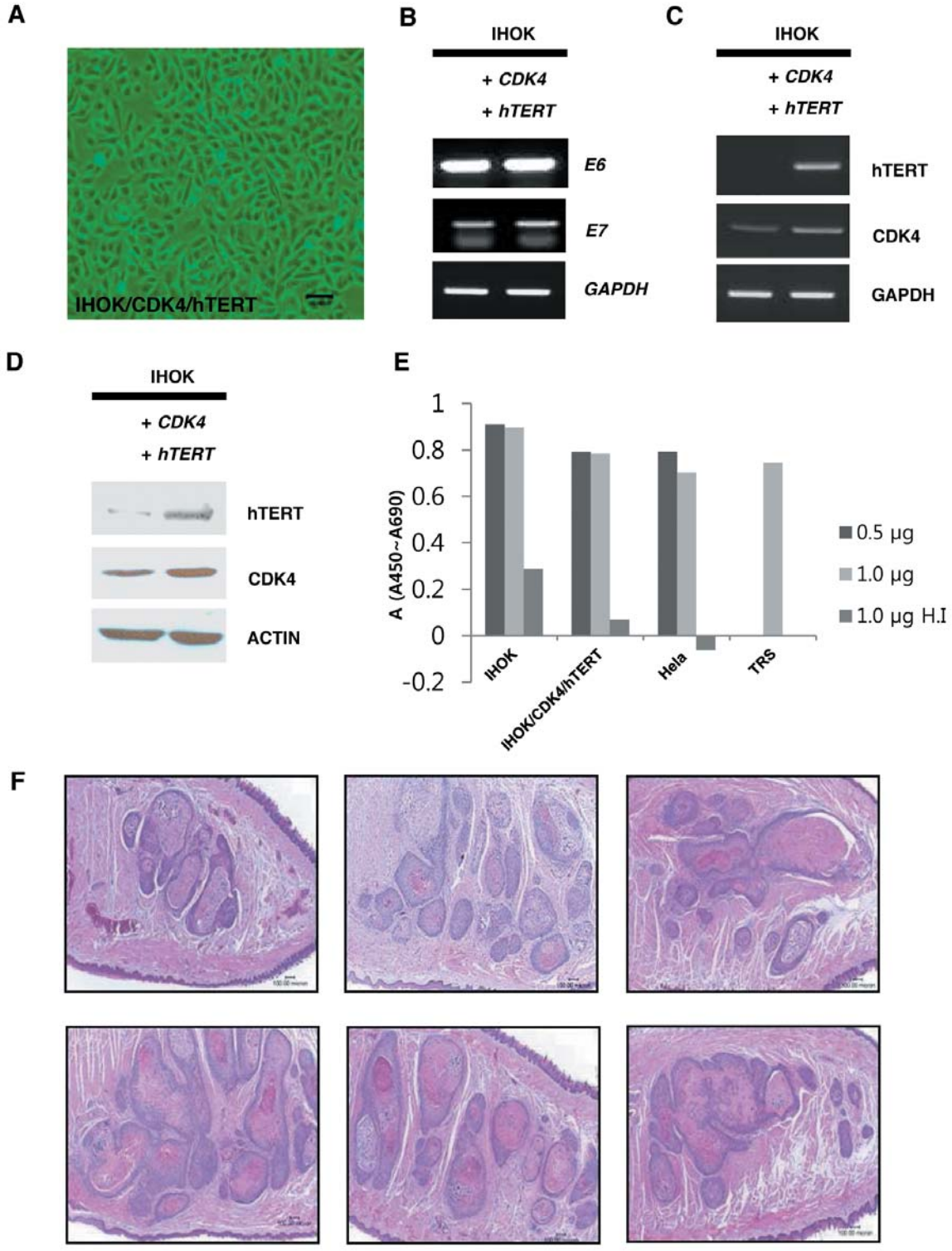

Figure 1. *Confirmation and tumorigenicity of IHOK/CDK4/hTERT cells. (A) Morphology of IHOK/CDK4/hTERT cells (×100) (B) HPV-16 E6 and E7 DNA expressions were analyzed by PCR. GAPDH was used as a loading control. (C) CDK4 and hTERT mRNA expressions were analyzed by RT-PCR. GAPDH was used as a loading control. (D) CDK4 and hTERT protein expressions were also analyzed by western blotting. Actin was used as a loading control. (E) Telomerase activity of IHOK and IHOK/CDK4/hTERT. Hela was used as a positive control cell line. TRS was used as a positive control cell line of PCR. H.I stands for heat-inactivated cell extracts. (F) Tongue tumors of IHOK/CDK4/hTERT cell-injected mice that formed SCC: all ten mice developed SCC (100%). Four out of 10 mice died before sacrifice due to obstructive tongue mass.*

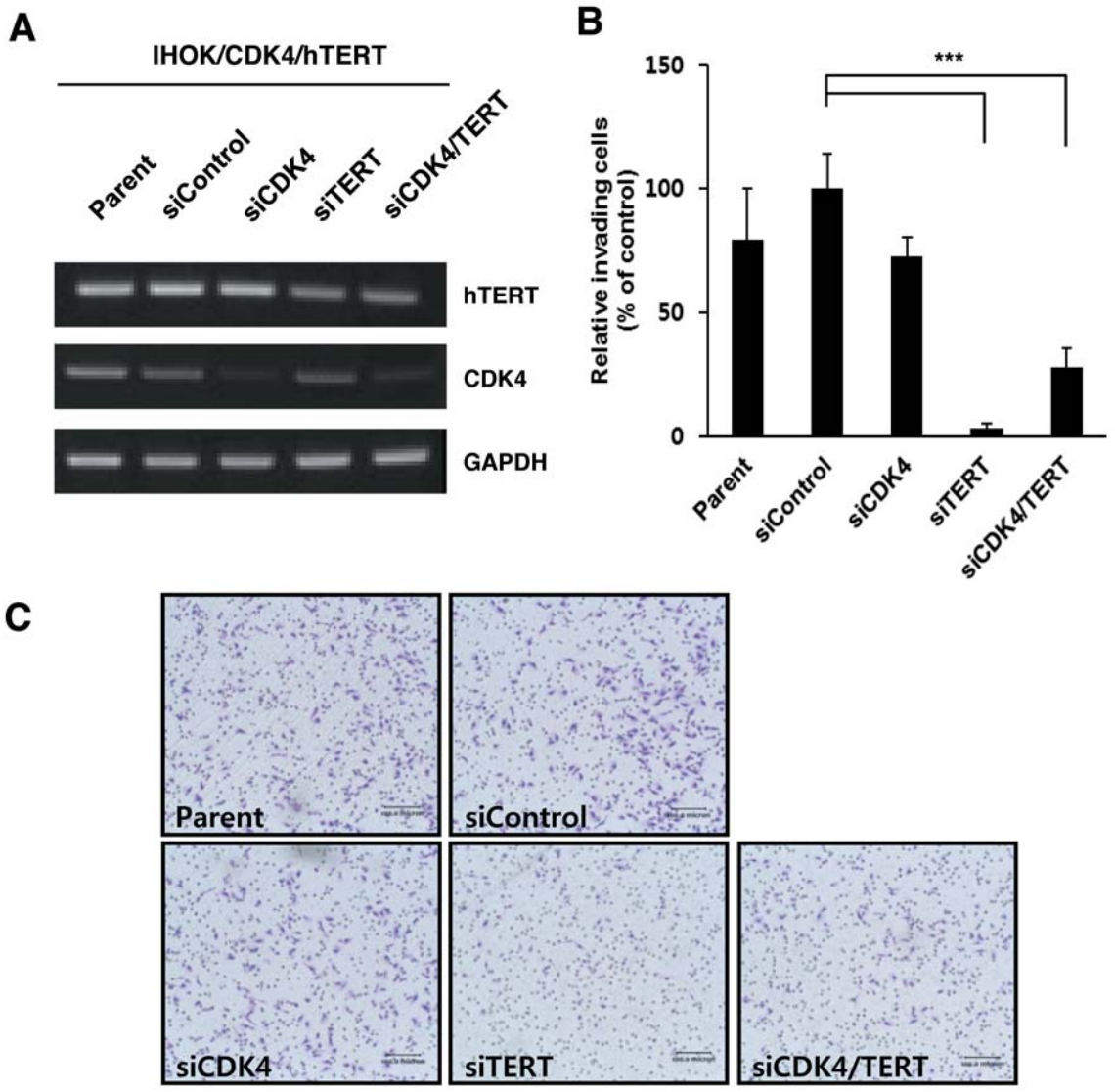

Figure 2. *Effects of CDK4 and hTERT knockdown by transient silencing in IHOK/CDK4/hTERT cells. (A) Transient siRNA-mediated knockdown of CDK4 and/or hTERT was detected by RT-PCR. (B, C) Invasiveness was evaluated by using a 24-transwell plate with a collagen-coated filter from control siRNA-treated, CDK4 siRNA-treated, hTERT siRNA-treated, and CDK4/hTERT siRNA-treated IHOK/CDK4/hTERT cells (means±SD of triplicate experiments: ***p<0.001).*

Results

IHOK/CDK4/hTERT cells represented HPV-16 E6/E7-infected OSCC. We examined HPV-16 *E6* and *E7* DNA infection in IHOK/CDK4/hTERT cells (Figure 1B). To confirm whether exogenous *CDK4* and *hTERT* were successfully expressed in IHOK/CDK4/hTERT cells, we measured CDK4 and hTERT mRNA and protein expression levels, respectively. IHOK/CDK4/hTERT cells showed much higher expressions of hTERT and CDK4 than those of IHOK cells (Figure 1C and D). Figure 1E confirmed telomerase activity of these cell lines. In order to examine whether IHOK/CDK4/hTERT cells harbored *in vivo* tumorigenicity, IHOK/CDK4/hTERT cells were injected into the dorsal tongues of nude mice. After 8 weeks, all 10 mice (100%) formed tumors in the IHOK/CDK4/hTERT cell-injected group. Four out of ten mice died during the experiment due to obstructive tongue masses. Histological examination of tongue masses from 6 mice showed invasive squamous cell carcinoma, confirming that genetically engineered IHOK/CDK4/hTERT cells represent HPV-16-infected OSCC cells (Figure 1F).

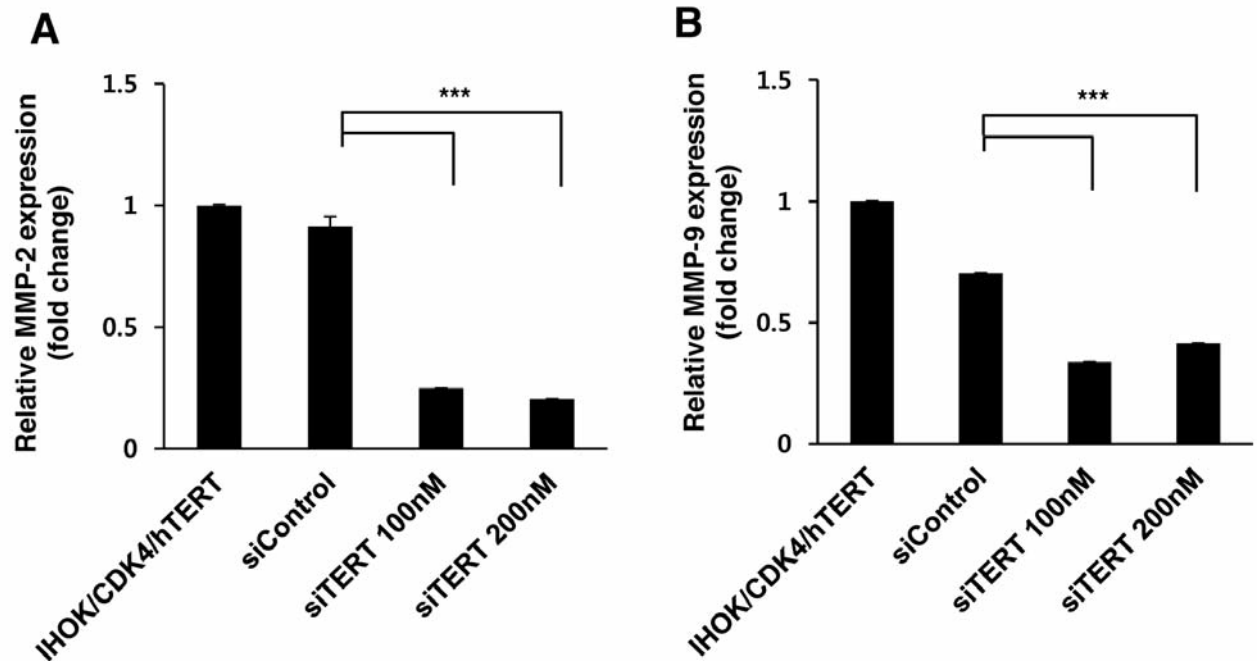

Figure 3. *Effects of siRNA hTERT on expression of MMP-2 and MMP-9 in IHOK/CDK4/hTERT cells. (A) MMP-2 and (B) MMP-9 expression was analyzed by real-time PCR from control siRNA-treated or hTERT siRNA-treated (100 nM or 200 nM) IHOK/CDK4/hTERT cells (mean±SD of triplicate experiments: ***p<0.001).*

Transient silencing of hTERT inhibits cell invasion. To evaluate whether knockdown of hTERT inhibits invasive activity, we measured invasive activity through the down-regulation of either CDK4 or hTERT in IHOK/ CDK4/hTERT cells. A reduction in the mRNA expressions of CDK4 and hTERT was observed 48 h post-transfection, indicating that siRNA knockdown of hTERT and CDK4 was effective (Figure 2A). The invasive activity was markedly reduced to 10% in hTERT-knockdown cells ($p<0.001$) (Figure 2B and C). Knockdown of both CDK4 and hTERT also led to the reduction of invasiveness ($p<0.001$) (Figure 2B and C). In contrast, CDK4-knockdown cells and controls showed no significant reduction of invasiveness. To confirm the effect of hTERT on invasiveness, we examined the mRNA expression levels of MMP-2 and MMP-9 after siRNA-mediated inhibition of hTERT in IHOK/ CDK4/hTERT cells. Expressions of MMP-2 and MMP-9 mRNA were significantly decreased in hTERT-knockdown cells (Figure 3). To evaluate whether knockdown of hTERT inhibits invasive activity in HPV-non-related OSCC, siRNA hTERT was transfected in HSC3 cells. As shown in Figure 4A, the mRNA expression of *MMP-2* and *MMP-9* were markedly reduced and concurrently showed the reduction of invasive activity *via* knockdown of hTERT expression (Figure 4B and C).

Discussion

In the present study, we established a genetically-manipulated OSCC cell line *via* overexpression of CDK4 and hTERT derived from HPV-16 *E6/E7*-transfected IHOK. IHOK/ CDK4/hTERT cells harbored strong *in vivo* tumorigenicity, supporting that these cells were transformed to squamous carcinoma cells. With these cells, we sought to determine whether knockdown of hTERT induced anti-invasive activity. In our data, hTERT-specific siRNA led to reduced invasive activity. Supporting this result, the expression of MMP-2 and MMP-9 was markedly reduced in siRNA hTERT-transfected IHOK/CDK4/hTERT cells. Knockdown of hTERT in HSC3 cells also showed the reduction of MMP-2 and MMP-9 expression, leading to a low invasive activity. Taken together, targeting hTERT can be contributable to treat both HPV-infected and HPV-nonrelated carcinomas.

Cyclin dependent kinases (CDKs) are major factors modulating cell cycle by forming cyclin-CDKs complexes (19). Hence, CDK4 is thought to be a core factor in the development of human cancer (20, 21). Although recent data have shown that CDK4-cyclin D1 affects cell migration and invasion through the interaction with filamin A (22), knockdown of CDK4 showed no reduction of invasive activity in IHOK/CDK4/hTERT cells in

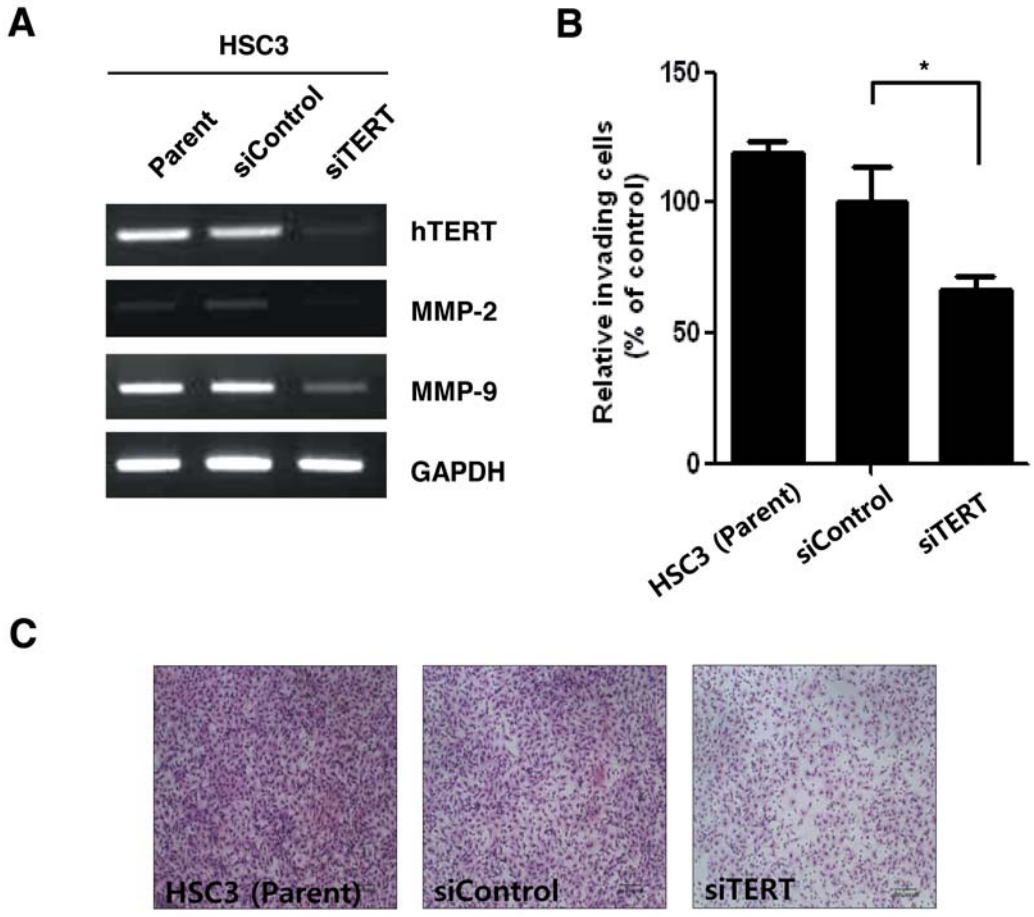

Figure 4. *Effects of siRNA hTERT on expression of MMP-2 and MMP-9 in HSC3 cells. (A) hTERT mRNA expression was analyzed by RT-PCR. MMP-2 and MMP-9 mRNA expression was also analyzed by RT-PCR from HSC3 cells, control siRNA-treated and hTERT siRNA-treated (100 nM) HSC3 cells. GAPDH was used as a loading control. (C) Invasiveness was evaluated by using 24-transwell plate with collagen-coated filter from HSC3 cells, control siRNA-treated and hTERT siRNA-treated HSC3 cells (means±SD of triplicate experiments: *p<0.05).*

our study. Given our data that knockdown of hTERT expression in IHOK/CDK4/hTERT cells maintained cell viability but its knockdown in IHOK cells caused total cell death (data not shown), the CDK4 expression in IHOK/CDK4/hTERT cells contributed to sustain their own cell cycle.

Apart from maintaining telomere length, hTERT induces migration and invasion (6, 7). In our data, hTERT-specific siRNA led to reduced invasive activity; however, transient knockdown of CDK4 expression had no impact on invasive activity. Consequently, we concluded that hTERT expression leads to cancer cell invasion in IHOK/CDK4/hTERT cells. Our results are consistent with other findings showing that hTERT helps tumorigenicity through promoting migration and invasion (7). A recent finding showed that hTERT activated MMP-9 in a NF-kB-dependent manner. However, the mechanism through which hTERT induces invasion remain incompletely understood (23).

In summary, we showed that knockdown of hTERT inhibited the invasive activity in hTERT-overexpressing immortalized oral keratinocytes and HSC3 cancer cells. These results provide a useful basis for the development of new therapeutic approaches in both HPV-infected and HPV-non-related carcinomas.

Conflicts of Interest

None of the Authors have any financial relationships with any organizations that would inappropriately influence the research findings.

Acknowledgements

This work was supported by the Mid-career Researcher Program (2009-0078630) and by the Basic Science Research Program (2009-0094027) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education and Science and Technology

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Received June 26, 2014
Revised August 1, 2014
Accepted August 6, 2014