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Original Articles

Human telomerase reverse transcriptase (hTERT) promotes cancer invasion by modulating cathepsin D *via* early growth response (EGR)-1

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ABSTRACT

Human telomerase reverse transcriptase (hTERT) contributes to tumor progression as well as maintaining telomere length, however, the mechanism by which hTERT promotes invasiveness is not yet completely understood. This study aims to unravel the precise mechanism through which hTERT promotes cancer invasion. We established an hTERT-overexpressed immortalized cell line (IHOK/hTERT). In orthotopic xenograft models, IHOK/hTERT harbors higher tumorigenicity than IHOK/Control. IHOK/hTERT showed much higher migration and invasion activities compared to IHOK/Control. IHOK/hTERT co-cultured with fibroblasts displayed increased invasion compared to IHOK/hTERT without fibroblasts. We screened for genes that play an important role in intermodulation between cancer cells and fibroblasts using a microarray and identified fibroblast activation protein (FAP). hTERT knockdown showed decreased expression of FAP and early growth response (EGR)-1, one of the transcriptional regulators of FAP in IHOK/hTERT and oral cancer cell line YD10B. Furthermore, EGR-1 knockdown in IHOK/hTERT and YD10B showed reduced invasion and reduced cathepsin D expression compared to Control-siRNA cells. Taken together, this study provides evidence that hTERT overexpression is responsible for the upregulation of the cysteine protease cathepsin D by regulating EGR-1 to activate invasiveness in cancer progression.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy in the world. Oral squamous cell carcinoma (OSCC) comprises approximately 10% of HNSCC, in general, but almost one-third in specific geographic areas [1,2]. Despite the advances in therapeutic approaches, an overall 5-year survival rate of OSCC patients has been estimated for the lowest 25% [2].

Infection with human papilloma virus (HPV) is one of the leading causes of HNSCC, especially cancer in the oropharynx and base of the tongue. OSCC is the second most common high risk-HPV (hr-HPV)-related cancer, and its incidence is gradually increasing [2,3]. hr-HPV causes human cancers by expressing two viral oncoproteins, E6 and E7. The expressions of E6 and E7 alone are not sufficient for cellular transformation, and additional genetic alterations are necessary for malignant progression [4]. E6 and E7 have multiple binding partners that exert oncogenic effects beyond the degradation of p53 and pRb. For example, E6, in concert with E6AP, induces telomerase activity through the activation of human telomerase reverse transcriptase (hTERT) *via* degradation of NFX1, a transcription repressor of hTERT, thus contributing to cellular immortalization [5].

The catalytic subunit of telomerase, hTERT, is overexpressed in approximately 90% of human cancer cells, suggesting that hTERT is indispensable for cancer progression [6]. In addition to maintaining the telomere length for cellular immortalization, hTERT has been shown to play an active role in tumor progression by inducing mobility, invasion, and anti-apoptosis of cancer cells, supporting independent roles for telomerase beyond telomere lengthening [7–9]. A recent finding provided evidence that hTERT is involved in invasion through the modulation of matrix metalloproteinase (MMPs) expression [10]. In our previous study, we also found that knockdown of hTERT reduced invasiveness in both hr-HPV- and non-infected OSCC through down-regulation of MMP2 and MMP9 expression [11]. MMPs play an important role in various physiological and pathological processes. In particular, MMPs are prime candidates for invasion and metastasis activities. Although several MMP inhibitors have been investigated in clinical trials for various cancers, none of these trials have demonstrated satisfactory efficacy, likely due to the lack of inhibitor specificity and unclear scientific mechanism [12–14]. Accordingly, it is necessary to discover an effective molecular target and supporting mechanism as a substitute for MMPs for current anticancer therapy.

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A recent finding showed that cathepsin, but not MMPs, is significantly associated with poor survival [15]. However, there are fewer studies that connect cathepsins with carcinogenesis compared with MMPs. Cathepsins are lysosomal cysteine proteases that degrade the extracellular matrix (ECM) and thus play active roles in the invasion and metastasis of cancer cells. Among the cathepsins, cathepsin D is an invasion promoter and plays a critical role in various cancers including OSCC [16,17]. According to our previous study [18], cathepsin D expression in cancer cells was increased by fibroblast stimulation in collagen gel-based co-cultured models, suggesting that cathepsin D expression is related to cancer invasion *via* crosstalk between cancer cells and stromal fibroblasts.

Here, this study aims at investigating the role of hTERT in invasion related to crosstalk between cancer cells and stromal fibroblasts. We demonstrate that hTERT induces invasion by modulating cathepsin D *via* early growth response (EGR)-1 in HPV-16 E6/E7-transfected immortalized human oral keratinocytes (IHOK) and HPV-not related OSCC cells. This new discovery will contribute to the development of a novel chemotherapeutic approach targeting hTERT.

Materials and methods

Cell culture

IHOK was established by transfecting the pLXSN vector containing the E6/E7 open reading frame of HPV-16 as previously described [19]. IHOK/hTERT and IHOK/Control were constructed by using plpc-hTERT and pLXRN (Clontech, USA) vectors, respectively. Each vector was transfected into a GP2-293 packaging cell line to produce retrovirus particles that were subsequently used to infect IHOK. Four types of OSCC cells (YD9, YD10B, YD32, and YD38) [20,21] and immortalized human gingival fibroblasts (hTERT-hNOF) were used for this study [22]. Details about the cell culture procedures are described in the Supplementary Materials and Methods.

Co-culture

IHOK/hTERT, YD10B, and siRNA-transfected cells were co-cultured with hTERT-hNOF to observe whether fibroblasts have an impact on protein expression. The Supplementary Materials and Methods are described in details.

Mouse orthotopic xenograft model

The animal studies were approved by the animal ethics committee at Yonsei University College of Dentistry. BALB/c male mice (16 ± 2 g, 4 weeks of age) were provided from Orient Bio Incorporation (South Korea). Cells (5×10^5) were injected into the dorsal tongue of 15 mice in each group. Three mice in the IHOK/Control-injected group died during the procedure. The mice were then sacrificed after 6 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. The tumor volume was assessed by two-dimensional measurements [23].

Immunofluorescence observation

Immunofluorescence staining was performed to detect protein expression of hTERT between IHOK/Control and IHOK/hTERT. The details were described in the Supplementary Materials and Methods.

BrdU incorporation assay

Cell proliferation was measured by the BrdU Flow Kit (BD Pharmingen™, UK) according to the manufacturer's instructions.

Telomerase repeat amplification protocol (TRAP) assay

Telomerase activity was measured by the Telomerase TeloTAGGG PCR ELISA (Roche, Switzerland) according to the manufacturer's instructions using 0.05 and 0.5 μg total protein.

Wound-healing migration assay

Cells (3×10^5) were seeded on 6-well plates and allowed to adhere overnight in growth media containing 1% FBS to achieve up to 90% confluence. The monolayer was scratched using a sterile 200 μL pipette tip. After 24 h, wound closure was evaluated by light microscopy (Olympus, Japan).

Invasion assay

Invasion assay was performed to compare the invasive activity between IHOK/Control and IHOK/hTERT. The Supplementary Materials and Methods are described in details.

Organotypic culture

Organotypic culture was performed to confirm the invasive activity between IHOK/Control and IHOK/hTERT. The Supplementary Materials and Methods are described in details.

Polymerase chain reaction (PCR)

Total DNA was extracted from each cell line using QIAamp DNA minikit (Qiagen, Germany). The primer sequences are listed in Table S1. DNA was amplified by using Accu Power Hot Start PCR Pre Mix (Bioneer, South Korea) with the following conditions: 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 40 s at 72 °C. The amplified products were separated on 1.0% agarose gel stained with 0.1 μg/mL of ethidium bromide, and photographed under UV light (Bio-Rad, USA).

Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was extracted from each cell line using a RNeasy plus mini kit (Qiagen, Germany), and complementary DNA was synthesized using the 2.5× RT-&GO™ Mastermix (MP Biomedicals, USA) according to the manufacturer's instructions. The primer sequences are listed in Table S1. Details about the procedures of RT–PCR are described in the Supplementary Materials and Methods.

siRNA transfection

Cells (1.5×10^5) were seeded in a 6-well plate for 24 h before transfection. hTERT-specific siRNA, EGR-1-specific siRNA, and control siRNA were the products of Bioneer Corporation (South Korea). Transfection of siRNA was performed using Lipofectamine RNAi MAX (Invitrogen, USA) according to the manufacturer's instruction. Total RNA and proteins were extracted 48 h after transfection. The sequences of siRNA are listed in Table S2.

Western blotting and zymography

Cells were lysed using a lysis buffer (Cell Signaling, USA) and used for western blotting. Collected conditioned medium was used for zymography. Details about procedures are described in the Supplementary Material and Methods.

Microarray data analysis

Microarray was performed to find altered gene expressions between IHOK/Control and IHOK/hTERT. The Supplementary Materials and Methods are described in details.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U* test to determine the statistical significance. All of the variables were tested in three independent experiments, and each experiment was performed at least in triplicate. The results are reported as the mean ± standard deviation (SD). The value of $p < 0.05$ was considered statistically significant.

Results

Construction of the hTERT-overexpressed cell line

To evaluate the functional significance and mechanism of hTERT on cancer invasion, we established hTERT-overexpressed immortalized cells (IHOK/hTERT). No significant change in morphology was observed between IHOK/Control and IHOK/hTERT (Fig. S1A). HPV-16 *E6* and *E7* DNA infection was confirmed in IHOK/Control and IHOK/hTERT by PCR (Fig. 1A). The mRNA expression levels of hTERT exhibited much higher in IHOK/hTERT than IHOK/Control, confirming exogenous hTERT was successfully expressed in IHOK/hTERT (Fig. 1B). The expression of hTERT was also confirmed by immunofluorescence staining (Fig. 1C and D). Significant difference in telomerase activity was observed between IHOK/Control and IHOK/hTERT when TRAP assay was performed using 0.05 μg total protein (Fig. 1E). No significant difference in proliferative activity was observed between the two cell lines when a BrdU incorporation assay