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p63-Mediated activation of the β-catenin/c-Myc signaling pathway stimulates esophageal squamous carcinoma cell invasion and metastasis

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ABSTRACT

The development of esophageal squamous carcinomas (ESC) results from numerous genetic alterations. Our previous study demonstrated that p63 is highly expressed in human ESC cells and stimulates their growth; however, the mechanism by which p63 regulates ESC cell adhesion and invasion remains unclear. In the present study, we further elucidated the underlying molecular mechanisms by which p63 regulates metastasis in ESC cells. Knockdown of p63 significantly diminished the invasion of ESC cell lines TE-8 and TE-12, whereas overexpression of p63 significantly increased the migration rates of BE3 and OE33 cells. The mRNA and protein levels of vimentin, twist, SUSD2, and uPA were significantly decreased in p63-knockdown ESC cells, while overexpression of p63 induced an increase in vimentin, SUSD2, and uPA. In addition, knockdown of p63 in ESC cells significantly reduced levels of β-catenin and c-Myc, while overexpression of p63 increased β-catenin, but reduced p-β-catenin level. Therefore, p63 regulates the migration and invasion of ESC cells through activation of the β-catenin/c-Myc pathway. Our results suggest that targeting p63 may constitute a potential therapeutic strategy for ESC.

Introduction

Studies of human cancers have demonstrated that most tumors arise from accumulation of genetic mutations. The development of esophageal squamous carcinoma (ESC) results from numerous changes in both genes and proteins. These multiple genetic alterations lead to increases in the rate of metastasis and invasion of ESC into surrounding tissues in the early stages thereof [1,2]; however, the biological roles of these changes in ESC remain unclear.

The p63 gene is a member of the p53 gene family [3,4] and has several isoforms, defined according to which transactivation domain is present in the N-terminal region, and by alternative splicing at the C-terminal region [3]. Various functions have been ascribed to p63 in normal squamous epithelia, including those of the esophagus [5]. A study on deletion of the p63 gene in mice revealed severe developmental defects during embryonic development [6,7], and many studies have reported that p63 plays an essential role in the maintenance of proliferative potential in epithelial stem cells [8–10]. Moreover, p63-knockout mice showed an abnormal persistence of ciliated cells in the esophagus [11], suggesting that p63 is required for esophageal differentiation and morphogenesis. Since p63 regulates normal epithelial development, it is also often found to be overexpressed in various squamous epithelia carcinomas [12–15]. In human esophageal squamous carcinoma (ESC), p63 protein is highly expressed [16,17]. High p63 expression has been shown to be strongly associated with an aggressive phenotype and metastasis in many cancers [18–20]. However, the relationship between p63 expression and invasion/metastasis in ESC has not yet been elucidated. Our previous study demonstrated that p63 is highly expressed in human ESC cells, and that p63 stimulates the growth of ESC cells by activation of the Akt signaling pathway [21]. However, the mechanism by which p63 regulates ESC cell adhesion and invasion is unknown. Therefore, in the present study, we further elucidated the underlying molecular mechanisms of p63 in the regulation of invasion and migration in ESC cells.

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Materials and methods

Cell culture and reagents

Two human esophageal squamous carcinoma cell lines, TE-8 and TE-12, and two human esophageal adenocarcinoma (EAC) cell lines, BE3 and OE33, were obtained from the University of Texas M.D. Anderson Cancer Center (Houston, TX, USA). All esophageal cancer cell lines were cultured in DMEM/F12 medium (Gibco Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco Life Technologies) and 100 μg/ml penicillin and 100 μg/ml streptomycin under standard conditions in a humidified atmosphere with 5% CO_2 at 37 °C. p63 SMARTpool siRNA and a negative control siRNA (empty vector, siCtrl) were purchased from Thermo Fisher Scientific (Walthan, MA, USA). Oligofectamine and Opti-MEM were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). β-catenin and phospho-β-catenin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), and p63, ΔNp63, TAp63, c-Myc, vimentin, Twist, SUSD2, uPA, and GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Wound healing assay

To determine the effect of p63 on esophageal cell migration, a wound healing assay was performed with endogenous p63-silenced cell lines and overexpressed p63 cell lines. p63 SMARTpool siRNA (50 nM and 100 nM) was transfected into TE-8 and TE-12 cells with 175 μl Opti-MEM as described previously [22]. As per the manufacturer's protocol, transfection was carried out using 4 μl of oligofectamine. Empty vector and p63 siRNA-transfected esophageal squamous carcinoma cells were grown to confluence. Transfection for overexpression of p63 in BE3 and OE33 cell lines was performed with Fugene-6 (Roche USA, South San Francisco, CA, USA) following the manufacturer's recommendations as described previously [22]. A wound was made through the monolayer using a 200-μl pipette tip. Wounds were measured over a time course to calculate the migration rate according to the following formula: percentage wound healing = ((wound length at 0 h) − (wound length at 24 h or 48 h))/(wound length at 0 h) × 100. The experiments were performed more than three times.

Matrigel invasion assay

In vitro cell invasion was performed using the BD Biocoat™ Matrigel™ Invasion Chamber (BD Biosciences, San Jose, CA, USA) with endogenous p63-silenced cell lines (TE-8 and TE-12) and overexpressed p63 cell lines (BE3 and OE33) according to the manufacturer's protocol. Briefly, the Matrigel coating chambers were rehydrated in 1 ml DMEM/F12 for 2 h in a humidified tissue culture incubator with a 37 °C, 5% CO_2 atmosphere prior to the experiments. Cells (2.5×10^4) were seeded onto matrigel-coated filters after 48 h of incubation. The filters were stained using a Diff-Quik kit (Sysmex Corporation, Kobe, Japan) and the number of traversed cells was counted on an inverted microscope (100×). For each membrane, five random fields were selected, and the invasion rates were determined by the percent invasion formula: percentage invasion = (mean number of cells invading through the matrigel insert membrane)/(mean number of cell migrating through the control insert membrane) × 100.

Real-time PCR

To quantify gene expression at the mRNA level, real-time RT-PCR analysis was performed. 1 μg of total RNA from each sample was subjected to reverse transcription using a Prime Script RT reagent kit (Takara) according to the manufacturer's protocol. Real-time PCR reactions were then carried out in a total of 10 μl of reaction mixture (1 μl of cDNA, 5 μl of 2 × SYBR Premix Ex Taq II, 0.4 μl of each 10 μmol/L forward and reverse primers, 0.2 μl Rox Reference Dye, and 3 μL of H_2O) in an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR program consisted of 40 cycles of 30 s at 95 °C, 15 s at 95 °C, and 1 min at 60 °C. Data was analyzed according to the comparative C_T method and was normalized to GAPDH expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product. Primers used for the analyses are listed in Table 1.

Western blot analysis

TE-8 and TE-12 cell lines were transfected with empty vector and p63 siRNA and Transfection for overexpression of p63 in BE3 and OE33 cell lines was performed with Fugene-6 (Roche USA, South San Francisco, CA, USA) following the manufacturer's recommendations. Total proteins were fractionated using ice-cold PRO-PREP™ (Intron Biotechnology, Seoul, South Korea), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), electrotransferred, and probed with p63, β-catenin, p-β-catenin, c-Myc, twist, uPA, and vimentin antibodies as described previously [23].

Statistical analysis

The experimental results presented in the figures were performed three or more times. The data from the wound healing assay, Matrigel invasion assay and mRNA levels were expressed as mean ± SEM. Comparisons between groups were performed using one- and two-way ANOVA with Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Expression of p63 regulates migration and invasion of TE-8 and TE-12 cells

We performed wound healing and Matrigel invasion assays to test whether p63 regulates ESC cell migration. We found that knockdown of p63 in TE-8 cells significantly reduced cell migration in a time- and dose-dependent manner (Fig. 1A). The migration rates with 100 nM of p63 siRNA in TE-8 cells were reduced by 30% at 24 h and 60% at 48 h, compared with that of the nonspecific siRNA-transfected cells (siCtrl). Another cell line, TE-12, also showed that p63 knockdown significantly inhibited cell migration (Fig. 1B). The effect was clearly observed at the higher concentration of p63 siRNA in a time-dependent manner (Fig. 1B). In addition, a significant anti-invasion effect was observed in the Matrigel invasion assay after 48 h of p63 siRNA (100 nM)-transfected cell lines TE-8 and TE-12 (Fig. 2A). We further investigated the role of p63 in ESC cell migration and invasion with two additional esophageal cancer cell lines (BE3 and OE33), which overexpressed p63. As shown in Fig. 1C, the migration rates were significantly increased by overexpression of p63 in a time-dependent manner in BE3 and OE33 cells. The Matrigel invasion assay also demonstrated significantly increased invasion effects in p63 overexpression cell lines BE3 and OE33 after 48 h of incubation (Fig. 2B). These results suggest that p63 promotes ESC cell migration and invasion.

p63 Promotes the mRNA expression of vimentin, twist, SUSD2, and uPA in ESC cells

To further confirm that p63 regulates molecular markers of invasion and migration, real-time RT-PCR was performed to investigate the molecular events involved in ESC cell migration. We

Table 1
Sequence of primers used for real-time RT-PCR.

Gene	Primer sequences	
β-catenin	F: 5′-GAGCCTGCCATCTGTGCTCT-3′	R: 5′-ACGCAAAGGTGCATGATTTG-3′
c-Myc	F: 5′-CAGCTGCTTAGACGCTGGATT-3′	R: 5′-GTAGAAATACGGCTGCACCGA-3′
Vimentin	F: 5′-CCCTCACCTGTGAAGTGGAT -3′	R: 5′-TCCAGCAGCTTCCTGTAGGT -3′
Twist	F: 5′-CGGGAGTCCGCAGTCTTA -3′	R: 5′-CTTGAGGGTCTGAATCTTGCT-3′
uPA	F: 5′-AGAATTCACCACCATCGAGA-3′	R: 5′-ATCAGCTTCACAACAGTCAT-3′
SUSD2	F: 5′-ATGGAGCAGCTCTCTTGGG-3′	R: 5′-CCACTGCACTGCTGGCT-3′
GAPDH	F: 5′-GTCTCCTCTGACTTCAACAGCG-3′	R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′