The role of HPV-18 E6 and E7 in a human basal cell carcinoma cell line

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Abstract: Basal cell carcinoma (BCC) is the most common skin cancer, but the pathogenic role of human papillomavirus (HPVs) in BCC is not still completely understood. In this study, we investigate the involvement of the integrated HPV-18 E6 and E7 in proliferation and senescence in a human BCC cell line. We had demonstrated HPV-18 E6 and E7 proteins in the BCC cell line. The inducible shRNA system was applied to suppress E6 and E7 expression and the cells were selected for further investigation. The effect of E6/E7 inhibition were analyzed by MTT, clonogenic assay and senescence-associated β-galactosidase staining. Our data show that repression of E6 and E7 in BCC cells result in proliferation inhibition and cellular senescence. Combined with the results of previous inducible shRNA inhibition system, we showed that HPV E6 downregulates p53 and E7 downregulates Rb proteins. Furthermore, integrated E6/E7 genes of HPV-18 upregulate the pro-inflammatory cytokines IL-6 and IL-8. These results suggest that HPV-18 involved in the tumorigenesis of BCC cell line. [Jen-Jung Cheng, Gwo-Tarng Sheu, Wen-Jun Wu, Meng-Hsuan Lin, Yu-Ping Hsiao. **The role of HPV-18 E6 and E7 in a human basal cell carcinoma cell line.** *Life Sci J.* 2013;10(4):2131-2134.] (ISSN:1097-8135) http://www.lifesciencesite.com. **284**

Keywords: basal cell carcinoma, human papillomavirus, pathogenesis

1. Introduction

High-risk human papillomavirus (HPV) has been associated with non-melanoma skin cancers but lack of a HPV-infected cell line to investigate the effect of HPV in carcinogenesis of skin cancers. Basal cell carcinoma (BCC) is the most common skin cancer and has an estimated annual incidence of 0.1-0.5%. Many trigger factors were implicated in sporadic BCC pathogenesis, such as UVR exposure, immunosuppression, and chemical carcinogens: ingestion of arsenic acid, medicine, pesticides, hydrocarbons, industrial oils, dyes, solvents, radiation, thermal burns, scarring, and viral carcinogenesis. Although viral infection has low percentage in BCC, investigations have shown HPV DNA was detected in 27.9% of BCC [1]. HPV 8, 18, and 5 could contribute to the development of non-melanoma skin cancers [2]. The incidences of HPV infection were five of 15 (33%) in BCC and four of 12 (33%) in SCC specimens [3]. Among the HPV-infected BCC, 23.1% were high-risk types of HPV (type 16 and 18) but lack of a HPV naturally infected cell line to investigate the role of HPV plays in the carcinogenesis of BCC [1]. Reuschenbach M et al., detected high risk types of HPV in 25% (7 / 28) of BCC from renal transplant recipients and in 11% (2 /18) from immunocompetent patients [4].

The pathogenic role of high-risk HPVs in nonmelanoma skin cancers still remain unclear, and literature data indicate that they might be at least cofactors in the development of certain cutaneous squamous cell carcinomas [5]. The role of HPV-18 in the tumorigenesis of BCC cell line was not clear. In this study, we investigate the involvement of the integrated HPV-18 E6 and E7 in proliferation and senescence in a human BCC cell line.

2. Methodology

2.1 Cell lines

The human BCC cell line (BCC-1/KMC) was established from human BCC derived from the undifferentiated type of BCC tumor arising from a thermal traumatic scar and was a gift from Dr. Chiang [6]. BCC cells were cultured on RPMI medium (GIBCO) containing 3% FBS. All cell lines were maintained at 37 ℃ in a 5% CO2-humidified atmosphere.

2.2 Selection of the Sh-E6 and Sh-E7 stable cell lines

The inducible shRNA of E6 and E7 were constructed by using pSingle-tTS-shRNA vector that annealed the shRNA oligonucleotides E6 upper strand(5'-tcgagGCAC GGAACTGAACACTTCATT CAAGAGATGAAGTGTTCAGTTCCGTGTTTTTTa) and lower strand (5'-agctt AAAAAACACGGAA CTGAACACTTCATCTCTTGAATGAAGAAGTGTT CAGTTCCGTGCc). The duplex DNA was restriction digested and ligated into the XhoI and Hind III sites to

generate Sh-E6 plasmid as described in user manual (Clontech). The Sh-E7 plasmid was constructed with E7 upper strand (5'-tcgagGCTCA GAGGAAGAAAACGATTTCAAGAGAATCGTTTT CTTCCTCTGAGTTTTTTa) and E7 lower strand (5'-gcttAAAAAACTCAGAGGAAGAAAACGAT TCTCTTGAAATCGTTTTCTTCCTCTGAGCc). The inserted sequences were confirmed by PCR amplification with tTs sh-RNA-F (5'-GAGGTA GGCGTGTAC GGTCA) and tTs sh-RNA-R (5'-AATACGCAAACCGCCTCTC) primers that an extra 50 bps was inserted when compared with the empty vector. The purified Sh-E6 and Sh-E7 plasmids were transfected with LipofectamineTM 2000 (Invitrogen) into BCC cells (1x105) that seeded on 35-mm dishes. After 24 h incubation, the transfected cells were re-seeded on 10-cm dishes with low cell density and treated with G418 (400 mg/ml) in fresh medium containing Tet System Approved FBS (Clontech). The selection was processed for weeks until single colony apparently observed. The well separated colonies were picked up and re-seeded in 96-well plate for proliferation followed by amplifying in 48-well, 24-well, 12-well and 6-well plates for further characterization.

2.3 Semi-quantitative RT-PCR analysis

To analyze the mRNA levels of E6 and E7, total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The purified RNA (2 μg) was reverse-transcribed to cDNA by MultiScribeTM Reverse Transcription System (ABI) using random primers. The amplified PCR products were analyzed with a 2% agarose gel. The Image J quantity software was downloaded from National Institutes of Health.

2.4 Cell viability assay (MTT assay)

Viability of BCC, Sh-E6 and Sh-E7 cells with or without Dox induction were determined using MTT colorimetric assay. Approximately 1×104 cells per well were seeded onto 24-well plates. After 24 h incubation, the cells were exposed to Dox in fresh medium. MTT (1 mg/ml; Sigma) were added to each well and cells were incubated at 37 ℃ for 2.5 h. 2-propanol solution were added per well to dissolve the water-insoluble formazan salt. The plates were shaken at 70 rpm at room temperature for 10 min. Finally, the absorbance was measured at 570 nm using an ELISA plate reader (Molecular Devices SPECTRA max 340 PC). Mean values were calculated from three independent experiments.

2.5 Senescence-associated β-Galactosidase staining

To determine whether E7 interference can induce the senescent phenotype, BCC and Sh-E7 cells were seeded in 35-mm dishes at a density of 1×105 cells. The cells were treated with Dox (1.5μg/ml) or PBS as negative control and incubated at 37℃ for 48 h. Cells were washed in PBS, then fixed at room temperature for 3–5 min in 2% formaldehyde/0.2% glutaraldehyde, washed with 1× PBS, and incubated at 37 ℃ with fresh staining solution consisting of 1 mg/ml 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-Gal, from a stock solution of 20 mg/ml in dimethylformamide) in 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl and 2 mM MgCl2. Staining was evident in 2-4h and maximal at 12–16 h.

3. Results

3.1 Expression of E6 and E7 viral proteins of HPV18 in BCC cells

In a previous experiment, we found that HPV18 genomic DNA was detected in a BCC cell line by PCR. Because the human cervical cancer cell of HeLa is also integrated with HPV18, therefore, we compared the HeLa cells with BCC cells to demonstrate the levels of E6 and E7 proteins by Western blot analysis (Fig 1). The lung cancer cell of A549 is negative for HPV18 and used as the negative control. The data confirm the integrated HPV18 in BCC cells is expressing E6 and E7 viral proteins.

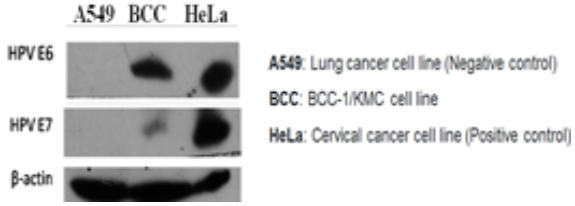

Fig. 1. Detection of HPV-18 E6 and E7 protein expression in A549, BCC, and HeLa cell lines

3.2 Viral E6 and E7 genes mediate the proliferation and senescence of HPV18-integrated BCC cells

The inhibition of E6 and E7 by Dox-induced shRNA resulted in Sh-E6-1 and Sh-E7-1 cells markedly reduced their viability at day-3 from 6.4-fold to 2.1-fold and 4.3-fold to 1.3-fold respectively, whereas the growth of BCC cells only slow down for 1 day by Dox treatment. The flatten shape morphology of Sh-E7-1 cells in Fig 3a indicated the possible development of cellular senescence with E7 inhibition. Therefore, we detected the expressed senescence-associated β-galactosidase (blue staining), a marker of cellular senescence in Sh-E7-1 cells (Fig 2). The results showed that Dox-induced Sh-E7-1 cells developed

22% of senescence cells and further demonstrated the activities of E6/E7 are essential for the growth and proliferation of host cells.

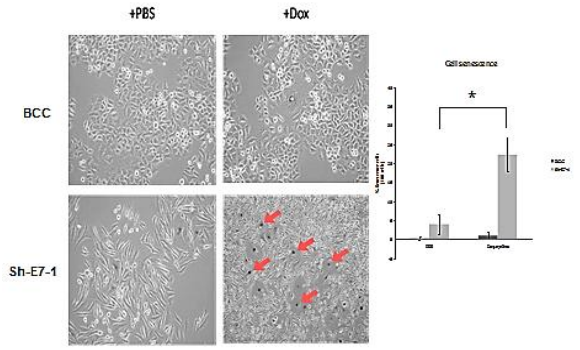

Fig. 2. The cell senescence of BCC cell line was increased after repressing E6 and E7 expression

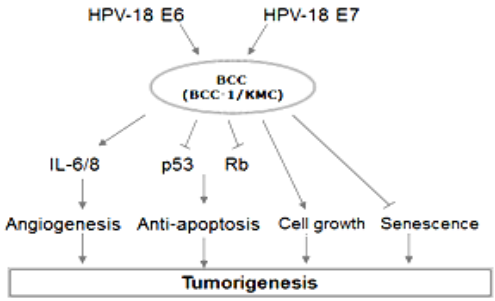

Fig. 3. HPV involved in the tumorigenesis of BCC cell line

4. Discussion

Human papillomaviruses (HPVs) have been reported to be associated with non-melanoma skin cancers [7-11]. High-risk HPVs can be detected in a proportion of non-melanoma skin cancers. Data on prevalence are inconclusive, but are essential to estimate the relevance of high risk-HPV, particularly with regard to prophylactic HPV vaccines for skin cancer prevention [4]. Paolini F et al. showed that p16INK4a and pAkt are over-expressed in BCC and suggested that HPV may exert a role in the carcinogenesis or biological property [5]. In this in vitro study, we established a cell model to elucidate the role of high risk type of HPV (type 18) in BCC. Our study investigated the naturally infected HPV-18 had a significant role in the tumorigenesis of this BCC cell line. We had showed that repression of HPV-18 E6 and E7 in BCC cells would upregulate tumor suppressor proteins of p53 and Rb *in vitro* [12]. Rb plays key roles in anti-apoptosis, cell differentiation, DNA replication, and cell senescence. The binding of E7 to Rb releases E2F and thereby deregulates the cell cycle [13]. p53 is involved in multiple processes including cell cycle regulation, the induction of apoptosis and DNA repair. The function of E6 is its ability to bind to the p53 tumour suppressor protein in conjunction with the cellular ubiquitin ligase E6AP and target p53 for degradation [14]. Repression of HPV E6 and E7 would also result in cell proliferation inhibition and cellular senescence in BCC cell line. When HPV-18 E6 and E7 expression were inhibited, the expression of angiogenetic cytokines IL-6 and IL-8 were coordinately repressed [12]. By inducible shRNA inhibition, we showed that HPV E6 downregulates p53 and E7 downregulates Rb proteins. Furthermore, integrated E6/E7 genes of HPV-18 upregulate the pro-inflammatory cytokines IL-6 and IL-8. These results suggest that HPV-18 involved in the tumorigenesis of BCC cell line (Fig.3).

Acknowledgements

This work was supported by Grants (CSH-2011-C-024) to Y-P Hsiao and (NSC-101-2320-B-040-006) to G-T Sheu.

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11/15/2013