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Original Research

Human papillomavirus 16 E2 interacts with neuregulin receptor degradation protein 1 affecting ErbB-3 expression *in vitro* and in clinical samples of cervical lesions☆

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Abstract The ErbB tyrosine kinase receptors play a key role in regulating many cellular functions and human papillomaviruses (HPVs) may interact with transductional pathway of different growth factor receptors. Here, these interactions were analysed in W12 cell line carrying HPV 16 genome and in clinical samples. W12 cells, in which HPV16 becomes integrated during passages, were utilised to detect viral and ErbB family expression at early (W12E) and late passages (W12G). Interestingly, a strong reduction of ErbB-3 expression was observed in W12G. Loss of the E2 and E5 viral genes occurs in W12G and this may affect ErbB-3 receptor expression. E2 and E5 rescue experiments demonstrated that only E2 gene was able to restore ErbB-3 expression. E2 is a transcriptional factor but the expression levels of ErbB3 were unaffected and ErbB-3 promoter did not show any consensus sequence for E2, thus E2 may interact in another way with ErbB3. Indeed, HPV 16 E2 can modulate ErbB-3 by interacting

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with the ubiquitin ligase neuregulin receptor degradation protein 1 (Nrdp-1) that is involved in the regulation of this receptor, via ubiquitination and degradation. E2 co-immunoprecipitated in a complex with Nrdp-1 leading to hypothesise an involvement of this interaction in ErbB-3 regulation. In addition, 90% of the clinical samples with integrated virus and E2 loss showed no or low ErbB-3 positivity, confirming *in vitro* results. In conclusion, the new discovered interaction of HPV-16 E2 with Nrdp-1 may affect ErbB-3 expression.

1. Introduction

Certain human papillomaviruses (HPVs) have been associated with some human cancers, most notably cervical cancer [1], which is the second most common cause of death from cancer among women worldwide and remains the major basis of mortality among women of reproductive age in developing countries. Strong epidemiologic and experimental evidences have linked 'high-risk' HPVs (i.e. HPV16 and HPV 18) infection to the development of cervical carcinoma [2].

The ErbB family of receptor tyrosine kinases regulates a large variety of biological processes including cell proliferation, migration, invasion and survival [3,4]. This family includes four members: ErbB-1 (epidermal growth factor receptor [EGFR], or HER-1), ErbB-2 (HER-2), ErbB-3 (HER-3) and ErbB-4 (HER-4). During the replicative cycle HPV may interact with several different pathways mostly by inactivating pRB and p53 tumour suppressors in order to maintain the proliferative status of the epithelium during differentiation. However, few studies evaluated whether HPV oncogenic proteins may affect the pathways of ErbB receptors in cervical cancer. HPV16 E6 and E7 cooperate with ErbB-2 to induce cellular transformation of human oral epithelial cells and E6 alone is able to mediate ErbB-2 stabilisation during the neoplastic transformation of human cervical keratinocytes [5–7]. HPV-16 E5 protein significantly affects ErbB-4-induced c-Jun expression in HPV16-infected cervical cells [8]. All the above mentioned studies were performed in presence of viral oncogene over-expression without the simultaneous expression of all viral genes. In addition, no data in clinical specimens were reported.

Our study was designed to evaluate the relationship among HPV viral proteins and ErbB receptors in a setting of physiological viral expression such as W12 cell line, an accurate model of HPV16 low-grade squamous intraepithelial lesion progression [9]. Indeed, early passage W12 cells (W12E) harbour episomal copies of HPV16 and express all early viral genes whereas late passage cells (W12G) contain only integrated HPV16 with loss of E2–E5 region of viral genome.

In the present report, decreased ErbB3 levels were observed during HPV integration, and, for the first time, an interaction of HPV16 E2 protein with the neuregulin receptor degradation protein 1 (Nrdp-1) was demonstrated. This interaction, in turn, may affect ErbB3 expression because Nrdp-1 is a Really Interesting New Gene (RING) finger E3 ubiquitin ligase involved in ErbB-3 degradation [10]. In addition, 17 cervical biopsies were analysed to confirm our observation *in vivo*. Data from clinical specimens showed that ErbB-3 expression levels decreased in association with HPV viral integration.

2. Patients and methods

2.1. Patients

The study included 43 consecutive formalin-fixed and paraffin-embedded (FFPE) cervical tissue specimens from patients referred to our institution. Five (12%) were CIN1, seven (16%) CIN2, 13 (30%) CIN3 and 18 (42%) were cervical cancers. Median age was 41 years (23–76). Only 17 out of 43 samples were HPV16 positive and were analysed for ErbB-3 expression. All tissue samples were collected for diagnostic purposes and studied in accordance with national ethical principles. The investigation protocol was approved by the review board of our institute.

2.2. Cell culture, MTT proliferation assay and HPV gene expression

W12 cell line was maintained in keratinocyte growth medium (Clonetics, Italy).

For MTT proliferation assay, W12E and W12G were seeded in 96-well-microplates, and epidermal growth factor (EGF)-starved for 24 h. At time zero 10 ng/ml EGF was added, and proliferation was measured at indicated intervals. Before each time point, cell monolayers were treated with 20 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) solution and incubated at 37 °C for 3 h.

In order to verify the status of viral genes at the moment of experimentation multiplex E2/E6 and E5 specific polymerase chain reaction (PCR) assays were performed according to previously described protocols [11]. Amplified products were detected in ethidium bromide stained agarose gel.