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Correlation between the presence of high-risk human papillomaviruses and Id gene expression in Syrian women with cervical cancer

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Abstract

Infection by high-risk human papillomaviruses (HPVs) is considered to be the central cause of invasive cervical cancer. Previously reported studies have shown that Id genes regulate cell invasion and metastasis in several human carcinomas including cervical cancer. In order to investigate the correlation between high-risk HPVs and Id genes in human cervical cancer, the presence of high-risk HPVs and their association with Id gene expression was examined using PCR methods and tissue microarray analyses in a cohort of 44 cervical cancer patients from Syria. This study showed that high-risk HPVs were present in 42 samples (95%) that represent invasive cervical cancers and that the most frequent high-risk HPV types in Syrian women were 33, 16, 18, 45, 52, 58, 35, 51 and 31. Furthermore, the expression of E6 oncoprotein of high-risk HPVs was found to correlate with overexpression of Id-1, but not of Id-2, Id-3 or Id-4 in the majority of invasive cervical cancer tissue samples. These data suggest that high-risk HPVs can enhance the progression of human cervical cancer through Id-1 regulation.

Keywords: Cervical cancer, high-risk HPV, Id genes, Syrian women
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Introduction

High-risk HPVs are important risk factors for human cervical cancer; approximately 96% of cervical cancers score positive for high-risk HPVs [1,2]. In addition, the presence of high-risk HPVs serves as a prognostic factor in early-stage cervical cancers, and are associated with vascular invasion, lymph node metastases and tumour size [2–5]. The E6 and E7 oncoproteins of high-risk HPVs, which are expressed in cervical cancers, inactivate p53 and pRb tumour suppressors, respectively [6]. E6 facilitates the degradation of p53 through its association with accessory protein E6-AP, a component of the ubiquitin proteolytic pathway [7]. E7 proteins of high-risk HPVs bind to Rb [8], as well as to other pocket proteins, such as p107 and p130 [9], leading to cell cycle deregulation. This results in genomic instability and has been implicated in the transformation of normal cells and the progression of cancerous cervical cells.

Id (inhibitors of differentiation and DNA binding) genes are members of the helix-loop-helix transcription factor family and have multiple functions including inhibition of differentiation, induction of proliferation and delaying replicative senescence [10,11]. Moreover, Id-1 and Id-3 have been indicated as potential oncogenes because they are overexpressed in several human cancers including cervical, colorectal, breast, pancreatic and prostate cancer [12–16]. The up-regulation of Id-1 is clearly associated with more aggressive behaviour of tumour cells, particularly in cervical cancers [12]. These studies suggest that Id genes play an important role in the progression of human cervical cancers.

This investigation aims to identify the specific types of high-risk HPV infections present in cervical cancers of Syrian women and their association with Id gene expression and tumour aggressiveness.

Materials and Methods

HPV detection and type specification

Formalin-fixed, paraffin-embedded blocks of cervical tumour samples were obtained from 44 Syrian patients with an average age of 57 (range 38–82) years. Formalin-fixed (buffered, neutral aqueous 10% solution), paraffin-embedded cervical tumour samples were supplied by the Department of Pathology, Faculty of Medicine at the University of Aleppo, Syria. The specimens and data used in this research were approved by the Ethics Committee of the Faculty of Medicine of Aleppo University. Five micrograms of purified genomic DNA (Qiagen GmbH, Hilden, Germany) were taken from each sample and analysed for HPVs by multiplex PCR targeted to the conserved L1 region of the viral genome using PGMY09/11 L1 primer pools [3,13]. In parallel, specific primers for the E7 gene were used to detect HPV types 16, 18, 31, 33, 35, 45, 51, 52 and 58, while specific primers for the gene encoding for glyceraldehyde-3-phosphate dehydrogenase were used as an internal control [17] (Table 1). PCR products were denatured in 0.13 N NaOH and hybridized to an immobilized HPV probe array using an extended reverse line-blot assay for HPV genotyping (Roche Molecular Systems, Inc., Alameda, CA, USA) of nine high-risk HPVs (types 16, 18, 31, 33, 35, 45, 51, 52 and 58), as classified by Begum *et al.* [3].

Tissue microarray

The tissue microarray (TMA) construction was performed as described by Kuefer *et al.* [18]. Cervical tumour samples were embedded in a virgin paraffin TMA block using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD, USA). Each block was assembled without prior knowledge of associated clinical or pathological staging information. An average of two TMA cores (range two to six) 1.0 mm in diameter were sampled from a cohort of 44 Syrian patients diagnosed with cervical carcinoma. After construction, 4-μm sections were cut and stained with haematoxylin and eosin to verify the histological diagnosis. Slides of the finished blocks were used for immunohistochemistry analysis.

TABLE 1. Gene-specific primer sets for E7 genes of high-risk HPVs used for PCR amplification

HPV types	Region	Primers
16	E7	5′-ATGCATGGAGATACACCTACATTGCAT-3′ 5′-GTTTCTGAGAACAGATGGGGCACAC-3′
18	E6	5′-GCTTTGAGGATCCAACACGG-3′ 5′-TGCAGCACGAATGGCACTGG-3′
31	E7	5′-GGGCTCATTTGGAATCGTGTG-3′ 5′-AACCATTGCATCCCGTCCCC-3′
33	E7	5′-TGAGGATGAAGGCTTGGACC-3′ 5′-TGACACATAAACGAACTGTG-3′
35	E7	5′-CTATTGACGGTCCAGCT-3′ 5′-TACACACAGACGTAGTGTCG-3′
45	E7	5′-CCC ACG AGC CGA ACC ACA G-3′ 5′-TCT AAG GTC CTC TGC CGA GC-3′
51	E7	5′-TAC GTG TTA CAG AAT TGA AG-3′ 5′-AAC CAG GCT TAG TTC GCC CAT T-3′
52	E7	5′-GCA GAA CAA GCC ACA AGC AA-3′ 5′-TAG AGT ACG AAG GTC CGT CG-3′
58	E7	5′-CGA GGA TGA AAT AGG CTT GG-3′ 5′-ACA CAA ACG AAC CGT GGT GC-3′

Primers specific for the glyceraldehyde-3-phosphate dehydrogenase gene, 5′-GAAGGC-CATGCCAGTGAGCT-3′ and 5′-CCGGGAAACTGTGGCGTGAT-3′, were used as an internal control.

Immunohistochemistry

Immunohistochemical procedures examining the expression of Id-1, Id-2, Id-3, Id-4 and E6 were carried out using the following standard procedures. The protein expression levels and immunolocalization of Id-1, Id-2, Id-3, Id-4 and E6 in the TMA of Syrian cervical tumour specimens were analysed by mounting 4-μm sections of the TMA on saline-coated slides (Sigma, St Louis, MO, USA), which were subsequently dried overnight at 37°C. The TMA sections were then deparaffinized in graded alcohol, rehydrated, and boiled in 10 mM citrate buffer (pH 6.0) for antigen retrieval. The TMA slides were subsequently incubated for 32 min at 37°C with primary polyclonal rabbit antihuman Id-1, Id-2, Id-3 and Id-4 antibodies (sc-488, sc-489, sc-490 and sc-491, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:25, or E6 (mouse monoclonal; clone C1P5; Calbiochem, ON, Canada) at a dilution of 1:20, using the NexES automated immunostainer (Ventana Medical System, Tucson, AZ, USA). The automated Ventana Medical System uses an indirect biotin–avidin system with a universal biotinylated immunoglobulin secondary antibody. Diaminobenzidine was used as a chromogen, and slides were counterstained with haematoxylin prior to mounting. All staining procedures were performed according to the manufacturers' recommendations. Negative controls were obtained using specific blocking peptides from Santa Cruz Biotechnology with anti-Id antibodies at a ratio of 10:1 and by omitting the specific primary antibody for E6.

Following immunohistochemistry, all TMA slides were manually scored for Id-1, Id-2, Id-3, Id-4 and E6 expression and categorized using a three-tiered system (0 = negative, 1 = weak, 2 = strong) by two independent observers.

Results

In order to categorize the presence of high-risk HPV in human cervical cancer in Syrian women, we investigated the presence of high-risk HPV types 16, 18, 31, 33 35, 45, 51, 52

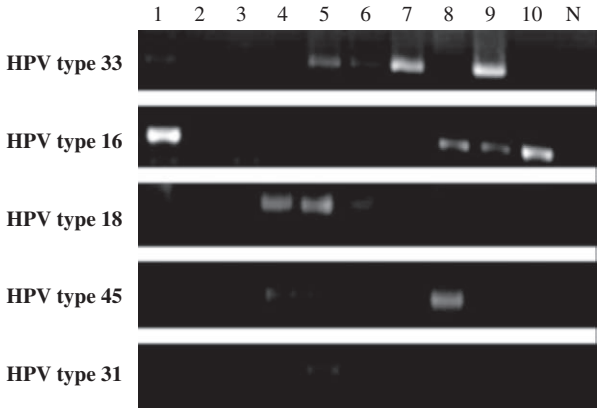

FIG. 1. Representative PCR reactions for E7 of high-risk HPVs in ten different cervical cancer tissue samples. E7 of high-risk HPV types (33, 16, 18 and 45) are present frequently in comparison with HPV type 31 in these patient samples (1–10; N, negative control).

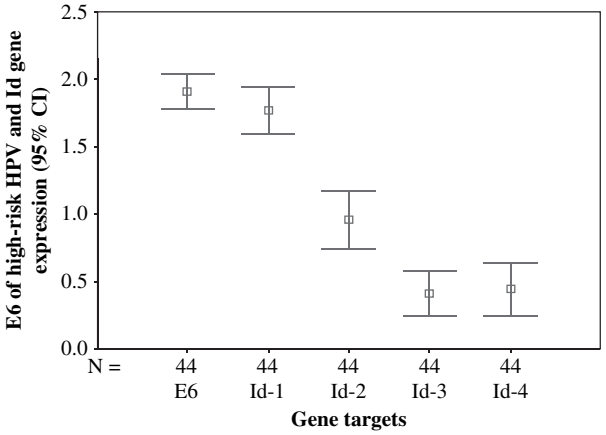

FIG. 2. Correlation between the expression of E6 of high-risk HPVs and of Id-1, Id-2, Id-3 and Id-4 in human cervical cancer tissue samples. E6 of high-risk HPV 16, 18, 31, 33, 35, 45, 51, 52 and 58 expression was compared with Id-1, Id-2, Id-3 and Id-4 expression in a cohort of 44 cervical cancer samples using immunohistochemistry as described in Materials and Methods. CIs (95%) show normalized mean protein intensity units of E6, Id-1, Id-2, Id-3 and Id-4 as determined by quantitative evaluation of immunohistochemistry. The mean is represented by square bars, ±SE. E6 expression of these high-risk HPVs is associated with Id-1 overexpression, but not with Id-2, Id-3 and Id-4, in these samples ($p < 0.001$). The presence of high-risk HPV types 16, 18, 31, 33, 35, 45, 51, 52 and 58 was confirmed by PCR using gene-specific primers for E6/E7 genes.

and 58 in a cohort of 44 cervical tumour samples from Syrian women by PCR analysis, using specific primers for the E7 gene (Table 1). The study revealed that 42 (95%) of the 44 samples were HPV positive and all of these positive specimens were co-infected with more than one HPV type. The most prevalent high-risk HPVs among the infected samples were types 33 (24/44), 16 (21/44), 18 (18/44), 45 (17/44), 52 (13/44), 58 (11/44), 35 (9/44), 51 (7/44) and 31 (5/44) (Fig. 1).

The association between the presence of high-risk HPV types 16, 18, 31, 33, 35, 45, 51, 52 and 58 and Id gene expression in relation to tumour aggressiveness in cervical cancer in these 44 Syrian women was then investigated. We examined the expression of the Id-1, Id-2, Id-3, Id-4 and the E6 oncoprotein in high-risk HPVs by immunohistochemistry in all the cervical tissue samples, using TMA methodology (Fig. 2). It was found that E6 expression correlated with Id-1 expression in 95% of human cervical cancers, which were invasive carcinomas in the majority of cases (Figs 2 and 3). Overexpression of Id-1 was detected in 36 patients, Id-1 expression was weak in four cases and absent in four cases.

By contrast, overexpression of Id-2 was observed in ten samples, weak expression observed in 22 cases and no expression of Id-2 was found in 12 cases. Genes Id-3 and Id-4 demonstrated strong expression in only one sample and weak expression in 16 and 13 cases, respectively, whereas no expression of these genes was found in the rest of the samples.

In order to confirm the association between E6/E7 of HPV types 16, 18, 31, 33, 35, 45, 51, 52, 58 and Id-1, the presence of E7 in these viruses was investigated by PCR using specific primers for this gene (Table 1). This analysis showed that high-risk HPV types 16, 18, 31, 33, 35, 45, 51, 52 and 58 were present in the majority of invasive cervical cancer tissues and their presence was associated with overexpression of Id-1 but not Id-2, Id-3 or Id-4 (Fig. 2).

Discussion

This is the first study to examine the relationship between the presence of high-risk HPVs and tumour aggressiveness in cervical cancer in Syrian women. Earlier studies reported that HPV types 16, 18, 31, 33, 35, 45, 52 and 58 are the most frequent high-risk HPVs in cervical cancers throughout the world [2]. Parallel studies have shown that HPV types 16, 18, 31, 33, 35, 45, 52 and 56 are the most common HPVs in women living in Europe and North America [2,19]. Similarly, studies on the presence of high-risk HPVs in North African and Middle Eastern women have revealed that HPV types 16, 18, 45, 35 and 33 are generally present in cervical cancer in these women [20–22]. In this study, we report that high-risk HPVs are present in 95% of cervical cancers in Syrian women.

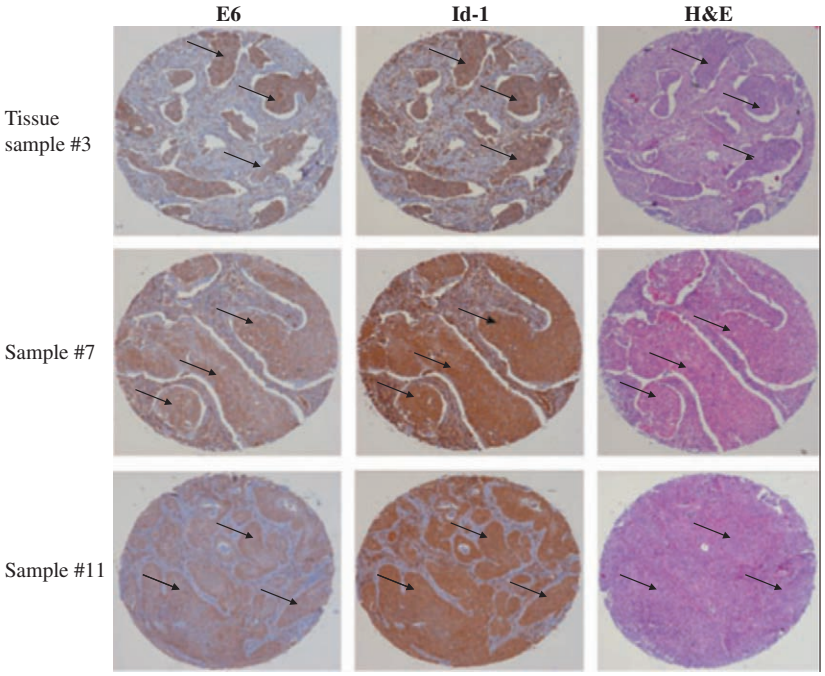

FIG. 3. Representative immunohistochemistry revealing E6 and Id-1 expression in cervical tumour tissue from high-risk HPVs. The three different cervical tumour tissue samples were prepared using tissue miscroarrays and bound E6 and Id-1 antibodies were visualized using diaminobenzidine staining. Structural features of the tumour tissue were revealed by haematoxylin and eosin staining. Although the levels of E6 and Id-1 expression vary between patients, there is a correlation between high levels of E6 expression and Id-1 overexpression (arrows) in tissue from individual patients. Magnification is 200×.

Moreover, HPV types 33, 16, 18, 45 and 52 are the predominant viruses of the high-risk HPV family in these cervical tumour tissues. Therefore, the prevalence of high-risk HPVs in cervical cancers of the Syrian women in this study is similar to those reported in other countries, especially the Middle East and North Africa. Nevertheless, we believe that future studies on a larger number of samples will be necessary to confirm the incidence of HPVs in cervical cancer in patients of Middle Eastern origin.

Evidence available to date suggests that persistent infection with high-risk HPVs is necessary for cervical precursor lesions to evolve into invasive carcinomas [2,20,23,24]. On the other hand, several studies have proposed that Id-1 and probably Id-3 genes play a crucial role in the progression of several human cancers, including cervical cancer [12,14,16]. This study reports for the first time that the presence of E6/E7 oncoproteins of HPV types 16, 18, 31, 33, 35, 45, 51, 52 and 58 is associated with Id-1 expression in invasive cervical cancer tissues from Syrian women. In support of these findings, Schindl *et al.* [12] found that Id-1, but not Id-2 or Id-3, is expressed in the majority of invasive cervical carcinomas. Moreover, in Schindl's study, patients with strong or moderate expression of Id-1 had a significantly shorter overall survival time. Recently, we demonstrated that the presence of high-risk HPVs is correlated with Id-1 expression in human invasive and metastatic breast cancer tissues [25], and that E6/E7 of HPV type 16 regulates Id-1 expression through the activation of its promoter [17]. The present study suggests that Id-1, but not Id-2, Id-3 or Id-4, is a downstream target of E6/E7 of HPV types 16, 18, 31, 33, 35, 45, 51, 52 and 58 in human cervical cancer cells.

In conclusion, high-risk HPV types 16, 18, 31, 33, 35, 45, 51, 52 and 58 were found in the majority of cervical cancers of Syrian women. Furthermore, HPV types 33, 16, 18, 45 and 52 were shown to be the most dominant types of HPV infection found in Middle Eastern women, based on the combination of this investigation and a study by Castellsagué *et al.* [20]. This study has also shown that Id-1 is a target of E6/E7 oncoproteins of HPV type 16, 18, 31, 33, 35, 45, 51, 52 and 58 in invasive cervical cancer cells. These findings provide a new basis for understanding the mechanisms of high-risk HPV infections and their relation to human cervical invasive carcinomas. Therefore, the development of new vaccines that target all of the high-risk HPVs identified in this study will be necessary to prevent cervical cancers and their progression. Current HPV vaccines cover only two high-risk (16 and 18) and two low-risk (6 and 11) types [26]. Moreover, further research is required to gain a better understanding of the association between HPV infection and cervical cancer progression.

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A.D. Darnel, D. Wang and L. Ghabreau contributed equally to this work.

Transparency Declaration

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