Impact of Cyclin D1 deregulation in HPV-mediated squamous intraepithelial lesions based on cell spots analysis

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Summary

Purpose: Human papillomavirus (HPV) involvement in cervical carcinogenesis represents a classical template of analyzing viral-mediated carcinogenesis. Our purpose was to investigate the role of abnormal cyclin D1 protein expression in HPV-mediated squamous intraepithelial lesions (SILs).

Methods: Eighty cases characterized as squamous intraepithelial lesions (SILs) and also borderline cases with molecularly proven HPV infection were examined. Using liquid-based cytology, we constructed 10 slides, each containing 8 cell spots. Immunocytochemistry (ICC) was performed using an anti-Cyclin D1 antibody. Digital image analysis was also implemented for evaluating objectively the protein expression levels on the corresponding stained slides.

Results: Cyclin D1 protein overexpression (moderate to high staining intensity values) was observed in 8/80 (10%) cell spots, whereas low expression rates were detected in 72/80 (90%) cases. Cyclin D1 overall expression was strongly associated with the HPV type group (HR-HPV) of the examined cases (p=0.001) and borderline with the cervical intraepithelial neoplasia (CIN) categorization (p=0.06). Concerning the influence of marker’s protein expression in SIL cytological categorization, no statistical significance was identified (p=0.10).

Conclusions: Cyclin D1 overexpression is observed in a subset of SILs developed by HR-HPV persistent infection in cervical epithelial host cells. Although SIL and CIN categorization seem to be not influenced by cyclin D1 expression levels, mechanisms of gene’s deregulation should be a promising molecular target for discriminating specific genetic signatures in the corresponding initial cervical neoplastic lesions.

Key words: cervical, intraepithelial, neoplasia, cytology, cyclin

Introduction

Extensive molecular analyses have shown that deregulation in crucial cell functions such as cell cycle balance, signaling transduction, apoptosis and angiogenesis leads a normal cell to its neoplastic and finally malignant transformation inducing the formation of cancerous microenvironment [1-3]. Concerning molecules involved in cell cycle control, activation of cyclins stimulates the progression of cell proliferation via interactions with specific catalytic cyclin-dependent kinases (CDKs) [4]. Cyclins D protein family regulate this process through phosphorylation of the retinoblastoma (Rb) protein. In particular, cyclin D1 is a protein of 36 Kd - also known as PRAD1 or bcl 1- encoded by the CCND1 gene located on chromosome 11 (11q13) and acts at the middle of G1 phase by activating CDK4 [5]. Several studies have already shown that the deregulated cyclin D1 is involved...
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in the pathogenesis of different histogenetic origin malignancies, such as breast, head and neck or gastrointestinal and pancreatic cancer [6-9].

Our aim was to investigate the role of abnormal cyclin D1 protein expression in HPV-mediated squamous intraepithelial lesions (SIL) regarding the influence of the marker in their biological behavior.

Methods

Study group

For the purposes of our study, 80 liquid-based (Cell Solutions, Menarini, It) cytological specimens of SILs and some atypical squamous cells of undetermined significance (ASCUS) cases -confirmed and categorized by the Bethesda 2001 (2014 revised) taxonomy system-were used. All these slides were stained by Papanicolaou staining protocol. The mean age of the examined women was 31.5 years (range: 19-53). The Medical School, University of Athens, Greece ethics committee consented to the use of these cytological specimens for research purposes, according to World Medical Association Declaration of Helsinki guidelines. Individuals’ informed consent was also implemented. The corresponding tissues were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. All lesions were classified according to the histological CIN typing criteria of World Health Organization (WHO). Clinicopathological data of the examined cases are demonstrated in Table 1.

Cell spot slides construction

Using liquid-based cytology, we constructed 10 slides, each containing 8 cell spots by rinsing the same quantity of every liquid-based specimen on the slide surface (spot diam: ~0.5 cm). Each slide contained two columns of four spots rows. We observed microscopically that all (n=80) examined SILs/ASCUS cases were represented by one spot (confirmation of the adequacy of the examined specimens) (Figure 1a). Microscopically normal liquid-based cervico-vaginal cell specimens were used as control group.

Antibodies and immunocytochemistry assay (ICC)

Protein expression levels of cyclin D1 were determined by the application of ready-to-use monoclonal antibody: anti-Cyclin D1 (clone DSC6-Novocastra, UK; dilution at 1:30). ICC for cyclin D1 antigen was carried out on the cell spots slides described above. En Vision IHC protocol (DAKO, Glostrup, Dk) was performed using an automated IHC staining system (Microscope Olympus BX-50, Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Measurements were performed in 5 optical fields per case and at a magnification of ×100. Using normal epithelia as control group, we character-sized cyclin D1 expression rates as low, moderate and high. Moderate and high expression levels were considered positive for protein overexpression. Total results are demonstrated in Table 1.

Statistics

Associations between cyclin D1 protein expression levels and pathological/molecular parameters were per-

Figure 1. Cyclin D1 protein expression analysis in SILs. a: A cell spot based cytological slide. Note 8 spots immunostained by applying anti-cyclin D1. b: LSIL case immunostained. Note dark and dense brown nuclear and peri-nuclear staining pattern for cyclin D1 expression. c: Digital image analysis of the same case. Red/green areas represent different levels of cyclin D1 expression (original magnification 100x).
formed by the application of Pearson chi-square ($\chi^2$) test (SPSS v 25.0 statistical software- Inc Chicago IL, USA). Total ICC results and also P values are described in Table 1.

**Results**

According to the protein analysis, all of the examined cases demonstrated cyclin D1 expression in different levels. Cyclin D1 protein overexpression (moderate to high staining intensity values) was observed in 8/80 (10%) cell spots, whereas low expression rates were detected in 72/80 (90%) cases. Cyclin D1 overall expression was strongly associated with the HPV type group (HR-HPV) of the examined cases ($p=0.001$) and borderline with the CIN categorization ($p=0.06$). Concerning the influence of marker’s protein expression in SIL cytological categorization, no statistical significance was identified ($p=0.10$). Furthermore, HPV type categorization (high to low risk) was strongly correlated to cytological taxonomy provided by Bethesda 2001, revised 2014 ($p=0.001$). Additionally, CIN categorization was strongly associated to SIL cytological estimation in the corresponding lesions ($p=0.001$). Normal cervico-vaginal cells demonstrated low cyclin D1 expression staining intensity values.

**Discussion**

In the current study we explored the role of cyclin D1 aberrant expression in cell spot slides containing a variety of SILs and some borderline cases cytologically described as ASCUS. Based on our results we detected moderate to high expression in a limited proportion of them. Cyclin D1 was associated with the HPV type group demonstrating overexpression in HR-HPV depended cases. Similar molecular analyses have shown that besides cyclin D1 other genes such as ovarian cancer gene 1 (OVCA1) are implicated in cervical cells genetic deregulation modifying the activity of cyclin D1 and p16 suppressor gene. A study focused on the OVCA1 gene activation showed that its protein overexpression was correlated to HR-HPV infection and increased mRNA levels were detected during the development of cervical lesions, particularly in the early stages [10]. Concerning cyclin D1 protein expression, some studies demonstrated controversial results. Based on an immunohistochemistry (IHC) assay, a research group observed that cyclin D1 expression showed a significant decrease in severe lesions including HSIL and CIN II/III correlated also with the aggressiveness of HPV types, especially HPV 16 [11]. In the same study, p16, p53, ki 67 and p21 analyses showed that increased protein expression was associated also with high grade lesions. Additionally, another study based on IHC analysis of uterine cervix adenocarcinomas showed that in HPV-16 positive cases a strong association with the cyclin D1 overexpression was assessed [12]. Furthermore, the researchers observed that Notch signaling proteins (JAG1/Notch-3) were influenced by cyclin D1 aberrant expression. Similarly, another study group concluded that the role
of cyclin D1 is critical for modifying the invasive potential in cervical adenocarcinomas [13]. Concerning interactions with other genes, there are molecular data implicating apoptotic proteins such as Nuclear Factor kappaB (NF-kB) family in the deregulation of cyclin D1 expression by stimulating its transcription in CINs and cervical carcinoma [14]. Based on a tissue microarray IHC analysis of several genes including hTERT, PIK3CA, hTERC, MYC, cyclin D1, BCL2, ZNF217 and p16, a study group observed that aberrant cyclin D1 expression was associated with the evolution of normal tissue to CIN I. They also suggested that myc and cyclin D1 overactivation are early genetic abnormalities in the progression of cervical carcinoma due to dysplastic epithelial lesions [15]. In contrast to these histo-molecular findings, two other studies suggest that cyclin D1 deregulation should be considered as a late genetic event in this process [16,17]. Another interesting observation regarding cyclin D1 aberrant expression in SILs and the corresponding CINs is the pattern of expression. A study group focused on this and reported that combined nuclear and cytoplasmic expression of the molecule seemed to be related to HPV high risk infection and also the aggressiveness of the lesion [18]. In our study, a subset of cases demonstrated also this expression profile. Finally, genetic studies focused on specific single nucleotide polymorphisms (SNPs) that modify cyclin D1 function associated also with genetic susceptibility to cervical cancer in populations, such as Chinese and Swedish (CCND1 rs9344), or Indian (G870A and G1722C), respectively [19-21].

In conclusion, cyclin D1 overexpression is observed in a subset of SILs developed by HR-HPV persistent infection in cervical epithelial host cells. Although SIL and CIN categorization seem to be not influenced by cyclin D1 expression levels, mechanisms of gene’s deregulation should be a promising molecular target for discriminating specific genetic signatures in the corresponding early stage cervical neoplastic lesions (SILs/CINs).

Acknowledgements

The authors would like to thank Mr George Vilas, Technologist in Dept of Pathology, Medical School, University of Athens, Greece, for providing the ICC procedure on cell spot and conventional (control) cytological slides.

Conflict of interests

The authors declare no conflict of interests.

References