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ORIGINAL ARTICLE

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 cor¬responding 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) exam¬ined 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 (I 6000-Biogenex, USA). This ICC protocol is based on a water soluble dextran polymer system, preventing the endogenous biotin reaction, which is responsible for the background in the stained slides. Briefly, the sections were incubated with the primary antibody for 30 min at room temperature and then incubated with the polymer for 30 min. The antigen-antibody reaction was visualized using 3-3,diaminobenzidine tetrahydrocloride (DAB) as a chromogen substrate. Finally, the corresponding slides were slightly counterstained with hematoxylin for 30 sec, dehydrated and mounted. For negative control slides, the primary antibodies were omitted. According to the manufacturer's guidelines, breast cancer tissue samples previously reported to strongly express cyclin D1 were used as positive controls of the nuclear staining pattern. Nuclear staining pattern was considered acceptable for the evaluation of ICC specificity (Figure 1b). Pale brown peri-nuclear cytoplasmic staining expression pattern was also observed in some cases.

Digital image analysis (DIA)

Cyclin D1 protein expression was evaluated by measuring the corresponding staining intensity levels provided by digital image analysis (Figure 1c). We performed DIA based on a semi-automated 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¬ized 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-

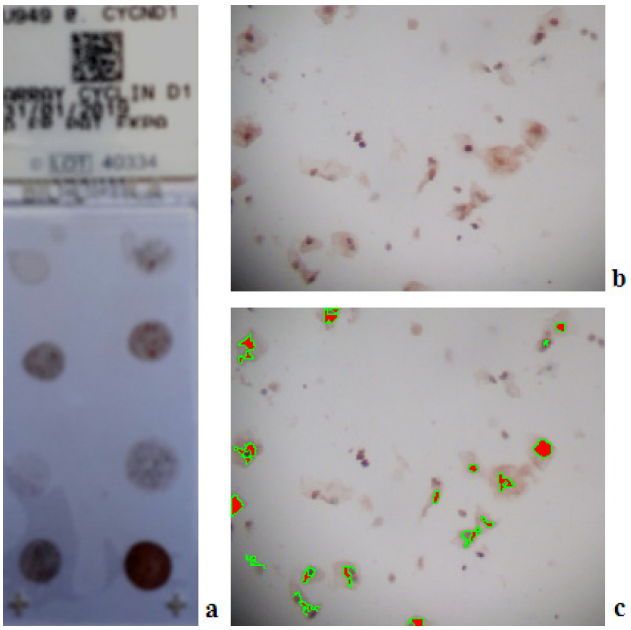

Figure 1. Cyclin D1 protein expression analysis in SILs. **a:** A cell spot based cytological slide. Note 8 spots immunostained by applying anti-cyclin D1. **b:** LSIL case immunostained. Note dark and dense brown nuclear and perinuclear staining pattern for cyclin D1 expression. **c:** Digital image analysis of the same case. Red/green areas represent different levels of cyclin D1 expression (original magnification 100x).

Table 1. Pathological/molecular features and total Cyclin D1 expression results

Clinicopathological parameters		*Cyclin D1*		*p value*
		OE	*LE*	
cases	(n=80) n (%)	8/80 (10%) n (%)	72/80 (90%) n (%)	
HPV type				0.001
HR	13/80 (16.2)	8/80 (10)	5/80 (6.25%)	
LR	67/80 (83.8)	0/80 (0)	67/80 (83.75)	
Cytological category				0.10
LSIL	66/80 (82.5)	6/80 (7.5)	60/80 (75)	
HSIL	6/80 (7.5)	2/80 (2.5)	4/80 (5)	
ASCUS	8/80 (10)	0/80 (0)	8/80 (10)	
CIN category				0.06
CIN I	73/80 (91.25)	5/80 (6.25)	68/80 (85)	
CIN II	5/80 (6.25)	2/80 (2.5)	3/80 (3.75)	
CIN III	2/80 (2.5)	1/80 (1.25)	1/80 (1.25)	

HR: High risk HPV, LR: Low Risk HPV, OE: Overexpression (Moderate to high expression) staining intensity values ≤138 at ≥50% stained nuclei, LE: Low expression (low expression) staining intensity values >146 at ≥50% stained nuclei, LSIL: Low grade squamous intraepithelial lesion, HSIL: High grade squamous intraepithelial lesion, ASCUS: Atypical squamous cells of undetermined significance, CIN: Cervical intraepithelial neoplasia

formed by the application of Pearson chi-square (x^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 cytologicaly 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's 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).

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Conflict of interests

The authors declare no conflict of interests.

References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell 2011;144:646-74.
2. Stratton MR, Campbell PJ, Futreal1 AP. The cancer genome. Nature 2009;458:719-24.
3. Polyak K, Haviv I, Campbell IG. Co-evolution of tumor cells and their microenvironment. Trends Genet 2009;25:30-8.
4. Loden M, Stighall M, Nielsen NH et al. The cyclin D1 high and cyclin E high subgroups of breast cancer: separate pathways in tumorigenesis based on pattern of genetic aberrations and inactivation of the pRb node. Oncogene 2002;21:4680-90.
5. Al-Aynati MM, Radulovich N, Ho J, Tsao MS. Overexpression of G1-S cyclins and cyclin-dependent kinases during multistage human pancreatic duct cell carcinogenesis. Clin Cancer Res 2004;10:6598-605.
6. Gillet C, Fantl V, Smith R et al. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res 1994;54:1812-7.
7. Loden M, Stighall M, Nielsen NH et al. The cyclin D1 high and cyclin E high subgroups of breast cancer: separate pathways in tumorigenesis based on pattern of genetic aberrations and inactivation of the pRb node. Oncogene 2002;21:4680-90.
8. Poch B, Gansauge F, Schwarz A et al. Epidermal growth factor receptor induces cyclin D1 in human pancreatic carcinoma: evidence for a cyclin D1-dependent cell cycle progression. Pancreas 2000;23:280-7.
9. Tsiambas E, Karameris A, Gourgiotis S et al. Simultaneous deregulation of p16 and cyclin D1 genes in pancreatic ductal adenocarcinoma: a combined immunohistochemistry and image analysis study based on tissue microarrays. JBUON 2007;12:261-7.
10. Tong R, Yang Q, Wang C, Bi F, Jiang B. OVCA1 expression and its correlation with the expression levels of cyclin D1 and p16 in cervical cancer and intraepithelial neoplasia. Oncol Lett 2017;13:2929-36.
11. Portari EA, Russomano FB, de Camargo MJ et al. Immunohistochemical expression of cyclin D1, p16Ink4a, p21WAF1, and Ki-67 correlates with the severity of cervical neoplasia. Int J Gynecol Pathol 2013;32:501-8.
12. Tripathi R, Rath G, Jawanjal P, Bharadwaj M, Mehrotra R. Cyclin D1 protein affecting global women's health by regulating HPV mediated adenocarcinoma of the uterine cervix. Sci Rep 2019;9:5019-23.
13. Balan R, Căruntu ID, Amălinei C. The immunohistochemical assessment of HPV related adenocarcinoma: pathologic and clinical prognostic significance. Curr Pharm Des 2013;19:1430-8.

14. Tilborghs S, Corthouts J, Verhoeven Y et al. The role of Nuclear Factor-kappa B signaling in human cervical cancer. Crit Rev Oncol Hematol 2017;120:141-50.
15. Costa C, Espinet B, Molina MA et al. Analysis of gene status in cervical dysplastic lesions and squamous cell carcinoma using tissue microarrays. Histol Histopathol 2009;24:821-9.
16. Bahnassy AA, Zekri AR, Saleh M et al. The possible role of cell cycle regulators in multistep process of HPV-associated cervical carcinoma. BMC Clin Pathol 2007;7:4-9.
17. Bahnassy AA, Zekri AR, Alam El-Din HM et al. The role of cyclins and cyclins inhibitors in the multistep process of HPV-associated cervical carcinoma. J Egypt Natl Cancer Inst 2006;18:292-302.
18. Carreras R, Alameda F, Mancebo G et al. A study of Ki-67, c-erbB2 and cyclin D-1 expression in CIN-I, CIN-III and squamous cell carcinoma of the cervix. Histol Histopathol 2007;22:587-92.
19. Wang N, Qian X, Wang S et al. CCND1 rs9344 polymorphisms are associated with the genetic susceptibility to cervical cancer in Chinese population. Mol Carcinog 2012;51:196-205.
20. Castro FA, Haimila K, Sareneva I et al. Association of HLA-DRB1, interleukin-6 and cyclin D1 polymorphisms with cervical cancer in the Swedish population--a candidate gene approach. Int J Cancer 2009;125:1851-8.
21. Thakur N, Hussain S, Kohaar I et al. Genetic variant of CCND1: association with HPV-mediated cervical cancer in Indian population. Biomarkers 2009;14:219-25.