The expression of cell cycle regulators in HPV - induced cervical carcinogenesis

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Abstract

HPV (human papillomavirus) infection is associated with cervical carcinogenesis through an interaction between HPV oncogenic proteins and cell cycle regulators. However, the exact pathogenic mechanisms are not determined yet and therefore in the present study we evaluated the possible role of A1, B1, D1, E1 cyclins in a cohort of cervical cancer and cervical intraepithelial neoplasia (CIN) patients with or without HPV infection.

In order to evaluate their role in cervical neoplasia, cervical tissue specimens from 140 women were investigated. HPV genotyping was performed using Linear Array HPV Genotyping Test (Roche) according to the manufacturer’s instructions. In order to evaluate the gene expression levels of A1, B1, D1, E1 cyclins, isolated RNAs were reversed-transcribed and assayed in Taq-Man qPCR (Applied Biosystems).

All abnormal biopsies presented different levels of cyclins expression regardless of the lesion severity. There were statistically significant differences between the levels of cyclins expression in CIN1 HPV positive and negative cases. In HPV positive samples, cyclins E1 and B1 were upregulated compared with the HPV negative biopsies (p=0.001/p=0.0032). In SCC (squamous cell carcinoma) cases, cyclin D1 expression was significantly downregulated in HPV positive cases as compared with HPV negative cases (p=0.0002). Similarly, there was a significant difference (p=0.005) in the expression of cyclin B1.

We conclude that infection of cervical mucosa by high-risk HPV leads to deregulation of the cell cycle via altering the expression level of certain genes. Our results also provide evidence that cyclin expression could help in early diagnosis of cervical carcinoma.

Keywords: cervical cancer, HPV, cyclins, cell cycle

Introduction

Cervical carcinoma is one of the most common types of cancer worldwide and one of the leading causes of death from cancer among women. High risk human papillomaviruses (hrHPV) are recognized as the sexually transmitted etiological agents of cervical neoplastic lesions and subsequently cervical cancer [1, 2]. The presence of HPV infection has been implicated in more than 90% of cervical cancers. Even with Pap screening programs, a significant number of women die from cervical carcinoma each year. There are more than 100 different genotypes of human papillomaviruses, and more than 30 different HPV types infect the human genital mucosa [3].

Although the majority of HPV infections clear spontaneously, persistent infection with hrHPV is a significant risk factor for cervical malignancy. Progress in cervical cancer research provided evidence that HPV E6 and E7 proteins can act as oncogenes. Expression of
E6 and E7 proteins of hrHPV induces immortalization of cells through their inhibitory effects on the tumour suppressor proteins pRb and p53 and disturbing cell cycle control [4, 5].

Recent data show an association between hrHPV genotypes and cell cycle regulators [6]. The cell cycle is governed by a family of cyclins, cyclin dependent kinases (CDKs) and their inhibitors (CDKIs) through activating and inactivating phosphorylation events. Key among these are p53, the cdk inhibitors (p15, p16, p18, p19, p21, p27) and Rb, all of which act to keep the cell cycle from progressing until all repairs to damaged DNA have been completed. The periodic appearance of cyclins and CDKs in distinct phases of the cell cycle suggests that they could be used as markers for cell proliferation in cervical malignancy [7]. The D-type cyclins reach maximum levels of expression and form functional kinase complexes with CDK4 or CDK6; during the mid-G1 phase, whereas cyclin-E is expressed and associated with CDK2 in an active complex near the G1-S boundary [8, 9]. Cyclin B is a member of the cyclin family of proteins whose levels vary during the cell cycle in order to activate specific CDKs required for the proper progression through the cell cycle. Cyclin B protein begins to increase during G2, peaks in mitosis, and is rapidly degraded before the cell cycle is completed. Cyclin E binds to G1 phase CDK2, which is required for the transition from G1 to S phase. The Cyclin E/CDK2 complex phosphorylates p27, tagging it for degradation, thus promoting expression of cyclin A, allowing progression to S phase [10, 11].

Defects in many of the molecules that regulate the cell cycle have been implicated in cancer [12].

HPV infection is associated with cervical carcinogenesis through an interaction between HPV oncogenic proteins and cell cycle regulators. However, the exact pathogenic mechanisms are not determined yet and therefore in the present study we evaluated the expression of A1, B1, D1, E1 cyclins in a cohort of women with cervical lesions and with or without HPV infection.

**Materials and methods**

**Study group.** 140 women (29-65 years old) who referred to the Gynecology Clinic for the management of abnormal cervical cytology were recruited with written informed consent. In order to evaluate the cyclins expression in cervical neoplasia progression, cervical tissue specimens from 60 squamous cell carcinoma patients who underwent hysterectomy were investigated. 30 patients with CIN1 and 30 patients with CIN2/3 lesions were included in the study. A total of 20 women with normal biopsies who underwent hysterectomy for other causes than cervical cancer (and without HPV infection) were included in the negative control group. The study protocol was approved by the local institution ethics committee.

**Nucleic acids isolation.** Total genomic DNA and RNA were isolated (using High Pure PCR Template, Roche and Trizol reagent, Life Technologies, respectively) from the tissue samples from patients enrolled in the study. DNA was released by lysing tissue specimens under denaturating conditions at elevated temperatures. Lysis was performed in the presence of proteinase K, chaotropic agent and detergent and was followed by isolation and purification of DNA over a column and elution with elution reagent. RNA was released maintaining its integrity, while Trizol reagent disrupted cells and dissolved cell components. Addition of chloroform followed by centrifugation separated the solution into an aqueous phase and an organic phase. RNA remained exclusively in the aqueous phase. After transfer of the aqueous phase, RNA was recovered by precipitation with isopropyl alcohol. The quality of isolated RNAs and DNAs was assessed by determining the concentration and purity using Nanodrop spectrophotometer and RIN (RNA Integrity Number) values.
**HPV DNA Detection and Genotyping.** HPV genotyping was performed using Linear Array HPV Genotyping Test (Roche) according to the manufacturer’s instructions. This test uses biotinylated primers to define a sequence of nucleotides within the polymorphic L1 region of the HPV genome of approximately 450 base pairs long. A pool of HPV primers present in the Master Mix is designed to amplify HPV DNA from 37 high- and low-risk human papillomavirus genotypes, including those considered a significant risk factor for precursor cervical lesions progression to cervical cancer. An additional primer pair targets the human β-globin gene to provide a control for cell adequacy, extraction and amplification. The PCR reaction and HPV detection and genotyping were performed according to the manufacturer’s instructions. In each experiment negative and positive controls were used in order to validate the PCR method. Denatured biotinylated amplicons were hybridized with specific oligonucleotide probes immobilized on a strip. Incubation with the chromogen gave a blue/dark blue precipitate and results were interpreted visually.

**RT-PCR assay.** Total isolated RNA was reverse-transcribed using Promega reagents. Briefly, 2.5 μg of RNA, 0.5 μg/μl oligodT primer and 10 mM dNTPs were mixed and incubated 5 minutes at 65°C. After cooling on ice, 4 μl MuMLV RT buffer (5x), 200 mM DTT and 40U RNase inhibitor (RNAsin) were added to the mix. After 2 minutes incubation at 37°C, 200U of MuMLV reverse transcriptase was added and the incubation proceeded for 60 minutes at 37°C and 15 minutes at 70°C. In order to determine cDNA quality, a RT-PCR assay for β2-microglobulin house-keeping gene was performed on all samples. RT-PCR assay was performed by using primers synthetised by Invitrogen (forward primer: 5’-ACCACAGTCCATGCATCAC-3’, reverse primer: 5’- TCCACTACCCT GATGCT GTA-3’, amplicon size 148 bp). The primers were designed to discriminate between genomic and cDNA yielding 2 different amplicons with 2 different molecular weights (genomic DNA-772 bp and cDNA-148 bp). Reaction mix consisted in 100 ng cDNA, PCR buffer 1x, 1,5mM MgCl2, 400 pmols of each primer, 200 μM of each nucleotide and 2 units of Taq polimerase (Promega reagents). Thermocycling conditions were: 95°C for 5 minutes, 85°C for 1 minute, 47°C for 1 minute, 72°C for 1 minute (35 cycles) and a final elongation at 72°C for 1 minute. The results obtained were analyzed after agarose gel electrophoresis (2%) and ethidium bromide staining, using UVP BioDoc-It analysis program, Biometra.

**Quantitative Real-time PCR Assays.** In order to evaluate the expression levels of A1, B1, D1, E1 cyclins in cervical cancer, real-time qPCR was performed. PCR primers and TaqMan probes targeting the selected genes were designed using Primer Express 2.0 software (Applied Biosystems). Amplification products were detected using ABI prism 7300 Real-Time PCR instrument and Sequence Detection System software. Initial template concentration was calculated from the cycle number when the amount of PCR product passed a threshold set in the exponential phase of the PCR reaction. Thermocycling conditions were: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. Each 25-μl reaction contained 1X TaqMan Universal MasterMix, forward and reverse primer (15 pmol each) and TaqMan probe (10pmol). DNA template was 100 ng. To check for amplicon contamination, every run contained at least one “no template” control in which nuclease-free water was substituted for template. The threshold cycles (Ct) were recorded for the target gene and reference in all the samples and the four targets were analyzed separately. As positive controls, cDNA from HeLa and CaSki cell lines were used. Samples were tested in duplicate in each Q-PCR assay and detected on the FAM channel. The results were quantified and normalized using β-actin house keeping gene detected on the VIC channel.

**Statistical analysis.** Ct values obtained from the duplicate tests were averaged for calculations. Mann-Whitney test were used to determine differences in Ct values between women with normal histology (reference group) and women with CIN1, CIN2/3 and SCC.
Results

**HPV DNA Detection and Genotyping.** In the present study 140 tissue specimens were selected for HPV DNA testing; of these, 60 originated from patients with squamous cell carcinoma (SCC), 30 from patients with CIN1 lesions, 30 from patients with CIN2/3 lesions and 20 from patients without cervical lesions. The presence of the most frequently found high-risk HPV in single infections in the study group is presented below, in table 1. Furthermore, our data revealed the fact that 66.6% (20/30 cases) of CIN1 patients were positive for HPV DNA, present in single or co-infections. The most prevalent HPV genotypes in this group of patients were HPV6 (20%), HPV11 (13.3%), HPV51 (13.3%) and HPV18 (6.6%) present in single infections. On the other hand, HPV31, 53, 54 were found in multiple infections. In CIN2/3 patients group, 80% (24/30 cases) of the women presented HPV DNA in single infection (66.5%) or co-infection (33.5%). HPV16 (33.3%) and HPV31 (20%) were the most prevalent high-risk genotypes in CIN2/3 patients. Regarding HPV infection in cervical cancer patients group, 86.6% (52/60 cases) of the patients presented either infection with a single high risk HPV genotype (60%) or a co-infection with multiple HPV genotypes (26.6%). HPV 16 genotype was present in 66.6% of the cases in single infection (40%) or co-infection with various genotypes (HPV 18, 45, 53). Regarding HPV 18 type, we also found it present in single infections (20% of cases) and in multiple infections (19.9%) (Tables 1 and 2).

Table 1. The presence of high-risk HPV in single infections in the study group

<table>
<thead>
<tr>
<th>HPV presence</th>
<th>CIN 1</th>
<th>CIN2/3</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV negative</td>
<td>33.3%</td>
<td>20.0%</td>
<td>13.3%</td>
</tr>
<tr>
<td>HPV 16</td>
<td>0.0%</td>
<td>33.3%</td>
<td>40.0%</td>
</tr>
<tr>
<td>HPV 18</td>
<td>6.6%</td>
<td>6.6%</td>
<td>20.0%</td>
</tr>
<tr>
<td>HPV 31</td>
<td>0.0%</td>
<td>20.0%</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

Table 2. The presence of HPV DNA in co-infections in the study group

<table>
<thead>
<tr>
<th>Lesion grade</th>
<th>HPV genotypes</th>
<th>presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC</td>
<td>HPV16,18,53</td>
<td>6.6%</td>
</tr>
<tr>
<td></td>
<td>HPV16,18</td>
<td>13.3%</td>
</tr>
<tr>
<td></td>
<td>HPV16,45</td>
<td>6.6%</td>
</tr>
<tr>
<td>CIN2/3</td>
<td>HPV16,58</td>
<td>5.6%</td>
</tr>
<tr>
<td></td>
<td>HPV45,58,68</td>
<td>6.6%</td>
</tr>
<tr>
<td>CIN1</td>
<td>HPV11,31,53,54</td>
<td>7.5%</td>
</tr>
</tbody>
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Cyclins mRNA expression. The aim of our study was to investigate the possible cyclin expression modifications in HPV positive and negative tissue samples. The data regarding the normalized cyclin gene expression in HPV positive vs. HPV negative cervical biopsies (CIN1, CIN2/3, SCC cases) are shown in figure 1. All abnormal biopsies presented different levels of cyclin expression regardless of the lesion severity. The cyclins expression was
double normalized using β-actin as house-keeping gene and the cyclin gene expression mean value in negative control group. There were statistically significant differences between the levels of cyclin expression in CIN1 HPV positive and negative cases. As shown below, we noticed the fact that cyclin A1 presented a significant downregulation (p=0.0025) in HPV negative samples. In HPV positive samples, cyclins E1 and B1 were upregulated compared with the HPV negative biopsies (p=0.001/p=0.0032). Regarding CIN2/3 cases, there were no significant differences in cyclins expression in HPV positive and negative patients. All the studied cyclins were upregulated with the mention that HPV positive cases were slightly more upregulated but we failed to find any statistically significant expression modifications. On the other hand, in SCC cases, cyclin D1 expression was significantly downregulated in HPV positive cases as compared with HPV negative cases (p=0.0002). Similarly, there was a significant difference (p=0.005) in the expression of cyclin B1.

Regarding the HPV positive group, our results showed significant differences between cyclin expression in CIN1, CIN2/3 and SCC patients. Cyclin A1 was significantly underexpressed in CIN1 patients as compared with CIN2/3 and SCC patients (p=0.002). Regarding cyclin E1 and B1, we also noticed differences in the expression patterns. Accordingly, we noticed an important upregulation of cyclin E1 and B1 in SCC group as compared with CIN1 and CIN2/3 women. An important aspect is cyclin D1 downregulation in SCC cases compared to CIN2/3 (p=0.005).

Figure 1. Normalized cyclin A1, E1, B1 and D1 gene expression in HPV positive vs. HPV negative cervical biopsies (CIN1, CIN2/3, SCC cases). The normalization was performed using β-actin house-keeping gene and the negative control group.)
Discussion

Studies demonstrated that HPV DNA presence is necessary for the development and persistency of cervical neoplasia and DNA disappearance anticipates the regression of neoplastic cells, even in advanced cervical lesions. Even though cervical lesions may be detected on cervical smears several years before the onset of invasive squamous carcinoma, there is no morphologic criterion that may predict if a cervical lesion will disappear or will progress toward cancer. High risk DNA HPV detection in such lesions selects women with high risk for cervical cancer development.

Literature data show that HPV 16, 18, 45, 31 and 33 are the most prevalent types found in cervical cancer, and researchers demonstrated that genotypes 16 and 18 account for over 70% of cervical cancers worldwide [1]. Our findings support the fact that the presence of HPV DNA in cervical cell specimens as well as the prevalence of HPV 16 and 18 genotypes could confirm them as significant prognostic factors in cervical cancer development. The detection of HPV 16 and 18 genotypes might play an important role in assessing prognosis of cervical lesion patients.

Despite its strong association with cervical cancer, HPV infection alone is not sufficient for the cervical epithelium to develop an invasive cervical cancer. Persistent HPV infection contributes to the development of squamous intraepithelial lesions, with viral oncoproteins facilitating the deregulation of cellular proliferation and the apoptotic process. Numerous researchers focused on the study of altered expression of G1 cyclins and CDKs because the major regulatory events leading to cell proliferation and differentiation occur within the G1 phase of the cell cycle [13, 14]. To our knowledge, this is the first study to investigate the expression of cell cycle regulatory genes in Romanian patients with precursor cervical lesions and cervical carcinoma at gene level.

G1 cyclins, as candidate proto-oncogenes, play a key role as a cellular regulator through direct interaction with retinoblastoma gene product (pRb) and cyclin-dependent kinase [15]. E6/E7 HPV modify cyclin expression therefore the rationale for selecting A1, E1, B1 and D1 cyclins genes is that E7 of the oncogenic HPV types binds to the pocket proteins (p105, p107, and Rb2/p130), leading to abrogation of their growth-suppressive properties. Similarly, E7 increases free E2F by disruption of E2F-pocket protein-complexes and phosphorylation and degradation of Rb, leading to anchorage-independent cell proliferation [16].

Because it is not clearly understood, there are controversial results on the role of cyclin D1 in cervical carcinogenesis and clinical outcome. The hypophosphorylated form of Rb, complexed with E2F serves as activator of cyclin D1 transcription by binding to its promoter. This drives cyclin D1 in the early and mid G1 phase of the cell cycle [17, 18]. Since D type cyclins and HPV E7 possess similar binding regions for pRb and pRb related pocket proteins, inactivation of pRb either by the cyclin/CDK complexes in G1 or by interaction with the high-risk HPV oncoprotein E7 may result in a decreased expression of cyclin D1. In this study, we found significantly lower cyclin D1 expression in HPV-positive cervical lesions compared to HPV-negative cases and normal cervical epithelium. Our data are in concordance with Bae et al. [19] who reported reduced cyclin D1 mRNA and protein expression in cases of CIN2/3 and SCC compared to normal control. Therefore, cyclin D1 expression may be a useful molecular marker in predicting unfavourable prognosis of cervical lesion progression.

On the other hand, other studies have also shown that in high-grade cervical precancerous lesions, cyclin B1 expression was up-regulated and persists into the upper epithelial layers [20, 21]. This suggests that cyclin B1 could play a crucial role in the early phase of cervical carcinogenesis. Our results are in concordance with this and furthermore indicated that cyclins B1, E1 and A1 are very often found to be overexpressed in cervical
cancer patients with an associated HPV infection and their expression is correlated with cervical lesion progression. As key cell cycle modulators of the G2-M transition, these cyclins are considered to play an important role in human neoplasia development [22, 23]. The slightly different levels of expression in patients with detected levels of HPV DNA observed in our study group sustain the idea according to which cyclin deregulation may play a role in uncontrolled proliferation and malignant transformation of the uterine cervix.

We noticed a close association between oncogenic HPV and cyclin A1 especially in CIN 1 and CIN2/3 cases. This could be explained by its complex up-regulation by both E7 (during the G1-S transition) and E6 (in G2-M transition) oncoproteins, making cyclin A active in both S phase and late G2 phase of the cell cycle [24, 25]. Not unexpectedly, cyclin A1 is a useful marker of cell proliferation most notably orchestrated by the capability of E7 to abrogate the inhibitory activity of p21CIP1/WAF1/SDI1 on CDK and proliferating cell nuclear antigen–dependent DNA replication necessary for the G1-S transition.

The interactions of viral proteins with host cellular proteins, particularly cell cycle proteins, are involved in the activation or repression of cell cycle progression in cervical carcinogenesis. Our results were consistent with the concept that upregulation of these cyclins by E6/E7 viral oncoproteins occurred in cervical cancer and their aberrant expression might play an important role in cervical carcinogenesis. In summary, this study demonstrated that cyclins mRNA expression was significantly increased in invasive cervical carcinomas compared with normal cervix. This change may play a role in uncontrolled proliferation and malignant transformation of the uterine cervix.

Conclusion

We conclude that infection of cervical mucosa by high-risk HPV leads to deregulation of the cell cycle via altering the expression level of certain genes. Our results also provide evidence that cyclin expression could help in early diagnosis of cervical carcinoma since alterations affecting the expression level of these genes occur at an early stage of cervical carcinogenesis. Therefore they could be used as possible markers for early detection of SCC and for monitoring patients with cervical dysplasia. However, further work on larger series of cases is needed in order to confirm the results of the present study.

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References


