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p14^{ARF} and p16^{INK4A}, two products of the same gene, are differently expressed in cervical intraepithelial neoplasia

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Abstract

Objective. To study the expression patterns of two different tumor suppressor proteins p16^{INK4A} and p14^{ARF} in cervical lesions. Both proteins are encoded by the same INK4A/ARF gene on chromosome 9p21. The expression patterns of these two proteins, both playing a central role in the cell cycle, were analyzed in detail in CIN, carcinomas, and normal epithelium to test the hypothesis that p16^{INK4A} positive cells also demonstrate p14^{ARF} expression.

Methods. Serial tissue sections of 9 CIN1 lesions, 10 CIN2 lesions, 12 CIN3 lesions, and 7 carcinomas were stained with monoclonal antibodies against p16^{INK4A} and p14^{ARF}. The short fragment polymerase chain reaction hybridization line probe assay was used to detect HPV.

Results. Normal epithelium was negative for both proteins. Marked immunoreactivity (++) for p16^{INK4A} and p14^{ARF} was observed in 5/7 carcinomas, 10/12 CIN3, and 1/10 CIN2 lesions and 0/9 CIN1 lesions. Simultaneous expression (+ or ++) was found in 19/22 CIN2/3 and not in CIN1 lesions. The fraction of p16^{INK4A}-stained cells increased with CIN-grade. Overexpression of p14^{ARF} was observed in a subpopulation of p16^{INK4A} positive cells, and exclusively found in lesions infected with high-risk HPV. In two CIN3 lesions with early stromal invasion, p14^{ARF} positivity was mainly found in the invasive cells. In carcinomas, all cells showed p16^{INK4A} expression, whereas p14^{ARF} was limited to the peripheral cells of the invasive tumor nests and individual migrating tumor cells.

Conclusions. Overexpression of p14^{ARF} is limited to a fraction of the p16^{INK4A}-expressing cells and therefore it is likely that p14^{ARF}− and p16^{INK4A} expression are not induced by the same mechanisms. Before expression of p14^{ARF} can be linked to invasion or invasive phenotype, larger series of (micro-) invasive squamous lesions need to be studied.

Keywords: p14^{ARF}; p16^{INK4A}; CIN; Cervical carcinoma; HPV; Invasive behavior

Introduction

High-risk human papillomavirus (HPV) types are involved in cervical carcinogenesis and integration of HPV in the human genome results in overexpression of the viral oncogenic proteins E6 and E7 [1–7]. The E6 protein binds to the p53 tumor suppressor protein which results in its degradation, and subsequently in the loss of genetic integrity [5–8]. The E7 protein inactivates the tumor suppressor protein pRb by hyperphosphorylation of pRb, and subsequently this results in a release of the transcriptional factor E2F from the pRb–E2F complex [5–7]. The E2F transcription factor allows the cell to enter the S-phase [9]. The ability of E2F to induce cyclin E, which in turn regulates CDK2 to enforce pRb phosphorylation, creates a positive feedback loop that helps contribute to irreversibility of the G1/S transition and to hyperproliferation of the affected cells [9].

E2F-accumulation also leads to induction of p16^{INK4A} activity, a member of the INK4 family of cell-cycle inhibitors [10–12]. Recently, it was shown that overexpression of p16^{INK4A} is highly associated with infection of high-risk HPV types, and that p16^{INK4A} may be used as a sensitive biomarker to identify dysplastic and neoplastic epithelial cells of the uterine cervix [13,14].

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The INK4A/ARF gene on chromosome 9p21 has two promoters and it encodes two completely different proteins, $p16^{INK4A}$ and $p14^{ARF}$ [15,16]. The latter does not bind to any of the cyclin–CDK complexes, but seems to have a tumor suppression function dependent upon the presence of p53. $p14^{ARF}$ binds to Mdm2 and stimulates its degradation which results in stabilization of p53 [16]. We expected an enhanced expression of $p14^{ARF}$ in high-grade CIN lesions and carcinomas, because $p14^{ARF}$ is up-regulated by E2F, and the inhibitory effect of p53 on the expression of $p14^{ARF}$ is lost due to degradation of p53 by the viral oncoprotein E6 [15]. Preliminary results indicated that, at least in some HPV-positive cell lines, $p14^{ARF}$ levels are indeed increased significantly [17]. Recently, it was shown that, in most cervical cancers and pre-malignant lesions infected with high-risk HPV, a marked overexpression of both $p14^{ARF}$ and $p16^{INK4A}$ is present [18].

The aims of this study were: (1) to investigate the relation between the expression of $p14^{ARF}$ and $p16^{INK4A}$ in normal cervical epithelium, cervical intraepithelial neoplasia (CIN), and carcinomas in order to test our hypothesis that these two proteins are co-expressed by dysplastic cells or tumor cells and (2) to assess the expression of $p14^{ARF}$ and $p16^{INK4A}$ in relation to the presence of high-risk HPV types.

Material and methods

Patients

Paraffin-embedded tissue sections of 31 CIN lesions (9 CIN1, 10 CIN2, and 12 CIN3) and 7 carcinomas obtained from the archives of the Institute of Pathology of the University Medical Centre, Nijmegen were used. Two of the 12 CIN3 lesions also exhibited early stomal invasion.

Four-micrometer thick hematoxylin and eosin-stained sections were used for grading of CIN lesions. All lesions were graded independently by two experienced pathologists according to the generally accepted criteria as CIN1, CIN2, CIN3, and carcinoma [19]. There was agreement concerning the CIN-grade between them in 29 cases (94%). In only 2 cases (6%), the diagnosis differed one CIN-grade and in these two cases a consensus diagnosis was reached by consulting a third pathologist. In addition, the proliferation fraction (Ki-67 labeling index, as previously described), was measured in immunohistochemically Ki-67-stained parallel sections to confirm the histomorphological grade of the CIN lesions [20,21]. The proliferation fraction was in accordance with the final CIN-grade in all cases.

Immunohistochemistry

Four-micrometer thick paraffin sections were mounted onto polylysine-coated slides and dried overnight at 58°C. Sections were dewaxed in xylene and endogenous peroxidase was blocked using H_2O_2 in methanol for 15 min and the slides were rinsed three times in phosphate-buffered saline (PBS, pH 7.4) for 5 min. The slides were placed in a citrate buffer (10 mM, pH 6.0) and heated in a household microwave oven (3 min at 850 W until boiling; followed by 10 min at 150 W). Subsequently, the sections were allowed to cool down to room temperature (RT) and slides were briefly washed with phosphate-buffered saline (PBS, pH 7.4). An indirect immunoperoxidase technique was used to visualize the $p16^{INK4A}$ and $p14^{ARF}$-expressing cells, following the next procedure. The slides were pre-incubated with 20% normal horse serum and then incubated with $p16^{INK4A}$ (clone 16PO4, Neomarkers, Fermont, CA, USA) 1:100 in PBS (60 min, RT) and $p14^{ARF}$ (clone 14PO2, Neomarkers, Fermont, CA, USA) 1:50 in PBS (60 min, RT), followed by incubations of horse anti-mouse and avidin–biotin complex (ABC). Negative controls were also performed using PBS (pH 7.4) instead of primary antibodies. Paraffin-embedded sections of HeLa and CaSki cervical cancer cell lines were used as positive controls for $p14^{ARF}$ and $p16^{INK4A}$. Normal cervical squamous epithelium was present in all slides and served as negative control. In our hands, this was never positive for either of the proteins. The peroxidase-labeled complex was developed with diaminobenzidine (DAB). All incubation steps were followed by three washes in PBS (5 min, RT). The slides were slightly counterstained with Mayer's hematoxylin, dehydrated in ethanol and xylene, and finally mounted.

Interpretation of $p16^{INK4A}$ and $p14^{ARF}$ staining

Both nuclear and cytoplasmic stainings were considered positive reactions for $p16^{INK4A}$. The percentages of $p16^{INK4A}$-expressing cells (%$p16^{INK4A}$) were estimated. The staining pattern for $p16^{INK4A}$ of CIN lesions and invasive carcinomas was graded as follows: *negative* (−) if <5% of the cells were stained, *positive* (+) if the percentage was in the range of 5–75%, and *diffusely positive* (++) if more than 75% of the cells were stained.

A different semi-quantitative scoring system was used for $p14^{ARF}$, because the expression pattern for this protein was less extensive than that of $p16^{INK4A}$. The percentages of $p14^{ARF}$ positive cells were estimated with a 40× objective. A nuclear or nucleolar staining was considered to be a positive reaction for $p14^{ARF}$. The staining pattern for $p14^{ARF}$ was graded as follows: *negative* (−) if <1% of cells were stained, *positive* (+) if the percentage was in the range of 1–5%, and *markedly positive* (++) if more than 5% of cells were stained. Cytoplasmic staining for $p14^{ARF}$ was not considered for judgment.

Short fragment polymerase chain reaction hybridization line probe assay (SPF-PCR-LiPA)

A serial 4-μm thick tissue section was put into a reaction tube and incubated overnight at 56°C in 200 μl of 10 mM Tris–HCl with 1 mM EDTA, 0.2% Tween-20, and proteinase K (0.3 mg/ml). Proteinase K was inactivated by 10 min incubation at 100°C. The sample was centrifuged for 10 min at 11,000 rpm and 10 μl was directly used for PCR analysis. A water blank control was processed with each batch of 10 samples.

Broad-spectrum HPV DNA amplification was performed using a short PCR fragment (SPF-PCR) assay [22,23]. The SPF-PCR system amplifies a 65 bp fragment of the L1 open reading frame, allowing for detection of at least 43 HPV types. Subsequent HPV genotyping was performed via a reverse hybridization line probe assay (LiPA), allowing for simultaneous typing of the following 25 genotypes: HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74. The combined SPF-PCR-LiPA system for detection and genotyping of HPV in one sample has been described in detail elsewhere [22]. This HPV detection test is highly sensitive, specific, and reproducible and has been clinically validated [22–24]. In order to avoid cross-contamination with HPV, the tissue specimens were processed separately.

Similar to a recently published epidemiologic classification of HPV types associated with cervical cancer, we classified the HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 as high-risk types. The HPVs 53 and 66 were classified as probable high-risk types, and HPVs 6, 11, 34, 40, 42, 43, 44, 54, and 70 as low-risk types [25].

Statistics

The data were analyzed by nonparametric statistical procedures because %$p16^{INK4A}$ and %$p14^{ARF}$ were not Gaussian distributed. The Spearman rank correlation coefficient r_s was used to assess the association between the four diagnostic groups (CIN1, CIN2, CIN3, and invasive carcinoma) and %$p16^{INK4A}$ and %$p14^{ARF}$. The Kruskal–Wallis one-way analysis of variance by ranks was used to test whether these three parameters were different for at least one of the four diagnostic groups. When a significant difference was found, distribution-free all-treatments multiple comparisons based on pair wise rankings with correction for tied observations were used to disclose which of the diagnostic groups differed significantly [26]. To test whether the measured parameters differed between cases infected and not infected with high-risk HPV, the nonparametric Mann–Whitney-*U*-test for unpaired observations was used. All analyses were performed with SPSS.