STUDIES ON THE EXPRESSION OF p16\textsuperscript{INK4A} mRNA IN CERVICAL DYSPLASIAS

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ABSTRACT

Cervical cancer is caused by a persistent infection with high-risk human papillomavirus (hr-HPV). Accurate grading of premalignant precursor lesions, referred to as cervical intraepithelial neoplasia (CIN) is important for clinical management of patients. A promising candidate marker to identify high-grade CIN lesions is the cellular protein p16 INK4a, an indirect indicator of cell cycle dysregulation commonly expressed in cervical dysplasias and carcinomas associated with hr-HPV. Like other cell cycle regulatory proteins, the diagnostic role of p16INK4A is still under consideration.

The aim of the current study was to investigate the expression of p16\textsuperscript{INK4A} mRNA by a quantitative reverse transcriptase PCR (qRT-PCR) in cervical specimens and evaluate the correlation between the p16\textsuperscript{INK4A} expression levels and the grade of CIN as well as hr-HPV genotypes. The qRT-PCR assay for the detection of p16\textsuperscript{INK4A} mRNA was developed and optimized. As an internal control, integrin gene was selected. The levels of p16\textsuperscript{INK4A} mRNA were investigated in 567 cervical specimens collected from women with confirmed cytological and/or histological diagnosis. The correlation between the p16\textsuperscript{INK4A} mRNA expression level and the grade of cervical cytological/histological alterations was statistically evaluated. The highest p16\textsuperscript{INK4A} mRNA expression level was observed in patients with high-grade cervical dysplasia. The lowest mRNA expression level was detected in cervical specimens collected from women with normal Pap smears. To investigate the correlation between the levels of p16\textsuperscript{INK4A} mRNA and the particular HPV genotype the multiplex PCR was employed that allowed detection of 16 hr-HPV genotypes. The correlation analysis revealed that the oncogenic potential of HPV but not a particular HPV genotype is associated with the increased levels of p16\textsuperscript{INK4A} mRNA in cervical specimens.

Keywords: cervical dysplasia, human papillomavirus, p16\textsuperscript{INK4A}, quantitative PCR

Introduction

Infections with high-risk human papillomavirus (hr-HPV) are causative factors in the development of many benign and cancer diseases of skin and mucosa. High risk HPV have been detected in 99% biopsy samples of cervical carcinoma (15). Cancer of the cervix is the second most common cancer in women worldwide (20). For the laboratory diagnostics of premalignant lesions and cervical cancer the cytological test (Pap smear) is commonly used. However, the success of the Pap smear test is limited with respect to sensitivity and specificity (14). Neither cytological test nor HPV DNA typing could indicate whether there will be a remission or progression to the invasive disease (2, 18). This emphasises the need for specific cellular biomarkers to aid objective CIN lesion grading, and to identify true high-grade dysplasia of the cervix (4).

It is known that the hr-HPV contributes to the neoplastic progression of infected cells predominantly through the action of two viral oncoproteins, E6 and E7. These proteins interact with various host regulatory proteins and influence the function or expression levels of host gene products, eventually leading to the disruption of the cell cycle (5). E6 and E7 respectively degrade two key cellular negative regulatory proteins - p53 and retinoblastoma protein (pRb) (17, 19). Due to the inactivation of pRb at the protein level, a specific cyclin-dependent kinase inhibitor, protein p16\textsuperscript{INK4A}, is released from negative feedback control. Normally, p16\textsuperscript{INK4A} decelerates the cell cycle by inactivating the cyclin-dependent kinase inhibitors, CDK4 and CDK6, which prevents complexing with cyclin D1, pRb phosphorylation, E2F liberation and induction of cell division. However, in the cases with inactivation of pRb by E7 the up-regulation of p16\textsuperscript{INK4A} occurs, resulting in paradoxically high levels of this tumour suppressor protein (1, 17). Thus, p16\textsuperscript{INK4A} represents an indirect indicator of cell cycle dysregulation associated with hr-HPV infection. Several studies examined p16\textsuperscript{INK4A} protein as a biomarker for dysplastic squamous and glandular cells of the cervix by immunocytochemical analysis (4, 9, 12). However, like other cell cycle regulatory proteins, the diagnostic role of p16\textsuperscript{INK4A} is still under
consideration. Although p16$^{\text{INK4a}}$ immunohistology is currently used as a surrogate marker for cervical dysplasias, many of the published data are confusing and contradictory. The discrepancies can be attributed to a multitude of factors operating at the molecular, technical and interpretative levels. Unresolved technical issues include the variables of tissue fixation, antibody dilution, antibody isotype and clone, and the sensitivity of the particular detection method (13).

In the current study, we have investigated the expression of p16$^{\text{INK4a}}$ at the mRNA level in a large number of cervical specimens by a quantitative reverse-transcriptase PCR and evaluated the correlation between the p16$^{\text{INK4a}}$ mRNA expression level and the grade of cervical dysplasia and HPV genotypes.

**Materials and Methods**

**Clinical specimens**

Cervical specimens were collected at the Institute of Oncology, Vilnius University (Vilnius, Lithuania) by cytobrush. In total, 567 women with known histological and/or cytological diagnosis were included into the study. The study was approved by the Vilnius Regional Committee of Biomedical Research (Lithuania, permission No. 158200-6-062-16). All specimens were divided into three groups according to the cytological and histological diagnosis: the group of specimens with normal limits/cervicitis (n=151), the group of specimens with cervical dysplasia according to the cytological diagnosis (n=198) and the group of specimens with cervical dysplasia according to the histological diagnosis (n=218). The group of specimens with cytological diagnosis included 55 cases of atypical squamous cells of undetermined significance (ASCUS), 21 cases of low grade squamous intraepithelial lesion (LSIL) and 122 cases of high grade squamous intraepithelial lesion (HSIL). The group of specimens with histological diagnosis included 8 cases of CIN1 (cervical intraepithelial neoplasia grade 1), 11 cases of CIN2 (cervical intraepithelial neoplasia grade 2), 68 cases of CIN3/CIS (cervical intraepithelial neoplasia grade 3 or carcinoma in situ), 82 cases of cancer (Ca) and 49 patients with CIS treated by cervical conization (CIS/Con).

**Extraction of RNA and DNA from clinical specimens and cDNA synthesis**

The extraction of RNA and DNA from clinical specimens was performed as previously described (3). The RNA extracted from the specimens was transcribed to copy DNA (cDNA) using Maxima$^{\text{TM}}$ First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific Fermentas, Vilnius, Lithuania) according to the manufacturer's instruction and stored at -70°C until use. The cDNA was used to determine the p16$^{\text{INK4a}}$ expression level by quantitative PCR (qPCR) as described below. The DNA extracted from the same clinical specimens was used to determine HPV DNA and HPV types as described below.

**HPV DNA detection and genotyping**

HPV DNA was amplified by wide-spectrum PCR using general primers GP5+/GP6+ and PGMY09/11 within the L1 open-reading frame (6, 8). The quality of isolated DNA was tested using specific primers of β-globin gene and prostate specific antigen (PSA) gene. As a positive control, the DNA extracted from HeLa cells was used. The specimens positive for HPV DNA were genotyped by in-house developed multiplex PCR-based systems using four sets of specific primers for 16 HPV types: 16, 18, 39, 58, 31, 33, 52, 56, 59, 68, 35, 45, 66, 51, 73 and 82 (16) As a positive control for HPV genotyping, plasmids containing HPV DNA of the respective HPV type were used. The amplification products were analysed by electrophoresis in 2% agarose gels and visualized using ethidium bromide.

**Development and optimization of quantitative RT-PCR**

The qPCR was run using Maxima$^{\text{TM}}$ Probe/ROX qPCR Master Mix 2x (Thermo Scientific Fermentas). Three pairs of p16$^{\text{INK4a}}$ specific primers were designed and tested: Inkrev-3 and Inkfor-3, Ink4for-1 and Ink4rev-1, Ink4for-2 and Ink4rev-2. The best results were obtained with Ink4for-2 and Ink4rev-2 primers. The amplified PCR product was of 147 bp in length. As an internal control, specific primers and probe for the integrin gene were used. Optimum concentrations of primers and probes as well as optimum annealing temperature was experimentally selected. Concentrations of primers and probes ranging from 0.15 to 0.4 μM and annealing temperatures ranging from 55.4 to 68.9°C were used. The qPCR was performed in a RotorGene 6000 (Qiagen, Germany). The optimum amplification conditions were 2 min at 50°C, 7 min at 95°C, 55°C at 30 sec, 55°C at 20 sec, 72°C at 20 sec for a total of 40 cycles.

**Quantitation of p16$^{\text{INK4a}}$ mRNA**

Recombinant plasmid pTZ57R with integrated p16$^{\text{INK4a}}$ gene fragment was constructed and used as a template for preparing RNR transcripts with the p16$^{\text{INK4a}}$ target sequences. The p16$^{\text{INK4a}}$ transcripts were further used as an amplification template to obtain standard curves for p16$^{\text{INK4a}}$ mRNA quantitation. The p16$^{\text{INK4a}}$ RNR transcripts were serially diluted 10-fold starting from 10$^7$ copies/reaction up to 10 copies. Each dilution was run in duplicate in Rotor-Gene 6000. The standard curves were created and the respective correlation
coefficients were calculated using Rotor-Gene 6000 detection software. The cDNA prepared from clinical specimens was used to determine the p16\(^{\text{Nk4a}}\) expression level in qPCR system. As a positive control for p16\(^{\text{Nk4a}}\) expression, cDNA prepared from HPV-positive HeLa (ATCC NO. CCL-2) and CaSki (ATCC NO. CRL-1550) cell lines was used. As an internal control, cDNA of integrin gene fragment synthesized using specific primers was used. All reactions were run in duplicate. The number of calculated copies of the p16\(^{\text{Nk4a}}\) gene were divided by a number of calculated copies of the internal control (integrin gene) and multiplied by the coefficient (x1000).

**Statistical analysis**
Statistical analysis of experimental data was performed using the statistical software SPSS version 13.0. Data correlations were tested by using the Spearman’s correlation coefficient. The Pearson \(\chi^2\) test was performed to compare the significance of the positive rates of high-risk HPV types and p16\(^{\text{Nk4a}}\) mRNA expression level in clinical specimens with different grade of cervical dysplasia. The p-value of 0.05 was considered statistically significant.

**Results and Discussion**
In total, 567 cervical cytological specimens collected from women with confirmed histological and/or cytological diagnosis were subjected to the study. In the first step, both DNA and RNA were isolated from clinical specimens. The isolated RNA was used to investigate the expression of p16\(^{\text{INK4a}}\), whereas the isolated DNA was used to determine the positivity for HPV and HPV typing.

To develop and optimize qPCR for studying the expression of p16\(^{\text{Nk4a}}\) at mRNA level, the recombinant plasmid pTZ57R with integrated p16\(^{\text{Nk4a}}\) gene fragment was constructed and used to obtain RNR transcripts. The transcripts of the p16\(^{\text{Nk4a}}\) target sequence were employed to design a standard curve for qPCR, which then was used as a reference standard for extrapolating quantitative information on p16\(^{\text{Nk4a}}\) mRNA levels in clinical specimens (Fig. 1). Selection of the house-keeping integrin gene as an internal control allowed standardization of the assay according to the quantity of cells and calculation of the copies of the p16\(^{\text{Nk4a}}\) gene in each clinical specimen. The calculated number of p16\(^{\text{Nk4a}}\) gene copies in 567 tested specimens varied from 599,558 to 49895,51. The correlation between p16\(^{\text{Nk4a}}\) mRNA expression levels and the degree of cervical cytological/histological alterations was statistically evaluated. For studying the correlation between the p16\(^{\text{Nk4a}}\) mRNA levels and cytological alterations, all specimens with known cytological diagnosis were divided into seven subgroups: 151 cases of normal limits/cervicitis, 8 cases of CIN1, 11 cases of CIN2, 68 cases of CIN3/CIS, 82 cases of Ca and 49 cases of CIS/Con. As shown in Fig. 2, the highest p16\(^{\text{Nk4a}}\) mRNA expression level was observed in CIN3/CIS specimens (mean 20182.76) and the lowest level in normal Pap smears (mean 599.56). Thus, enhanced p16\(^{\text{Nk4a}}\) mRNA expression was associated with high-degree cervical dysplasia.

![Fig. 1. Standard curves of the quantitative PCR obtained by a dilution series of the positive control (RNA transcripts of the p16\(^{\text{Nk4a}}\) target sequences) ranging from 10\(^7\) to 10 of initial RNA template copies per reaction](image)

For studying the correlation between the p16\(^{\text{Nk4a}}\) mRNA levels and histological alterations, all specimens with known histological diagnosis were divided into seven subgroups: 151 cases of normal limits/cervicitis, 8 cases of CIN1, 11 cases of CIN2, 68 cases of CIN3/CIS, 82 cases of Ca and 49 cases of CIS/Con. As shown in Fig. 3, the highest level of p16\(^{\text{Nk4a}}\) mRNA was determined in CIN3/CIS specimens (mean 20182.76) and the lowest level in
Fig. 2. Mean values and confidence intervals of p16\textsuperscript{ink4a} mRNA copies in the group of cervical specimens with confirmed cytological diagnosis

Norm- specimens with no cytological changes in Pap smear, ASCUS- atypical squamous cells of undetermined significance, LSIL- low-grade squamous intraepithelial lesions, HSIL- high-grade squamous intraepithelial lesions

Fig. 3. Mean values and confidence intervals of p16\textsuperscript{ink4a} mRNA copies in the group of cervical specimens with confirmed histological diagnosis

Norm- specimens with no histological changes in biopsy, dysplasia- unknown grade of dysplasia, CIN1- cervical intraepithelial neoplasia grade 1, CIN2- cervical intraepithelial neoplasia grade 2, CIN3/CIS- cervical intraepithelial neoplasia grade 3 or carcinoma in situ, Ca- cervical cancer, CIS post con- the subgroup of patients treated by cervical conization

In summary, the correlation analysis of p16\textsuperscript{ink4a} mRNA expression and cytological/histological alterations revealed a clear association between the enhanced p16\textsuperscript{ink4a} mRNA levels and high-grade cervical dysplasias. These results are in line with previous studies that reported enhanced expression of p16\textsuperscript{ink4a} protein in dysplastic squamous and glandular cells by immunocytochemical analysis (4, 7, 9, 10, 12). On the other hand, our study demonstrated that expression levels of p16\textsuperscript{ink4a} mRNA (and consequently the levels of p16\textsuperscript{ink4a} at protein level) may decrease during tumour progression thus leading to the possible false-negative results of the immunodetection.

As the disruption of the cell cycle leading to the oncogenic transformation of cervical cells is caused by hr-HPV infection, we have investigated the correlation between the p16\textsuperscript{INK4A} mRNA expression levels and the particular hr-HPV genotype. To determine the infectivity with HPV and identify hr-HPV types, we have used DNA isolated from 567 cervical cytological specimens.

To evaluate the relevance of DNA isolation from clinical specimens, the amplification of β-globin gene and PSA gene was performed using specific primers. Only 324 clinical specimens were found to be positive for either β-globin or PSA DNA that confirms the sufficient quality of the isolated DNA (data not shown). Therefore, 324 specimens out of 567 were further analysed for HPV DNA by two PCR-based systems: PGMY09/11 and GP5+/GP6+ (6, 8). The numbers of HPV DNA-positive specimens detected by PGMY09/11 and GP5+/GP6+ primer systems were 175/324 (54%) and 230/324 (70.9%), respectively. In total, 275 specimens positive for HPV DNA were found. They were further analysed for HPV types by using a multiplex PCR system developed in-house (16). The multiplex PCR system consisting of 4 sets of specific primers allowed detection of 16 hr-HPV types (HPV-16, -18, -39, -58, -31, -33, -52, -56, -59, -68, -35, -45, -66, -51, -73 and -82). Fifty-seven specimens out of 275 (21%) were positive for HPV-16. These data are in line with previous cohort studies indicating that HPV-16 is the most prevalent hr-HPV type (11, 21). Two and more hr-HPV types were identified in 15 out of 275 specimens (5.4%). More than one-half of the specimens (165/275) were negative for any of the tested hr-HPV types, which suggests that these specimens were infected with low-risk (non-oncogenic) HPV types. The correlation between the particular hr-HPV genotype and the type of cytological and/or histological lesions was analysed. The majority of specimens with confirmed cervical cancer were positive for one or more hr-HPV types (data not shown). However, no evidences on the correlation between a particular hr-HPV genotype and the type of pathology were obtained. This indicates that all hr-HPV genotypes may cause pathology.

The results of hr-HPV genotyping were compared to the expression levels of p16\textsuperscript{INK4a} mRNA. It was determined that all hr-HPV types cause the increased p16\textsuperscript{INK4a} mRNA expression, however, the correlation between the particular hr-HPV type and the p16\textsuperscript{INK4a} mRNA level is obscure (Fig. 4). This suggests that all hr-HPV types may dysregulate the cell cycle by a similar molecular mechanism. Therefore, it can be concluded that the oncogenic potential of HPV but not a particular hr-HPV type is the main cause of enhanced p16\textsuperscript{INK4a} mRNA expression indicating the dysregulation of cell cycle and leading to high-grade cervical lesions.
Conclusions
The study confirms the diagnostic relevance of p16\textsuperscript{INK4a} as a cellular marker for high-grade CIN. We have demonstrated that p16\textsuperscript{INK4a} mRNR expression levels correlate with high-grade cervical neoplasia, such as HSIL and CIN3/CIS, but is not specific to the particular hr-HPV type.

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REFERENCES