**p16**<sup>ink4a</sup> Is a Surrogate Marker for High-Risk and Malignant Cervical Lesions in the Presence of Human Papillomavirus

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**Key Words**
Cervical cancer · Human papillomavirus · In situ hybridization · **p16**<sup>ink4a</sup> · Squamous intraepithelial lesions

**Abstract**

**Objective:** To strengthen the role of high-risk human papillomaviruses (HPVs) in the development of cervical cancer, and the association between HPV and the cell cycle inhibitor gene **p16**<sup>ink4a</sup> in cervical carcinogenesis. **Methods:** In this study, the association between **p16**<sup>ink4a</sup> and the presence of HPV DNA in cervical lesions was investigated in a total of 177 cervical biopsies classified as benign (n = 42), low-grade squamous intraepithelial lesions (LSIL; n = 34), high-grade squamous intraepithelial lesions (HSIL; n = 48) and invasive cancer (n = 53). **p16**<sup>ink4a</sup> expression was analyzed by immunohistochemistry while HPV DNA was localized by in situ hybridization. **Results:** No significant association was found between **p16**<sup>ink4a</sup> expression and HPV presence in benign and LSIL samples. In HSIL and invasive cancer, a significant association was found between **p16**<sup>ink4a</sup> overexpression and the presence of HPV DNA. **Conclusion:** Immunohistochemical **p16**<sup>ink4a</sup> expression is associated with HPV infection in HSIL and cervical cancer, suggesting a role of **p16** as a biomarker of HPV-associated cervical lesions.

**Introduction**

Human papillomavirus (HPV) is a definite etiological agent for cervical neoplasia [1]. Integration of high-risk HPV sequences into the cell genome causes disruption of the E2 gene and subsequent overexpression of viral E6 and E7 oncoproteins that abrogate p53 and Rb tumor suppressor functions, respectively, and lead to loss of cell cycle control [2, 3]. HPV is thus a sensitive marker for identifying patients at risk for cervical neoplasia. Detection of HPV subtypes in cervical lesions would be very useful in distinguishing patients at risk for progression to cervical neoplasia.

The **p16**<sup>ink4a</sup> protein is a tumor suppressor protein that inhibits the cyclin-dependent kinases which phosphorylates the retinoblastoma protein. Overexpression of **p16**<sup>ink4a</sup> in cervical cancer is believed to be due to the inactivation of Rb by HPV E7 oncoprotein. Many groups of investigators have studied the expression pattern of **p16**<sup>ink4a</sup> in dysplastic squamous and glandular cervical tissue sections and smears [4–7]. **p16**<sup>ink4a</sup> is now widely accepted as a sensitive and specific marker of cervical squamous dysplastic cells and a valuable adjunct in cervical cancer screening. No data are however available for Indian cervical lesions regarding the association between **p16**<sup>ink4a</sup> and HPV status. India has over two thirds of the world’s cervical cancer burden [8]. Together with the age-
adjusted rate and ranking, the world pattern of cervical cancer clearly indicates that cervical cancer is predominantly a problem of poor socioeconomic status. Moreover, women in India have a higher life time risk to get cervical cancer, which is double the risk compared to the data worldwide.

In many countries, HPV testing has already been incorporated into screening programs generally as an adjunct to existing cytologic screening programs. Among the methods used for the detection of molecular markers, in situ hybridization (ISH) offers the unique advantage of visualizing and even quantifying clinically relevant molecules in a morphological context [9]. The application of nucleic acid targeting and signal amplification technologies to ISH has helped out detection of as few as one or two copies of DNA molecules [10]. The purpose of the present study was to provide data from India to strengthen the association between p16\textsuperscript{ink4a} and the presence of HPV in high-grade cervical lesions and cervical cancer tissue. In addition, it is also attempted to emphasize the importance of the ISH technique in the detection of HPV infection in clinical samples.

**Patients and Methods**

**Patient Samples**

Cervical tissue samples were obtained from patients attending the Surgical Oncology Clinics of the Regional Cancer Center, Thiruvananthapuram. The study was approved by the Institutional Review Board and Ethical Committee of the Regional Cancer Center. Biopsies were collected during colposcopic examination or loop electrosurgical excision procedures. Benign cervical tissue samples were obtained from patients undergoing hysterectomy for non-malignant conditions. Tissues were fixed in 10% buffered formalin and embedded in paraffin wax. Five-micrometer-thick sections were cut and stained with hematoxylin-eosin for histological examination. A total of 177 tissue samples were analyzed, including 42 benign cervical tissues, 34 low-grade squamous intraepithelial lesions (LSIL), 48 high-grade squamous intraepithelial lesions (HSIL) and 53 invasive cervical cancer samples.

**p16\textsuperscript{ink4a} Immunohistochemistry**

Serial sections were taken from each sample. Immunohistochemistry was done using mouse monoclonal anti-p16 antibody (Novocastra, UK) and a supersensitive polymer-HRP immunohistochemical detection system (Biogenex, USA). Briefly, sections were deparaffinized in xylene and then rehydrated through graded series of alcohol. Antigen retrieval was performed by heating the sections in 10 mM citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked by incubating sections in 0.3% hydrogen peroxide in methanol for 20 min. After blocking non-specific binding by incubation with 3% BSA, primary antibody was added and incubated for 1 h at 37°C. For detection, sections were incubated with super-enhancer solution for 30 min followed by polymer-HRP conjugate for 30 min at 37°C. The reaction product was visualized using diaminobenzidine as chromogen.

**Evaluation of Immunostaining**

p16\textsuperscript{ink4a} immunostaining was considered positive when either nucleus or cytoplasm or both showed positive staining. The staining was scored as follows: negative (<1% positive), sporadic (isolated cells positive, <5%), focal (<25% of the cells positive) and diffuse (>25% cells were positive).

**HPV ISH**

Samples were analyzed for HPV DNA by ISH using HPV-biotinylated DNA probes (DakoCytomation, Denmark) and a tyramide signal amplification system (DakoCytomation GenPoint ISH Detection System). The sections were subjected to proteinase K digestion followed by heating in citrate buffer (pH 6.0) in a microwave oven. Biotinylated HPV DNA probe was added to the sections, and denaturation of the probe and target DNA was performed by heating at 95°C for 10 min. The sections were incubated at 4°C overnight for hybridization. The slides were incubated in a stringent wash solution at 55°C for 30 min. The bound probe was identified by incubation with the detection system which involves tyramide signal amplification, followed by visualization using diaminobenzidine as chromogen substrate. All samples were analyzed using a HPV wide spectrum probe followed by analysis of positive samples with type-specific probes (HPV 6/11, 16/18, 31/33/35).

**Evaluation of ISH Signal Patterns**

Cervical cancer cell lines SiHa and HeLa, known to contain HPV integrated into the cell genome, were used to standardize ISH. The ISH signal patterns were noted as nuclear dot-like or punctuate and nuclear diffuse positivity.

**Statistical Analysis**

The data were analyzed using SPSS statistical software. Mann-Whitney and Wilcoxon nonparametric tests were applied to compare immunohistochemical expression of p16\textsuperscript{ink4a} in various grades of cervical lesions. Fisher’s exact test was used for comparisons between the histological groups for p16\textsuperscript{ink4a} and HPV types. p values were two sided, and the level of significance was set at 0.05.

**Results**

Immunohistochemical expression of p16\textsuperscript{ink4a} and HPV ISH are summarized in table 1.

**p16\textsuperscript{ink4a} Immunohistochemistry**

p16\textsuperscript{ink4a} expression was observed in both nucleus and cytoplasm with varying degrees of intensity. Representative expression patterns of p16\textsuperscript{ink4a} in benign and LSIL samples are shown in figure 1. Of the 177 cervical samples, p16\textsuperscript{ink4a} distribution was negative in 43 (24.3%), sporadic in 28 (15.8%), focal in 10 (5%) and diffuse in 96
(54.2%) cases. In benign or non-neoplastic epithelium, 72.1% of the samples were negative and only 10 of 42 (35.7%) showed sporadic staining. In the LSIL group, p16ink4a immunoreactivity was observed in 28 of 34 cases of which 14 were sporadic, 7 focal and another 7 with diffuse staining pattern. Figure 2 shows representative expression patterns of p16ink4a in HSIL and cervical cancer samples. Of the 48 samples in the HSIL group, 45 showed positivity for p16ink4a and 88.8% showed a diffuse staining pattern. Positivity was seen in the lower two thirds of the squamous epithelium in the CIN 2 lesions and in the full thickness of the epithelium in CIN 3 lesions. Among

### Table 1. Summary of cervical lesions evaluated by p16ink4a immunohistochemistry and HPV ISH

<table>
<thead>
<tr>
<th>Histology</th>
<th>p16 expression</th>
<th>HPV type:</th>
<th>negative</th>
<th>sporadic</th>
<th>focal</th>
<th>diffuse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>neg. 16/18</td>
<td>neg. 6/11</td>
<td>16/18</td>
<td>neg. 16/18</td>
<td>neg. 16/18</td>
</tr>
<tr>
<td>Benign</td>
<td>31</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LSIL</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>HSIL</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1. p16ink4a immunostaining patterns showing sporadic expression in benign cervical epithelium (a, b), focal expression in LSIL (c–e) and diffuse expression in LSIL (f).
the malignant lesions, 50 of 53 were positive for p16\textsuperscript{ink4a}; 98% of these positive samples showed a diffuse pattern. Statistical analysis showed a positive correlation between p16\textsuperscript{ink4a} immunostaining and histologic grade of the lesion ($r = 0.567, p = 0.000$). The frequency of expression patterns of p16\textsuperscript{ink4a} in various grades of cervical lesions studied is shown in table 2.

**HPV ISH**

HPV ISH signals were observed as punctuate or dot-like nuclear positivity in the majority of cases while a few showed diffuse nuclear positivity along with punctuate positivity. Figure 3 shows the positive ISH signal patterns in various grades of cervical lesions: 87 out of 177 samples were positive for HPV wide spectrum probe. All of the benign lesions were negative for HPV. HPV DNA was seen in 47 malignant samples, 37 HSIL and 3 LSIL samples. Of HPV type-specific probes, 37 HSIL and 42 cancer cases were positive for the HPV type 16/18 probe. Three of the LSIL were positive for the HPV type 6/11 probe while 1 of the malignant samples was positive for the HPV type 31/33/35 probe. The majority of samples showed ISH positivity as punctuate or dot-like signals while few showed diffuse nuclear positivity. The results showed a
strong correlation with histologic grades of the lesions ($r = 0.751, p = 0.000$).

$p16^{ink4a}$ immunoreactivity was compared with the HPV status of the individual samples. None of the benign lesions was positive for HPV. Forty-one cases were negative for both $p16^{ink4a}$ and HPV. Of the 96 cases which exhibited strong diffuse staining for $p16^{ink4a}, 22$ were negative for HPV and 73 were positive for high-risk HPV 16/18. Two samples which were HPV 16/18 positive showed sporadic $p16^{ink4a}$ staining. Samples positive for HPV 6/11 showed sporadic p16 positivity and were of the LSIL group. None of the advanced lesions showed positivity for the HPV 6/11 probe. The results indicate a strong correlation between the presence of HPV 16/18 and strong/diffuse $p16^{ink4a}$ expression in HSIL and invasive cervical cancer tissue ($r = 0.504, p = 0.000$). The frequency of results of $p16^{ink4a}$ immunohistochemistry and HPV ISH is graphically represented in figure 4.

**Table 2.** $p16^{ink4a}$ immunohistochemical staining patterns in cervical lesions

<table>
<thead>
<tr>
<th>Histology</th>
<th>$p16^{ink4a}$ expression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>negative</td>
<td>sporadic</td>
</tr>
<tr>
<td>Benign</td>
<td>31</td>
<td>10</td>
</tr>
<tr>
<td>LSIL</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>HSIL</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>43</strong></td>
<td><strong>28</strong></td>
</tr>
</tbody>
</table>

**Discussion**

Molecular analysis of complex interactions of deregulated oncogenes with various cellular protein complexes has led to the identification of new biomarkers in dysplastic and neoplastic cells [11]. One such marker is the cyclin-dependent kinase inhibitor $p16^{ink4a}$ in cervical cells, which may be responsible for the maintenance of the cell cycle and apoptosis. $p16^{ink4a}$ is a protein product of the cyclin-dependent kinase inhibitor 2 (CDK2) gene, which binds to and inhibits CDK4- or CDK6-mediated phosphorylation of pRb [12]. Abnormalities in the cell cycle...
regulatory machinery and disruption of cell cycle control are common features in many cancers. While some studies have reported p16ink4a overexpression to be related to cancer development [13–18], others have shown absence or low expression of p16ink4a with tumor progression [19–22]. In the present study, p16ink4a positivity patterns were found to correlate with tumor progression in the cervical samples studied. Some studies on cervical cancer have demonstrated that p16ink4a may serve as a marker for differentiating neoplastic lesions from hyperplastic or reactive lesions [5, 23]. In benign or non-neoplastic lesions, p16ink4a immunoexpression showed a sporadic pattern in positive cases while the majority of these lesions were negative.

HPV E6 and E7 proteins have been considered to be responsible for the malfunction of p16ink4a [24]. The E6 and E7 oncoproteins of low-risk HPVs have lesser oncogenic potential than those of the high-risk types. Thus E6/E7 proteins seem to be inactivated in low-risk HPV infection, resulting in lower levels of p16ink4a immunoexpression. Thus, p16ink4a immunoexpression may be distinctive in low- and high-risk HPV infection. The diffuse and strong staining pattern associated with high-grade lesions and the sporadic pattern associated with benign lesions could be attributed to the presence of the low- or high-risk type of HPV. However, some lesions with HPV positivity were p16ink4a negative or sporadically positive, suggesting that the presence of HPV alone does not give rise to p16ink4a positivity and normal function of p16ink4a may also take place in the absence of HPV.

For the detection of nucleic acid sequences, ISH has proven very powerful for revealing gene expression in the morphological context. The integrated HPV genome is detected as a punctuate signal in the nuclei of infected cells by ISH, whereas the episomal genome is detected as a diffuse signal throughout the nuclei [25–27]. In benign or non-neoplastic lesions, p16ink4a immunoexpression showed a sporadic pattern in positive cases while the majority of these lesions were negative.

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have consistently been associated with over 90% of cervical carcinomas and found in 50–80% of precancerous lesions [32]. Studies using sensitive techniques such as ISH with tyramide signal identification, which identifies the topographical distribution of HPV in cervical biopsies, might offer unique insight into the process of cervical carcinogenesis. The detection of HPV by ISH was considered insensitive and imprecise earlier due to limitations using radioisotopes as well as nonspecific labels. In any case, the detection limit is reported to be 10–50 viral copies per cell and the viral copies in HPV-infected cells is mostly 1–10 copies [27]. This led to the development of more sensitive methods in ISH [33, 34]. The commercially available ISH detection system used in this study is based on the catalyzed amplification of positive hybridization signals using biotin-tyramide complexes. The same or similar systems are now being used in several studies to identify HPV sequences in cervical lesions with high sensitivity. These systems are capable of detecting single viral copies in routinely fixed material [27].

Association of p16ink4a overexpression to malignant tumors was first reported by Sakaguchi et al. [35] and Sano et al. [7]. Subsequent studies have demonstrated that a negative or low level of p16ink4a expression is associated with LSIL, whereas p16ink4a overexpression is associated with HSIL and cervical cancer [4, 36]. In the present study, significant overexpression of p16ink4a was found in HSIL and invasive cancer samples compared to benign and LSIL samples. A significant association between p16ink4a expression in lesions and high-risk HPV types has also been reported [6, 23]. Our results showed an association of p16ink4a overexpression in HSIL and invasive cervical cancer, which were positive for high-risk HPV (type 16/18) DNA, highlighting the possible potential of p16ink4a as a marker for high-risk type HPV-related lesions and cervical cancer. The detection of p16 overexpression and high-risk HPV signals was useful in predicting the progression of CIN [37, 38]. Omori et al. [39] observed that similar observations correlate not only with the grade of CIN but also with CIN 2 progression.

In conclusion, we suggest that HPV 16/18 are involved in p16ink4a overexpression associated with HSIL and cervical cancer. Moreover, this study highlights that p16ink4a immunohistochemical evaluation could be useful as a marker for progressive and HPV-related lesions of the uterine cervix. No apparent correlation was found between the presence of HPV and p16ink4a expression in benign and LSIL, suggesting that HPV itself is not directly
involved in cell cycle-regulatory pathways that control cervical tumor progression. Further studies are needed to determine whether the viral oncoproteins are involved in the deregulation mechanism of the cell cycle in cervical cancer.

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**References**


