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Original Research Article

# A STUDY OF HPV, P16 EXPRESSION AND OXIDATIVE STRESS IN CERVICAL CANCER PATIENTS

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ABSTRACT

**Background:** Cancer of the uterine cervix is one of the most common cancers among women worldwide and second only to breast cancer in incidence and mortality. Epidemiological and molecular studies have shown that human papillomavirus (HPV) infection is the most important factor for cervical carcinogenesis. The p16INK4a is a cyclin-dependent kinase inhibitor that decelerates the cell cycle by inactivating the cyclindependent kinases involved in the phosphorylation of the retinoblastoma protein (RB). Expression of E6 and E7 oncogenes of high-risk (HR) human papillomavirus (HPV), affecting the RB-p16 pathway, leads to p16 upregulation. Oxidative stress is an imbalance between the prooxidant-antioxidant system. A decrease in the level of antioxidants generated free radicals, which leads to DNA damage, causing dysfunction and disease. It is caused by a disturbed oxidant-antioxidant balance in favor of oxidants, leading to excessive generation of free radicals, particularly reactive oxygen species (ROS), and biological damages. Superoxide anion (O2•−), hydrogen peroxide (H2O2), and hydroxyl radical (• OH) are kinds of ROS that are produced by partial reduction of atmospheric O2.

**Material and Methods:** This study is hospital based study examined at the Department of Pathology and Biochemistry at Tertiary Care Teaching Hospital. On a population of 70 consecutive patients who were diagnosed with PCV, and subsequently treated at Hospital. To get two groups with a significant difference in survival, patients with PCV were divided into short-term survivors (dying within ⩽2 years of diagnosis) and long-term survivors (surviving ⩾8 years after diagnosis). All tumour biopsies were fixed in buffered formaldehyde, paraffin-embedded and diagnosed on haematoxylin and eosin-stained tissue sections. For the present study, four sections from each archived tumour biopsy were prepared and used for histological diagnosis and immunohistochemistry (thickness: 4 μm). Sections for haematoxylin and eosin staining were prepared before and after each section to confirm tumour representativity.

**Results:** HPV types and status in correlation with clinical parameters and expression of p16. Sixty-five out of 70 patients with PCV could be evaluated for HPV status. 16 were positive for high-risk HPV and 49 were HPV negative. The majority (10 out of 16, 62.5%) of HPV-positive patients were positive for HPV16. The others were positive for HPV45 (2 patients, 12.5%), HPV18 (1 patient, 6.2%), HPV35 (1 patient), HPV56 (1 patient), and HPV68 (1 patient). Human papillomavirus positivity was significantly correlated with strong p16 expression (p= 0.045). In all, 9 out of the 49 HPV-negative patients were negative for p16 immunostaining, while the remaining 83% showed

varying expression: 32 out of 49 (65.3%) showed moderate or strong p16 expression. In our study, Oxidative marker such as MDA was 3.23 ± 0.51 nmol/mL. Antioxidant marker such as Glutathione peroxidase 7.23 ± 3.41 µmol/ml. SOD was 671.12+39.58 (U/gHb).

**Conclusion:** The vast majority of HPV positive vaginal cancers showed p16 overexpression, suggesting active involvement of HPV in the malignant transformation process. HPV vaccines will help prevent some of the primary female genital cancers associated with HPV type 16. More indepth studies are needed to understand the molecular carcinogenesis pathway in these p16-negative tumors and to improve outcomes for this population.

**Keywords:** Human papillomavirus, Cervical intraepithelial neoplasia (CIN), p16, Ki-67, p63.

## INTRODUCTION

Cancer of the uterine cervix is one of the most common cancers among women worldwide and second only to breast cancer in incidence and mortality.[1] Epidemiological and molecular studies have shown that human papillomavirus (HPV) infection is the most important factor for cervical carcinogenesis. Recent extensive studies have revealed the existence of more than 100 different HPV genotypes of which about 30 can infect the uterine cervical epithelium.[2] Each HPV subtype has been shown to have a different carcinogenetic potential on cervical epithelial cells and has been subdivided into high- (HR) and low-risk (LR) types.[3]

Although the persistence of viral infection and the capability of viral DNA integration into the host cellular DNA are closely associated with malignant transformation, the reasons for the difference in the oncogenic potential of each HPV subtype remains unresolved.[4] It has been suggested that the oncoproteins encoded by the E6 and E7 viral oncogenes, which bind important host regulatory proteins (ie p53 and retinoblastoma protein (RB)), play a pivotal role in cervical carcinogenesis.[5] In fact, p53 protein is degraded following E6 binding, whereas hypophosphorylated RB is functionally inactivated and degraded by E7 binding. In particular the E7 gene product from various HPV types has different efficiency both in RB binding and degradation.[6]

E2F-1 accumulation, a consequence of RB phosphorylation, leads to p16 upregulation which allows the control of the kinase activity through a feedback inhibition mechanism.[7] Since p16 expression is negatively controlled by an active RB, reduction or loss of the hypophosphorylated protein can induce p16 overexpression. Consequently, HPV E7 RB inactivation may result in the enhanced expression of p16 which appears to be a marker of E7 gene activity. Therefore the immunohistochemical (IHC) evaluation of p16 protein, known to be widely detected in cervical carcinomas, could improve conventional histopathological diagnosis of preneoplastic diseases, thus increasing interobserver agreement in the identification of lowgrade lesions.[8]

However, only a few studies have focused their attention on the potential role of this protein also in identifying those low-grade lesions caused by HR-HPV infection, which could be at higher risk of transformation.[9]

Chemoradiation is known to improve the survival of patients with cervical cancer. Oxidative stress is an imbalance between the prooxidant-antioxidant system. A decrease in the level of antioxidants generated free radicals, which leads to DNA damage, causing dysfunction and disease.[10] It is caused by a disturbed oxidant-antioxidant balance in favor of oxidants, leading to excessive generation of free radicals, particularly reactive oxygen species (ROS), and biological damages. Superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($^{\bullet}OH$) are kinds of ROS that are produced by partial reduction of atmospheric $O_2$.[11]

The present study aimed at evaluating p16 overexpression in parallel with HPV infection and Oxidative Stress In Cervical Cancer Patients, in a series of 70 cervical biopsies comprising normal tissues, preneoplastic and neoplastic lesions. The potential association between p16 and HPV infection was investigated to verify whether p16 upregulation could be a valuable tool in the identification of dysplastic lesions caused by HR-HPV genotypes.

## MATERIAL AND METHODS

This study is hospital based study examined at the Department of Pathology and Biochemistry at Tertiary Care Teaching Hospital. On a population of 70 consecutive patients who were diagnosed with PCV, and subsequently treated at Hospital. To get two groups with a significant difference in survival, patients with PCV were divided into short-term survivors (dying within $\leqslant$2 years of diagnosis) and long-term survivors (surviving $\geqslant$8 years after diagnosis). All tumour biopsies were fixed in buffered formaldehyde, paraffin-embedded and diagnosed on haematoxylin and eosin-stained tissue sections.

For the present study, four sections from each archived tumour biopsy were prepared and used for histological diagnosis and immunohistochemistry (thickness: 4 $\mu$m). Sections for haematoxylin and eosin staining were prepared before and after each

section to confirm tumour representativity. Primary carcinoma of the vagina diagnoses, as well as the representativity of the sections used for immunohistochemical studies, were reviewed and confirmed by two pathologists at the Department of Pathology. The histopathologist reviewed and confirmed the histopathological grade and the International Federation of Gynecology and Obstetrics (FIGO) stage before immunohistochemistry was performed. Histopathological evaluation was done according to the World Health Organization and staging was performed according to the FIGO staging system, based on the original clinical records and new histopathological results.

**Immunohistochemistry for p16$^{INK4A}$ and MIB-1**

Immunohistochemical staining was performed with the CINtec Histology Kit according to the manufacturer's recommendations using the Dako Autostainer. This kit contains Tris EDTA buffer ( × 10) pH 9, intended for epitope retrieval. A Coplin jar filled with epitope retrieval buffer, diluted 1 : 10 with distilled water, was placed in a water bath and heated to 95–99 °C. Deparaffinised sections were then incubated for 10 min while maintaining a temperature of 95–99 °C. Jars with slides were removed from the water bath and left to cool at room temperature for 20 min, followed by washing for 5 min in Wash Buffer diluted 1 : 10 with distilled water. The automated procedure began with 1 × rinse (1 × rinse equals 4 min) in wash buffer diluted 1 : 10 with distilled water (Wash Buffer ( × 10).

Endogenous peroxidase activity was abolished by incubating slides for 5 min in a peroxidase-blocking solution. Slides were rinsed 1 × after which 200 $\mu$l of the primary antibody against the p16$^{INK4a}$ protein clone E6H4 was dropped onto each slide, followed by incubation for 30 min. Slides were rinsed 1 × . Reaction products were visualised by incubating slides for 30 min with a visualisation reagent (a horseradish peroxidase/goat-anti-mouse immunoglobulin- labelled dextran polymer) and, after 2 × rinses, incubated for 10 min in a 1 : 40 solution of DAB chromogen (3.3′-diaminobenzidine) in DAB buffered substrate, also from the CINtec Histology Kit. Slides were then washed in distilled water for 1 min and counterstained for 2 min in Harris Hematoxylin solution diluted 1 : 2 with distilled water. After 2 min of washing in water, slides were dehydrated in ethanol to xylene and mounted in a water-free permanent mounting medium with mounting glass. Tissue sections containing cervical cancer were used as positive controls for p16$^{INK4a}$, while negative controls consisted of incubated doublet slides in the negative control reagent contained in the kit, instead of primary antibodies.

Immunostaining was independently evaluated by two observers and was considered as positive for p16$^{INK4a}$ when both observers agreed that the nuclei were clearly stained. In addition, cells with a distinct cytoplasmic immunoreaction were scored as positive. Image analysis was carried out as previously described. Immunohistochemistry results were scored based on both staining intensity and percentage of immunoreactive epithelial cells. Scoring criteria for p16$^{INK4a}$ were no expression (negative); weak expression (<30% positive cells); moderate expression (31–50% positive cells); and strong expression (>50% positive cells). Samples scored as moderate or higher were considered as positive for p16$^{INK4a}$.

To detect the Ki-67 antigen, we used the monoclonal mouse antibody (clone MIB-1). The Ki-67 antigen is a marker for mitotically active cells and is expressed in the nuclei of growing cells. Tumour biopsy sections were deparaffinised, rehydrated, and microwave treated in target retrieval solution diluted 1 : 10 with distilled water for 2 × 5 min at 500 W. Thereafter, slides were subjected to the autostainer procedure and treated together with the p16$^{INK4a}$ slides as described above. Ki-67 positivity was scored with respect to nuclear staining and with attention to heterogeneity in distribution as follows: <10% positive cells; 10–50% positive cells; and >50% positive cells.

**Sample collection and processing**

Approximately 5 mL of blood was obtained through venipuncture under aseptic condition in a clean, plain, red topped dry vial. Blood was kept for 10 min before centrifugation. Serum was separated from the blood through centrifugation at 3000 RPM for 10 min and stored in an eliquote at −20 °C until required for analysis. In both groups, MDA, SOD, and GSH were measured using a modified spectrophotometric method; 8-OHdG was estimated using a competitive sandwich ELISA assay.

**Laboratory analysis**

Lipid peroxidation was estimated by the formation of Thiobarbituric acid reactive substances (TBARS) described by Satoh K, 1978. The samples were deproteinized with 15% TCA (Trichloroacetic acid) and then treated with 0.375% TBA (Thiobarbituric Acid). The mixture was heated in a boiling water bath for 15 min. It was then cooled to room temperature and centrifuged at 3500 rpm for 10 min, and formed TBARS was measured at 535 nm. Total thiobarbituric acid-reactive materials (TBARS) was expressed as MDA. The value is expressed as nmol/ml.

The test of GSH was based on development of a yellow color when 5′5′ dithiobis (2- nitrobenzoic acid) was added to sulphhydryl compounds. The color thus developed was fairly stable for about 10 min and the reaction was read at 412 nm. Two tenth of whole blood was added to 1.8 ml distilled water. 3 ml of the precipitating solution was mixed with the hemolysate. The mixed was allowed to stand for approx. 5 min and the filtered. 2 ml of the filtrate was added to 8 ml of phosphate solution in a test tube. 1 ml of DTNB solution was added. A blank was prepared with 8 ml of the phosphate solution, 2 ml of diluted precipitating solution and 1 ml of the DTNB reagent. Absorbance was taken

within 30 s at 412 nm. The value is expressed in nM/L. (Ellman's method. 1959).

SOD assay by the pyrogallol autoxidation method was carried out following the procedure of Marklund and Marklund (1974). The reaction was initiated by the addition of 100 µl of freshly prepared 2·6 mM pyrogallol solution in 10 mM HCl to attain a final concentration of pyrogallol of 0·13 mM in the assay mixture.

**Ethical Considerations**

The present study followed the Declaration of Helsinki's, with participant confidentiality & privacy maintained. Informed consent is obtained from all participants before enrollment in the study.

## RESULTS

In table 3, HPV types and status in correlation with clinical parameters and expression of p16. Sixty-five out of 70 patients with PCV could be evaluated for HPV status. 16 were positive for high-risk HPV and 49 were HPV negative. The majority (10 out of 16, 62.5%) of HPV-positive patients were positive for HPV16. The others were positive for HPV45 (2 patients, 12.5%), HPV18 (1 patient, 6.2%), HPV35 (1 patient), HPV56 (1 patient), and HPV68 (1 patient). Human papillomavirus positivity was significantly correlated with strong p16 expression (p= 0.045). In all, 9 out of the 49 HPV-negative patients were negative for p16 immunostaining, while the remaining 83% showed varying expression: 32 out of 49 (65.3%) showed moderate or strong p16 expression. [Table 3]

In table 4, Oxidative marker such as MDA was 3.23 ± 0.51 nmol/mL. [Table 4]

In table 5, Antioxidant marker such as Glutathione peroxidase 7.23 ± 3.41 µmol/ml. [Table 5]

In table 6, SOD was 671.12+39.58 (U/gHb). [Table 6]

**Table 1: Patient and tumour characteristics**

| Parameters | Frequency | Percentage |
|---|---|---|
| **Histology** | | |
| Squamouscell carcinoma | 63 | 90 |
| Adenocarcinoma | 3 | 4.3 |
| Smallcellcarcinoma | 4 | 5.7 |
| **Histopathological grade** | | |
| Well differentiated | 06 | 8.6 |
| Moderately differentiated | 36 | 51.4 |
| Poorly differentiated | 28 | 40 |
| **FIGO stage** | | |
| I | 36 | 51.4 |
| II | 14 | 20 |
| III | 12 | 17.1 |
| IV | 08 | 11.4 |
| **Tumour size** | | |
| <4cm | 31 | 44.3 |
| 4–8 cm | 26 | 37.1 |
| >48cm | 13 | 18.6 |
| **Tumour localisation** | | |
| Upper third | 38 | 54.3 |
| Lower third | 15 | 21.4 |
| Allother locations | 17 | 24.3 |

**Table 2: Characteristics of tumour**

| Growth pattern | Frequency | Percentage |
|---|---|---|
| Exophytic | 26 | 37.1 |
| Ulcerating | 38 | 54.3 |
| Endophytic | 06 | 8.6 |
| **Regional metastasis (inguinal node metastasis)** | | |
| Yes | 7 | 10 |
| No | 63 | 90 |
| **Distant metastasis** | | |
| Yes | 14 | 20 |
| No | 56 | 80 |

**Table 3: p16 expression in relation to HPV status and different HPV types**

| | | HPV positive N (%) | |
|---|---|---|---|
| **p16expression** | **HPV negative N(%)** | **HPV16** | **Other HPV types** |
| None | 8 (16.3) | 1 (6.2) | |
| Weak (430%) | 09 (18.4) | | |
| Moderate (30–50%) | 14 (28.6) | 2 (12.5) | 2 (12.5) |
| Strong (450%) | 18 (36.7) | 7 (43.7) | 4 (25) |
| Total | 49 (100) | 16 (100) | |

**Table 4: Oxidative marker among patients**

| Parameters | Case (mean ± SD) |
|---|---|
| Malondialdehyde (nmol/mL) | 3.23 ± 0.51 |

**SD = Standard deviation; µmol/ = millimole per litre; N = total number of patients; N = 70.**

**Table 5: Antioxidant marker among patients**

| Parameters | Control (mean ± SD) |
|---|---|
| Glutathione peroxidase (µmol/ml) | 7.23 ± 3.41 |

**SD = Standard deviation; µmol/ = millimole per litre; N = total number of patients; N = 70.**

**Table 6: Biochemical parameters in cases and control group**

| Parameter | Case (N=70) |
|---|---|
| | Mean±SD |
| SOD (U/gHb) | 671.12+39.58 |

## DISCUSSION

Many studies have focused on the prognostic factors for CIN, including p16, Ki-67, p63, HPV L1, CK17, and CK8, but the value of these factors is controversial. A diffuse p16-staining pattern is detected immunohistochemically in almost all highgrade precancerous cervical lesions and cervical carcinoma, whereas a reactive condition-p16-negative staining or in the lesion cells-shows only sporadic staining.[10-17] Negri et al performed p16 immunohistochemical staining on CIN1 lesions and found that the CIN1 cases with diffuse p16 staining had a significantly higher tendency to progress to a higher-grade lesion than did the p16-negative cases.[18]

sMurphy studied p16INK4A as a marker for cervical dyskaryosis and found that all formalin-fixed, paraffin wax-embedded sections with either strong nuclear or cytoplasmic staining were considered positive; a certified pathologist then graded all sections qualitatively according to the following arbitrary scale: 0 (no positive staining), 1 (<10% positive staining), 2 (10-50% positive staining), and 3 (>50% positive staining). de Sanjose et al have used this scale, and we initially tried to use it to score the p16 stained slides. However, p16 exhibited a strong diffusely stained pattern in our study, and instead of the 0-3 scale, we considered grade 3 as positive in the statistical analysis of our small number of specimens. Ki-67 expression is greater in CIN samples than in those from normal and metaplastic epithelium, and Ki-67 staining is stronger in high-grade CIN than in lowgrade CIN. Therefore, Ki67 is usually used to differentiate and grade CIN.[19]

Kruse applied Ki-67 immunoquantification using a computerized image analysis system to predict the progression of early CIN. They classified the early CIN biopsies as low risk or high risk according to a Ki-67 model, which comprised the stratification index and the percentage of positive nuclei in the middle-third layer of the epithelium. In our current study, the number of p16- or Ki67-positive patients was not significantly greater in the persistent group than in the spontaneous regression group, indicating that the p16- and Ki-67-expression patterns did not predict the high-risk group. However, the number of patients with p16- or Ki-67 negative expression was significantly greater in the regression group than in the persistent group. Thus, p16- or Ki-67-negative expression was helpful in identifying the low-risk group CIN1 patients whose lesions did not progress to a higher grade.

A few previous studies have analyzed p63 expression in CIN and reported that p63 expression increases in high-grade CIN.[20] Quade performed p63 immunohistochemical staining on samples from CIN patients and reported that p63 typically localized to the basal and parabasal cells in CIN1 and that the p63-staining pattern extended to the middle and upper layers in samples from patients with CIN 2 or CIN 3. Martens reported similar results for the p63 immunohistochemical staining of samples from CIN patients. In our current study of CIN 1 patients, the p63 expression pattern did not differ significantly between the regression group and the persistent group, suggesting that p63 was of no value as a prognostic factor for early CIN; although all 7 patients who were negative for p63 expression were in the spontaneous regression group, this was not statistically significant.

Malondialdehyde, an end product of lipid peroxidation, is highly cytotoxic, acts as a tumor promoter. The level of MDA in cervical cancer patients is significantly increased. There is a significant rise in the serum MDA concentration in patients with cervical cancer.[21] Moreover, previous studies also demonstrated that MDA levels in the blood of different types of cancer patients were significantly higher when it was compared with healthy controls.[22] Similarly the result of this study showed that level of lipid peroxidation was significantly higher in serum of the FIGO stages of all the four groups as compared to the healthy control group. Therefore, oxidative stress is considered to be a predominant factor even in the initial stage of carcinogenesis.

Glutathione peroxidase (GPx) catalyzed the conversion of glutathione (GSH) to glutathione disulphide (GSSH) while its reduction is catalyzed

by glutathione reductase from GSSH to GSH.[23] They act as secondary antioxidants and protects the cell from many cytotoxic and carcinogenic agents by scavenging reactive oxygen species. A high GSH level is needed to restore the sufficient concentration of antioxidants and to stimulate the scavenger enzymes indispensable to counteract the damaging actions of free radicals.[24] Mukund et al. demonstrated the level of glutathione in patients with cervical cancer and a control healthy women. The results showed that the level of GSH in patients with cervical cancer were significantly lower than the healthy control women.[25] We also observed a significant decline in GSH level in patients of cervical cancer which could be considered as an adaptive response to oxidative stress.

SOD is one of the primary antioxidant enzyme which is widely distributed in all the cells but present in higher amount in red blood cells. SOD protects the cell against lipid peroxidation and catalyzes the dismutation of superoxide anions into oxygen and hydrogen peroxide. SOD enhanced its activity compensatively when there is increased production of superoxide anions. Inflammation in the cervix increases the production of SOD which in turn increases intracellular hydrogen peroxide, thus creating an environment favorable for DNA damage and for initiation and progression of cancer. Our study demonstrated that activity of SOD in blood of all the examined patients group were significantly increased compared to healthy control group. Our result showed statistical significance for patients in the advanced disease group as compared to healthy subjects. Demirci et al.[26] also showed an increase in SOD activity in cervical cancer patients, compared to the control group.

## CONCLUSION

The malignant transformation process. HPV vaccines will help prevent some of the primary female genital cancers associated with HPV type 16. More in-depth studies are needed to understand the molecular carcinogenesis pathway in these p16-negative tumors and to improve outcomes for this population. Our data suggested an imbalance between oxidant-antioxidant status of patients in our study. This imbalance plays an important role in the pathogenesis and progression of cervical cancer though the involvement of these parameters is altered in oxidative stress. The supplementation of antioxidants may reduce the progression of disease in cervical cancer patients.

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