p53 and bcl2 expression in malignant and premalignant lesions of uterine cervix and their correlation with human papilloma virus 16 and 18

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Abstract

Background and Objective: Persistent high risk human papilloma virus (HPV) infection is probably the best predictor of increased risk of cervical cancer, but expression of certain markers of cell proliferation and apoptosis have been studied. The present study was conducted to evaluate the expression of p53 and bcl2 in premalignant and malignant lesions of cervix and its correlation with HPV type 16 and 18. Materials and Methods: The study comprised of 35 cases (including 24 prospective cases and 11 retrospective cases) of premalignant and malignant lesions of the cervix. Slides were stained with Hematoxylin and Eosin and p53, bcl2 (immunohistochemistry), HPV 16 and HPV 18 (in situ hybridization). Results: p53 positivity was seen in 8/19 (42.1%) cases of cervical intraepithelial neoplasia (CIN) and 8/16 (50%) cases of carcinoma cervix, the difference not significant statistically. The difference in bcl2 expression in CIN versus carcinoma cervix (84.21% vs. 43.75%) was statistically significant (P = 0.030). There was no significant difference between p53 and bcl2 expression and the stage and grade of the tumors. Seven out of 19 cases of CIN (36.84%) were positive for HPV 16/18 infection and 8/16 cases (50%) of carcinoma cervix were HPV positive (P = 0.628). Conclusions: No significant association was found between HPV 16/18 infection and p53 and bcl2 expression in premalignant and malignant lesions of uterine cervix. Although, bcl2 staining showed a significant difference between CIN and carcinoma cervix, a larger case series is required to assess the association between HPV infection and overexpression of p53 and bcl2 proteins in these lesions.

Key words: bcl2, cervix, human papilloma virus, p53

Introduction

Carcinoma cervix is the most common cancer in females in developing countries like India and is the second most common cancer following breast cancer in females from developed countries.[1] Persistent high risk human papilloma virus (HPV) infection is probably the best predictor of increased risk of cervical cancer but expression of several other biomarkers of cell proliferation and apoptosis including p53, bcl2, Ki-67, mdm2, cyclin D1, cytokeratin, cyclin E, p16 etc., have been studied.[2] More than 200 types of HPV have so far been identified on deoxyribonucleic acid (DNA) sequence analysis. Approximately, 80-90% of cervical carcinomas contain DNA sequences of specific HPV subtypes, especially those of HPV 16 and 18.[1,5] Recent molecular biology data on the natural history of HPV infection and cervical cancer suggest that viral infections interferes with the mechanism of cellular growth, DNA repair and immunologic responses. Inactivation of p53 gene by E6 oncoprotein of HPV has been proven.[2,6] The association between p53 expression and progression of cervical cancer is not well-understood due to contradictory reports.[3,7,8] Bcl2 is an intracellular membrane protein which prevents apoptotic cell death. Overexpression of bcl2 can block p53 mediated G1 arrest and co overexpression of c myc and bcl2 can inhibit p53 induced apoptosis.[3] Bcl2 overexpression is present in premalignant and malignant lesions of cervix. It has been suggested that bcl2 may play a vital role in a relatively early stage of cervical tumorigenesis in association with bax expression and HPV infection.[8] Bcl2 positivity has also been shown to confer a better 5 year survival rate and prognosis.[5,8]

The present study was to evaluates the expression of p53 and bcl2 in premalignant and malignant lesions of the cervix by immunohistochemistry and its correlation with HPV type 16 and 18 (by in situ hybridization technique). The p53 and bcl2 expression has been correlated with the clinical stage and histologic grade of the lesion.

Materials and Methods

The study comprised of 35 cases of premalignant and malignant lesions of the cervix. All consecutive cases received over a 2 year period (from January 2005 to April 2007) were retrieved from the records of Department of Pathology of our institute, comprising of 24 prospective cases and 11 retrospective cases. The nature of specimens
included both cervical biopsies as well as hysterectomy specimens. The clinical details of the patients including age, clinical signs and symptoms, International Federation of Gynaecology and Obstetrics stage and histological diagnosis and grade of lesion were documented.

The study was approved by institutional ethical committee and written consent was obtained from all the patients. The lesions comprised of cervical intraepithelial neoplasia (CIN) grade 1, 2, 3 and invasive carcinoma of cervix of different histologic types.

Paraffin blocks of these patients were retrieved and five sections of 3-5 µ thickness were taken on poly L lysine coated slides. These slides were stained with Hematoxylin and Eosin and p53, bcl2, HPV 16 and HPV 18. p53 and bcl2 immunohistochemistry was put on tissue sections using Avidin Biotin technique (labeled streptavidin biotin + kit or labeled streptavidin-biotin + kit). p53 staining gave brown nuclear reactivity. A case was considered positive if more than 10% nuclei were stained.

In situ hybridization involves the specific hybridization of a labeled nucleic acid probe to complementary target sequences in tissues followed by visualization of the location of the probe. In the present study, the DAKO in situ hybridization detection system K0601 was used, which utilizes alkaline phosphatase conjugated streptavidin to localize biotinylated probes. Positive signals corresponding to the areas of hybridization appeared as blue or purple regions within individual cells of the tissue. Three patterns of staining were seen:

- As diffuse homogenous staining present throughout the nucleus indicative of episomal virus
- As punctate dots within the nucleus indicative of integrated virus
- As mixed pattern indicative of both episomal and integrated virus.

Statistical analysis was performed using SPSS software version 13 (IBM) \( P < 0.05 \) was considered to be significant.

**Results**

The study comprised of a total of 35 cases of CIN and carcinoma cervix, including both prospective and retrospective cases. Out of the total of 35 cases, CIN comprised of 19 cases including nine cases of CIN I, eight cases of CIN II and two cases of CIN III whereas the rest of the 16 cases were carcinomas of the cervix (including 12 cases of squamous cell carcinoma, three cases of adenocarcinoma and one case of adenosquamous carcinoma with adenoid cystic differentiation).

The patients ranged in age from 22 to 65 years. The mean age of patients diagnosed with carcinoma was higher (46.13 years) compared to CIN (37.16 years) and the difference was statistically significant \( (P = 0.033) \). The most common symptom in patients with CIN was discharge per vaginum whereas in cases with carcinoma cervix, it was bleeding per vaginum [Table 1]. The patients with carcinoma cervix had a significantly higher parity in comparison to patients with CIN (5.19 vs. 3.05; \( P = 0.004 \)) [Table 2]. Among the carcinoma cervix cases, 10 belonged to stage IB, four belonged to stage IIA and two were stage IIB. None of the cases were stage III or IV.

Most of the cases of carcinoma cervix were moderately differentiated (nine cases), followed by well-differentiated (6). However, one case of adenosquamous carcinoma was not graded.

p53 immunostaining was nuclear and its location generally paralleled the extent of dysplasia within the epithelium. p53 positivity was seen in 8/19 (42.1%) cases of CIN and 8/16 (50%) cases of carcinoma cervix [Figure 1]. However, there was no significant difference in p53 expression in the premalignant and malignant lesions of the uterine cervix \( (P = 0.301) \). CIN showed an increasing percentage positivity of p53 expression with increasing grade of the lesion (CIN I 2/9 cases (22.2%); CIN II 4/8 cases (50%) and CIN III 2/2 cases (100%); \( P = 0.110 \)).

p53 expression in stage IB carcinoma cervix was 6/10 cases (60%), in 2/4 cases (50%) of stage IIA and none of the cases of stage IIB carcinoma. As the stage advanced from IB to IIB, the p53 expression reduced \( (P = 0.301) \). There was no significant difference between p53 expression and the grade of the tumors (well differentiated vs. moderately differentiated 50% vs. 55.56%; \( P = 1.00 \)), p53 expression in various categories of lesions is depicted in Table 3.

### Table 1: Clinical presentation of all patients

| Case          | Post coital bleeding | Discharge per vaginum | Bleeding Asymptomatic | Total no. of cases |
|---------------|----------------------|------------------------|-----------------------|-------------------|
| CIN I         | 0                    | 5                      | 2                     | 2                 | 9                 |
| CIN II        | 1 (                  | 3                      | 2                     | 2                 | 8                 |
| CIN III       | 0                    | 1                      | 0                     | 1                 | 2                 |
| SCC           | 4 (                  | 2                      | 6                     | 0                 | 12                |
| Adenocarcinoma| 2 (                  | 1                      | 0                     | 0                 | 3                 |
| Adenosquamous | 0                    | 0                      | 1                     | 0                 | 1                 |

CIN=Cervical intraepithelial neoplasia, SCC=Squamous cell carcinoma

### Table 2: Distribution of cases in relation to parity

| Parity | CIN | Carcinoma cervix |
|--------|-----|------------------|
| 0-2    | 7   | 1                |
| 3-5    | 11  | 10               |
| 6-8    | 1   | 2                |
| >8     | 0   | 3                |

CIN=Cervical intraepithelial neoplasia
The immunostaining of bcl2 was cytoplasmic, localized to the basal layer in CIN [Figure 2]. A very high percentage of CIN cases (16/19 that is 84.21%) expressed bcl2. On the other hand less than half the cases of carcinoma cervix (43.75%; 7/16) showed bcl2 expression and the difference was statistically significant ($P = 0.030$). Among CIN, bcl2 positivity reduced with increasing grade (CIN I – 88.9%; CIN II – 87.5% and CIN III 50%; $P = 0.373$). Among carcinomas, a higher percentage of adenocarcinomas were positive for bcl2 compared with squamous cell carcinomas (66.67% vs. 33.33%) [Figure 3]. Bcl2 expression in carcinoma cervix increased as the stage advanced from IB to IIB (IB ‑ 30%, IIA ‑ 50% and IIB 100%), but the difference was not statistically significant ($P = 0.182$). bcl2 expression in various categories of lesions is depict in Table 4.

Although bcl2 positivity was higher in well differentiated carcinoma than in moderately differentiated carcinoma (50% vs. 33.33%); it was not statistically significant ($P = 0.622$).

In the present study, 10 cases showed both p53 and bcl2 expression (28.57%), six cases were p53 positive but bcl2 negative (17.14%). Thirteen cases (37.15%) were positive for bcl2 but p53 negative. The remaining six cases (17.14%) were both bcl2 and p53 negative. However, no statistical association was seen between p53 and bcl2 expression ($P = 1.00$).

Seven out of 19 cases of CIN (36.84%) were positive for HPV 16/18 infection and 8/16 cases (50%) of carcinoma cervix were HPV positive [Table 5]. There was no significant difference between HPV status of various cervical lesions ($P = 0.628$). 6/7 HPV 16/18 positive CIN cases showed a diffuse intranuclear staining pattern [Figure 4] while one case showed an intranuclear dot like pattern. 7/8 HPV 16/18 positive carcinoma cases showed an intranuclear dot like pattern while the remaining one case showed a mixed pattern. Significantly higher number of carcinoma cases showed an intranuclear dot like staining pattern ($P = 0.003$).
Among the cervical carcinomas, HPV 16 was detected exclusively in squamous cell carcinomas. HPV 16 was positive in two cases of CIN I, four cases of CIN II and one case of CIN III. HPV 18 was detected in fewer cases of both premalignant and malignant lesions of cervix as compared to HPV 16. HPV 18 was positive in two cases of CIN II, three cases of squamous cell carcinoma and two cases of adenocarcinoma. Only five cases showed coinfection with HPV 16 and 18 which included two cases of CIN II and three cases of squamous cell carcinoma.

In the present study, p53 expression was seen in eight HPV positive cases and eight HPV negative cases. No significant statistical association could be found between HPV infection and p53 expression both in CIN (P = 0.377) and in carcinoma cervix (P = 1.00). 10 cases were HPV positive and bcl2 positive (28.57%). However, 13 cases which were HPV negative were bcl2 positive (37.14%). Therefore, no significant statistical association could be found between HPV infection and bcl2 expression in both premalignant and malignant lesions of the cervix. Correlation of p53 and bcl 2 expression with HPV infection in CIN and carcinoma cervix is depicted in Table 6.

## Discussion

Carcinoma cervix is the most common cancer in females in India.[1] Persistent HPV infection has been implicated in around 80-90% of cervical cancers.[2] The various modalities required to stage a carcinoma cervix include. Numerous biomarkers have been studied in CIN and carcinoma of the uterine cervix. Not many studies have established a correlation between HPV infection and the overexpression of the commonly used biomarkers such as p53 and bcl2. This study was undertaken to study the significance of the expression of these biomarkers and their correlation with HPV infection in premalignant and malignant lesions of the cervix.

A total of 35 cases of cervical intraepithelial lesions and carcinoma of the cervix were included in the study. The patients ranged in age from 22 to 65 years. The patients of carcinoma had a significantly higher parity in comparison to patients of CIN (P = 0.006).

In the present study, overall p53 immunoreactivity in CIN was 42.10%. Jeffers et al.[9] and Dellas et al.,[10] using similar technique found comparable positivity rates between 40% and 50% respectively. In contrast, higher positivity rates ranging between 60% and 80% were found by other authors[3-5,7-11] some of whom also included cases under follow-up for genital HPV infection.[3] In our study, 50% of the carcinoma cervix cases showed p53 positivity. The results were in accordance with those found by several other authors.[12-14] However, many authors have found lower positivity rates ranging between 10% and 30% respectively.[15-17] In contrast, Grace et al.[17] and few other workers[11,18,19] found much higher rates of p53 expression ranging between 69% and 100% in squamous cell carcinomas of the cervix.
We found that as the stage of carcinoma advanced, p53 expression declined. However, there were no patients with stage III and IV. The difference between p53 expression and stage of carcinoma cervix was not statistically significant ($P = 0.301$). Similar results were obtained by Wootipoom et al.\(^\text{(19)}\) and Helland et al.\(^\text{(20)}\) who also did not find any significant correlation between the tumor stage and p53 expression. In the present study, there was no significant difference between p53 expression and the grade of the tumors ($P = 1.00$). A study done by Dellas et al.\(^\text{(10)}\) and Avall-Lundqvist et al.\(^\text{(16)}\) found that the well differentiated tumors showed a higher p53 expression than the poorly differentiated tumors and that this association was statistically significant. Wootipoom et al.\(^\text{(19)}\) showed that tumors with higher grade had lower levels of p53 protein expression and it was statistically insignificant.

In the present study, the bcl2 expression reduced with increasing grade of CIN but the difference was not significant ($P = 0.373$). On the contrary, most other studies\(^\text{(5,7,8)}\) showed an increasing expression of bcl2 with the rising grade of CIN but only Saegusa et al.\(^\text{(5)}\) and Grace et al.\(^\text{(7)}\) found the difference to be statistically significant. We found a trend that as the stage advanced, the expression of bcl2 increased, but it was not statistically significant ($P = 0.182$). This was in accordance with Munakata et al.\(^\text{(21)}\) and Wootipoom et al.\(^\text{(19)}\) and contrary to that observed by Tjalma et al.\(^\text{(25)}\) In our study, 50% well differentiated and 33.33% moderately differentiated carcinomas were bcl2 positive, but the difference was not statistically significant ($P = 0.622$). Similar results were observed by Tjalma et al.\(^\text{(22)}\) and Wootipoom et al.\(^\text{(19)}\). However, Aletra et al.\(^\text{(23)}\) found that bcl2 was more frequently expressed in well-differentiated tumors compared to poorly differentiated tumors, the association being statistically significant.

In the present study, 10 cases showed both p53 and bcl2 expression, five cases were p53 positive but bcl2 negative. Thirteen cases were positive for bcl2 but were negative for p53. The remaining five cases were negative for both bcl2 and p53. However, no statistical association was seen between p53 and bcl2 expression in premalignant and malignant lesions of uterine cervix ($P = 1.00$ for both CIN and carcinoma). This was in accordance with Crawford et al.\(^\text{(20)}\) Dimitrakakis et al.\(^\text{(8)}\) found that coexpression of p53 and bcl2 was most frequently seen in invasive squamous cell carcinoma than in other histologic subtypes ($P < 0.01$). However, Rajkumar et al.\(^\text{(17)}\) noted that there was an inverse correlation between p53 and bcl2 expression. We found HPV 16/18 positivity in 22.22% CIN I, 50% each in CIN II, CIN III, squamous cell carcinomas and 33.33% adenocarcinomas. Similar positivity ranging between 35% and 55% were obtained using in situ hybridization by al-Saleh et al.\(^\text{(4)}\) Choudhury and Singh\(^\text{(23)}\) and Jeffers et al.\(^\text{(9)}\). On the other hand, a study by Dellas et al.\(^\text{(10)}\) and Menon et al.\(^\text{(24)}\) found a much higher incidence of HPV infection (75-100%). Other authors using polymerase chain reaction, restriction fragment length polymorphism or Hybrid capture II found higher positivity rates varying between 70% and 95% for HPV 16 and HPV 18 infection in cases of CIN and carcinoma cervix.\(^\text{(5,7,12)}\) The higher rates have been reported because of the use of more sensitive methods of HPV detection. In our study, a significantly higher number of carcinoma cases showed an intranuclear dot like staining pattern ($P = 0.003$). A similar staining pattern of HPV 16/18 was observed by Menon et al.\(^\text{(26)}\) and Choudhury and Singh.\(^\text{(21)}\)

In the present study, no significant statistical association could be found between HPV infection and p53 expression ($P = 0.377$ for CIN and 1.00 for carcinoma cervix) which was in accordance with Jeffers et al.\(^\text{(9)}\) and Rajaram et al.\(^\text{(13)}\). On the contrary, Dellas et al.\(^\text{(10)}\) found a significant correlation between HPV infection and p53 immunoreactivity. We also did not find any significant statistical correlation between HPV infection and bcl2 expression. This was contrary to the findings of Saegusa et al.\(^\text{(5)}\) and Grace et al.\(^\text{(7)}\)

### Conclusions

To summarize, no significant association was found between HPV 16/18 infection and p53 and bcl2 expression in premalignant and malignant lesions of uterine cervix. However, bcl2 staining showed a significant difference between CIN and carcinoma cervix. A larger case series is required to assess the association between HPV infection and overexpression of p53 and bcl2 proteins in these lesions. Bcl2 immunostaining may be used as a diagnostic marker to differentiate malignant lesions from the premalignant lesions but its role as a prognostic marker needs to be further evaluated.

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