Original Article

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Comparative Evaluation of SOX2 and p16 Expression in Intraepithelial Neoplasia and Invasive Cancer of Cervix

Pathology Section

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ABSTRACT

Introduction: SRY (Sex determining region Y)-Box Transcription Factor 2 (SOX2), a transcription factor functioning as a stem cell marker has been studied in many cancers for its role as an oncogene. This study evaluates the expression of SOX2 and protein 16 (p16) expression in cervical with the intent to establish their role as a diagnostic biomarker.

Aim: To evaluate the nature of SOX2 expression in cervical cancer and in intraepithelial lesions of cervix and compare it with the expression of p16 with the intent to establish its role as a diagnostic biomarker.

Materials and Methods: This study was a retrospective observational study conducted in the Department of Pathology, Chettinad Hospital and Research Institute, Chennai, Tamil Nadu, India from October 2018 to September 2019. Archival blocks for study were collected from cases between January 2012 to December 2017. Immunohistochemistry for SOX2 and p16 on 61 cases of cervical lesions including SCC, Low-grade Squamous Intraepithelial Lesion (LSIL), High-grade Squamous Intraepithelial Lesion (HSIL) and normal cervix were done. A chi-square analysis was used to determine the relationship of SOX2 and p16 expression in different lesions and compared the same. All collected data was tabulated and analysed by Statistical Package for Social Sciences (SPSS) version 23.0 and was compared by chi-square tests.

Results: In the total 61 cases (majority with LSIL, n=21, 34.43%, SCC were 19 (31.15%), HSIL were 20 (32.79%) and adenosquamous carcinoma were 1 (1.64%), SOX2 (p-value <0.001) and p16 (p-value 0.0016) showed over-expression in SCC and HSIL with significant p-value, LSIL showed low expression. SOX2 and p16 expression was limited to the basal one-third in LSIL cases, whereas it was expressed up to two-third or full thickness in HSIL cases. Also, SOX2 and p16 had a significant relationship with p-value=0.001. SOX2 was sensitive for SCC with 84.21% sensitivity and p16 was sensitive for HSIL with 90% sensitivity.

Conclusion: Both SOX2 and p16 show increasing expression as the lesion progresses from low grade dysplasia to high grade dysplasia and invasive cancer and can complement each other to make a definitive diagnosis.

Keywords: Biomarker, Cervical cancer, High-grade squamous intraepithelial lesion, Immunohistochemistry, Low-grade squamous intraepithelial lesion, Stem cell marker

INTRODUCTION

Cervical cancer is the fourth common cancer globally and second commonest gynaecologic cancer in women of India with a burden of high cancer- related deaths in developing countries like India [1]. Every year, 134,420 women are newly diagnosed with cervical carcinoma and 72,825 deaths occur [2]. The reasons for the high mortality are due to late presentation, lack of early diagnosis and immediate treatment, disease recurrence, radio and chemo resistance and ineffective treatment options for advanced disease. The foremost important aetiologic factor for cervical cancer is infection by High-Risk Human Papilloma Virus (HR-HPV) which target Cancer Stem Cells (CSC). Cervical cancers which carry these HPV types exhibit poor response to treatment with chemotherapy, and display impaired chemotherapy-induced apoptosis [3]. The CSCs are a minor population of tumour cells with tumourigenic potential of cancer, including chemo and radioresistance [4]. Therefore, markers expressed by these CSCs can be valuable targets for predicting prognosis and for developing new therapeutic targets. One such cervical stem cell marker is SOX2. The SOX2 is sex-determining region Y (SRY)-box 2. The SOX2 belongs to SOXB1group. It is a transcriptional factor which plays an important role in maintaining self renewal or pluripotency of undifferentiated Embryonic Stem Cells (ESCs) [5]. Several recent studies conducted have shown that SOX2 is involved in the carcinogenesis including invasion and metastasis of many human malignant tumours. The SOX2 expression is higher in cervical cancer cells than in normal cervical cells. The SOX2 expression in cervical cancer is associated with poor prognosis including poor survival and chemo-resistance [6,7]. The p16INK4A, is a tumour suppressor protein that inhibits CDK4 and CDK6 which have oncogenic properties. Therefore, p16 was thought to be a negative regulator of cell proliferation. The p16INK4A gene was initially found to be down regulated in many tumour cell lines, suggestive of its nature as a tumour suppressor gene. However, over expression of p16 has been implicated in apoptosis, cell invasion and angiogenesis which are all related to cancer [8]. Its over expression has been observed in many human tumours [9-11]. The p16 over expression in tumours is now being used as a diagnostic tool and is said to be directly associated with infection by high-risk HPV types [12]. Therefore, p16INK4A over expression serves as a surrogate biomarker of HPV infection. For these reasons, it is imperative to develop an effective biomarker which will act as a diagnostic and a prognostic marker. This study evaluates the nature of SOX2 expression in cervical cancer and in intraepithelial lesions of cervix and compares it with the expression of p16 with the intent to establish its role as a diagnostic biomarker.

MATERIALS AND METHODS

This study was a retrospective observational study conducted in the Department of Pathology, Chettinad Hospital and Research Institute, Chennai, Tamil Nadu, India for a duration of one year from October 2018 to September 2019. Approval for the study was obtained from the Institutional Human Ethics Committee (133/IHEC/06-18). Histology proven cases of intraepithelial neoplasia of the cervix and invasive cervical cancer from January 2012 to December 2017 were

retrieved from the files in the Department of Pathology. Formalin-fixed and paraffin-embedded tissue blocks of these cases were retrieved from the archives. The clinical details of these cases were obtained from the Medical Records Department (MRD) of the institute.

Inclusion criteria: Complete diagnostic and clinical evaluation data of biopsy proven cases of intraepithelial neoplasia and SCC of cervix reported during January 2012 to December 2017 were included in the study, along with their formalin-fixed and paraffin-embedded tissue blocks. Data of 10 cases of normal cervix were also included for comparison with cases.

Exclusion criteria: Cases with no complete clinical details or non availability of paraffin blocks, adenocarcinoma of cervix were excluded from the study.

Finally, a total of 61 cases were included in the study. Of these 21 were LSIL, 20 were HSIL, 19 were SCC of cervix and one case of adenosquamous carcinoma. Ten cases of normal cervix were included in the study for the purpose of comparing them with the immunohistochemistry of the cases.

Immunohistochemistry

The tissue blocks obtained were reembedded and three sections were cut at 3.5 microns. One section was used for Haematoxylin and Eosin (H&E) staining and the other two sections were used for SOX2 and p16 staining. Haematoxylin and eosin staining of the cases were done to confirm the histological diagnosis and appropriate areas for performing immunohistochemistry were marked. Positively charged hydrophobic slides were used for Immunohistochemistry (IHC) sections. The SOX2 staining was done using rabbit monoclonal antibody (PR071-3ml RTU, PathnSitu; Clone-EP103) and p16 staining was done using mouse monoclonal antibody (PM143-3ml RTU, PathnSitu; Clone- G175-405). After deparaffinisation and hydration, the slides were subjected to antigen retrieval by pressure-cooking for 20 min.

Endogenous peroxidase activity was neutralised using peroxide block placement on the slides for 10 minutes at room temperature. The slides were then incubated with primary monoclonal antibody of SOX2 and p16 for 45 min at room temperature. This was followed by incubation with target binder (PolyExcel target binder, PathnSitu) for 10 minutes and application of secondary antibody (PolyExcel Poly HRP; PathnSitu) for 10 minutes at room temperature. Chromogen reaction was developed after exposure of the material to DAB (Diaminobenzidine) (PolyExcel Stunn DAB Chromogen; PathnSitu) for five minutes. Finally, haematoxylin was used as a nuclear counterstain. These cases were stained in 10 batches, each batch had one normal cervix, one positive control for SOX2 (glial tissue) and one positive control for p16 (SCC).

Assessment of SOX2 Expression

All slides were evaluated for both the intensity of the staining and the percentage of positive cells. The SOX2 shows nuclear positivity. The intensity of the staining was classified as strong (3), moderate (2), weak (1), and negative (0) and the percentage of positive cells was classified as <10% staining (0), 10-25% staining (1), 26-50% staining (2), 51-75% staining (3) and >75% staining (4). The histological score was defined as the percentage of positive cells score multiplied by the intensity of the staining score. According to the histological score, all slides were divided into 0 (negative), 1-4 (weakly positive), 5-8 (positive), 9-12 (strongly positive). Negative and weakly positive were taken as negative expression, positive and strongly positive were taken as positive expression [13].

Assessment of p16 Expression

All slides were evaluated for both the intensity of the staining and the percentage of positive cells. The p16 shows nuclear and cytoplasmic positivity. The intensity of the staining was classified as strong (3), moderate (2), weak (1), and negative (0) and the percentage of positive cells was classified as <1% staining (1), 1-10% staining (2), 11-33% staining (3), 34-66% staining (4) and >66% staining (5). The histological score was obtained by adding the percentage of positive cells score and the intensity of the staining score. According to the histological score, all slides were divided into 0-2 (low expression), 3-5 (moderate expression), 6-8 (over expression) [14].

STATISTICAL ANALYSIS

The frequencies and percentage analysis of various parameters were done using SPSS software version 23. Relationship of SOX2 and p16 with histopathology and comparison of SOX2 with p16 were done using chi-square analysis. The p-value <0.05 was considered statistically significant.

RESULTS

The study revealed the following findings. Of the 61 cases, majority of them had LSIL (n=21, 34.43%) [Table/Fig-1]. The distribution of the cases is given in the [Table/Fig-1]. Apart from the 61 cases, also included were 10 cases of normal cervix for the purpose of IHC staining which was done in 10 batches with one case of normal cervix for each batch of IHC. Scoring of the lesions based on SOX2 showed that majority of them were positive for SOX2 (n=33, 54.09%) [Table/Fig-2]. Of the 10 cases of normal cervix, none of them showed positive expression for SOX2. The 4/10 cases of normal cervix showed positive cells in the basal layer and had a score ranging from 2-3 which was considered negative. Scoring of the lesions based on p16 showed that 24 cases had over expression (39.34%) [Table/Fig-3]. Of the 10 cases of normal cervix, p16 was not expressed in any of the cases.

Histopathology	Frequency	Percentage (%)
SCC	19	31.15
Adenosquamous carcinoma	1	1.64
HSIL	20	32.79
LSIL	21	34.43

[Table/Fig-1]: Histopathology of the cervical lesions.
SCC: Squamous cell carcinoma; LSIL: Low-grade squamous intraepithelial lesion; HSIL: High-grade squamous intraepithelial lesion

Expression of SOX2	Frequency	Percentage (%)
Negative (0-4)	28	45.9
Positive (5-12)	33	54.09

[Table/Fig-2]: Expression of SOX2.
SOX2: Sex-determining region Y (SRY)-box 2

Expression of p16	Frequency	Percentage (%)
Low expression	23	37.70
Moderate expression	14	22.95
Over expression	24	39.34

[Table/Fig-3]: Expression of p16.
p16: protein 16

Relationship between histopathology and expression of SOX2 & p16: The SOX2 staining was seen in 16/19 cases of SCC, 1/1 case of Adenosquamous carcinoma, 15/20 cases of HSIL and only 1/21 cases of LSIL [Table/Fig-4]. There was a significant relationship between histopathology and SOX2 [Table/Fig-4]. The p16 over-expression was seen in 14/19 SCC cases, 10/20 HSIL cases and 0/21 LSIL cases. The 4/19 SCC cases, 2/20 HSIL cases, 17/21 LSIL cases showed low p16 expression. A total of 10/10 Normal cervix showed loss of expression of p16. The 1/19 SCC case, 8/20 HSIL cases, 4/21 LSIL cases and 1/1 adenosquamous carcinoma showed moderate expression for p16 [Table/Fig-5]. There was a significant relationship between histopathology and p16 expression [Table/Fig-5]. Relationship between SOX2 and p16 expression showed significant p-value by chi-square analysis

[Table/Fig-6]. Statistical analysis showed that SOX2 was sensitive for SCC with a sensitivity of 84.21% and p16 was sensitive for HSIL with a sensitivity of 90% [Table/Fig-7].

Histopathology	SOX2 expression		Total	Chi-square test p-value
	Negative	Positive		
Normal cervix	10	0	10	χ^2=36.19 **p=0.000348**
LSIL	20	1	21	
HSIL	5	15	20	
Squamous cell carcinoma	3	16	19	
Adenosquamous carcinoma	0	1	1	
Total	38	33	71	

[Table/Fig-4]: Relationship between histopathology and SOX2 expression.

Histopathology	Histopathology			Total	Chi-square test p-value
	Low expression	Moderate expression	Over expression		
LSIL	17	4	0	21	x^2=39.099 **p=0.001620**
HSIL	2	8	10	20	
Squamous cell carcinoma	4	1	14	19	
Adenosquamous carcinoma	0	1	0	1	
Total	23	14	24	61	

[Table/Fig-5]: Relationship between histopathology and p16 expression.

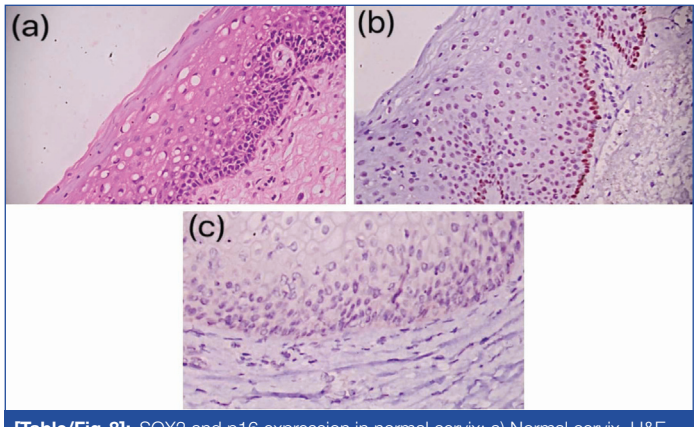
SOX2 and p16 expression		P16 expression			Total	Chi-square test p-value
		Low expression	Moderate expression	Over expression		
SOX2 expression	Positive	3	10	20	33	x^2=29.02 p=0.001186
	Negative	20	4	4	28	

[Table/Fig-6]: Relationship between SOX2 and p16 expression.

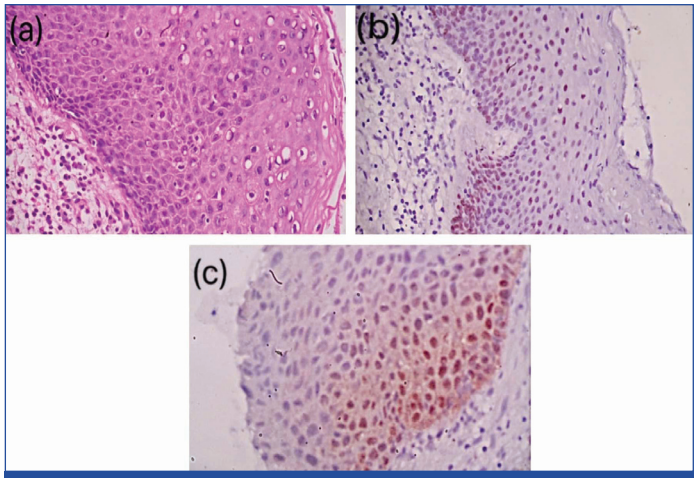
Histopathology	SOX2		p16 expression	
	Sensitivity	95% CI	Sensitivity	95% CI
LSIL	4.76%	0.12 to 23.82	19.05%	5.45 to 41.91
HSIL	75%	50.90 to 91.34	90%	68.30 to 98.77
SCC	84.21%	60.42 to 96.62	78.95%	54.43 to 93.95
Adenosquamous carcinoma	100%	2.5 to 100	100%	2.5 to 100

[Table/Fig-7]: Sensitivity and Specificity of SOX2 and p16 expression.

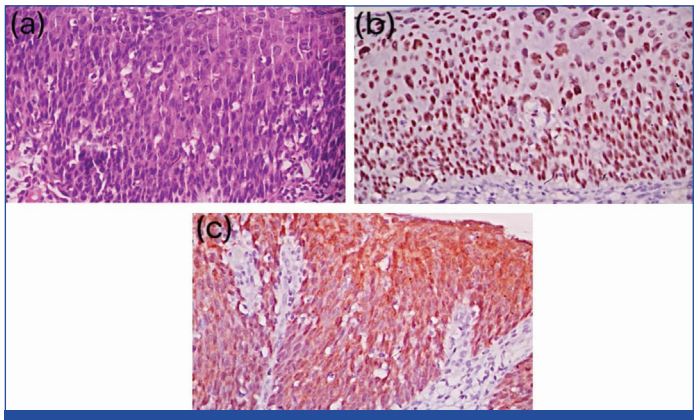
Among the normal cervix, staining was limited to the basal layer of the epithelium [Table/Fig-8]. The SOX2 and p16 staining was limited to lower one-third of the epithelium in LSIL [Table/Fig-9] cases and up to two-third or full thickness in HSIL [Table/Fig-10] cases. The SCC cases showed intense positivity for SOX2 and p16 [Table/Fig-11].



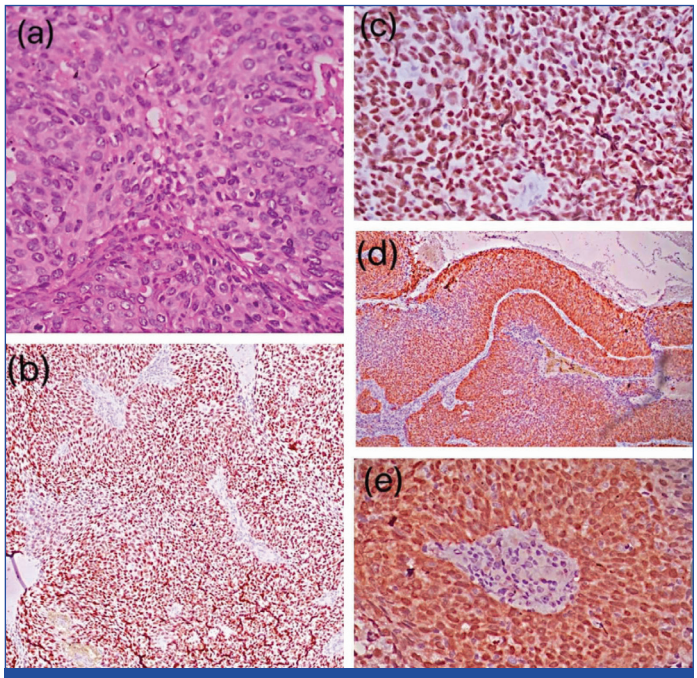
[Table/Fig-8]: SOX2 and p16 expression in normal cervix; a) Normal cervix. H&E 40x; b) SOX2 expression. Only the basal layer of the cervix shows positive cells. IHC 40x; c) p16 expression. No positive cells seen. IHC 40x.



[Table/Fig-9]: SOX2 and p16 expression in LSIL; a) LSIL. H&E 40x; b) Sox2 expression. Staining is seen in the lower third of the epithelium. IHC 40x; c) p16 expression. Staining is seen in the lower third of the epithelium. IHC 40x.



[Table/Fig-10]: SOX2 and p16 expression in HSIL; a) HSIL showing full thickness dysplasia. H&E 40x; b) SOX2 expression. Staining is seen along full thickness of the epithelium. IHC 40x; c) p16 expression. Staining is seen along full thickness of the epithelium. IHC 40x.



[Table/Fig-11]: SOX2 and p16 expression in Squamous cell carcinoma of cervix; a) SCC. H&E 40x; b) SOX2 expression. Intense positivity is seen. IHC 10x; c) SOX2 expression. Intense positivity is seen. IHC 40x; d) p16 expression. Intense positivity is seen. IHC 10x; e) p16 expression. Intense positivity is seen IHC 40x.

DISCUSSION

Many studies have been conducted on SOX2 expression in cervical lesions [6,13-15]. But this study was done to understand the relationship between SOX2 and p16. From the results in this study, it is seen that SOX2 and p16 expression is significantly higher in

SCC compared to HSIL and LSIL and among the intraepithelial lesions the expression is significantly more in HSIL.

Expression of SOX2 in cervical lesions: The results show that SOX2 expression is significantly higher in SCC closely followed by HSIL and low in LSIL. The SOX2 staining was limited to lower one-third of the epithelium in LSIL cases and up to two-third or full thickness in HSIL cases. Among the normal cervix, none of them showed increased expression. Study by Jing J and Zheng PS, on the expression of SOX2 showed that SOX2 over-expression was seen in cervical SCC [15]. Similar results were seen in other studies like Hou T et al., and Yang Z et al., [6,13]. However, they did not include precancerous lesions of the cervix. Studies by Kim BW et al., and Ji J et al., on the significance of SOX2 and OCT4 showed that OCT4 and SOX2 expression was elevated in premalignant and malignant cervical cancers compared to normal cervix suggesting that SOX2 and OCT4 may be involved in the pathogenesis of cervical SCC [16,17]. These results are concordant with the present study.

Expression of p16 in cervical lesions: The p16 expression is significantly higher in SCC compared to HSIL and LSIL. The LSIL shows low expression of p16. The p16 staining was limited to lower one-third of the epithelium in LSIL cases and up to two-third or full thickness in HSIL cases. A study by Lesnikova I et al., showed that, dysplastic cervical epithelium showed higher expression of p16, and this increased with increasing Cervical Intraepithelial Neoplasia (CIN) grade [14]. Also, a small number of cases of CIN showed negative expression. This may be attributed to the fact that few cases of CIN can regress spontaneously, thus p16 serving as a predictive marker for progression to invasive cancer. Similar to this study, a few cases of CIN showed negative expression for p16 in the present study. Study by Sarma U et al., showed increased p16 expression in high grade lesions compared to low grade lesions and non dysplasia cases [18]. Similar results were seen in other studies by, Kory S et al., and Kanthiya K et al., [19,20]. According to the studies by Klaes R et al., Tsoumpou I et al., Kishore V and Patil AG, Tan GC et al., Srivastava S, Kumari K and Vadivelan AA, and Pandey A et al., normal cervix had low expression of p16 and the expression increases from low grade lesions to high grade lesions and invasive cancer [21-27]. A study by Gupta R et al., showed that there is progressive increase in the percentage of positive cells as well as the staining intensity through increasing grades of cervical dysplasia and invasive cancer [28]. Study by Indu VP et al., concluded that combined use of H&E and p16 immunohistochemistry can significantly elevate the accuracy of interpreting and grading cervical lesions and may be incorporated into routine diagnostic/screening for cervical cancer [29].

Relationship between SOX2 and p16: The present study shows that SOX2 and p16 have significant relationship; cases which show high SOX2 show high p16 and vice versa. Analysis of the sensitivity of the markers showed that SOX2 is sensitive for SCC with a sensitivity of 84.21% and p16 is sensitive for HSIL with a sensitivity of 90%. A definite cut-off value for SOX2 and p16 could not be obtained because of a smaller number of cases included in this study. The study by Wolsky RJ et al., showed that SOX2, p16 and Ki67 expression were limited to the basal one-third of the cervix in LSIL cases, whereas HSIL cases showed expression up to two-third or full thickness [30]. These results were comparable to the present study.

Limitation(s)

The smaller number of cases which were included in the study and the lack of follow up details of the cases. In the present study, the prognosis could not be established because proper follow-up of the cases were not available due to the lack of surgical Oncology Department in the Institution.

CONCLUSION(S)

The SOX2 and p16 are significantly expressed in SCC of cervix and the expression increases with the grade of dysplasia from low grade to high grade precancerous lesion and in SCC. Also, few cases of precancerous lesions showed loss of expression of p16 suggesting the possibility of regression of the infection. In such case p16 can serve as a prognostic marker to predict the progression of the lesion. Comparing the expression of SOX2 with that of p16 showed that both the markers show positive relationship, with SOX2 having more sensitivity for detecting SCC of cervix and p16 have more sensitivity for detecting HSIL cases. To our knowledge this is the first study to correlate SOX2 with p16 and establishing sensitivity for each. If studies are done on a larger population with good follow up details, then the nature of SOX2 can be established. These markers in future can be used as targets for therapy.

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