Evaluation of expression of p16^{INK4a} & Survivin in squamous neoplasm of cervix- A 3 years study

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ABSTRACT

Objective: To explore the expression of p16^{INK4a}(p16) & survivin in cervical intraepithelial neoplasm (CIN) & cervical squamous neoplasm.

Materials & Methods: A 3years study was undertaken in MGM Hospital, Warangal, all the cervical & hysterectomy specimens were studied. By considering both exclusion & inclusion criteria 46 cases were taken for p16INK4A & survivin expression & data was analysed.

Results: The p16^{INK4A} & survivin expressions showed the presence of statistical significance in cervical cancer, CIN1, CINII, CINIII and a normal cervical tissue & the comparison also revealed statistical significance among group. P16^{INK4A} showed increase intensity with grade (CINI = 28.57%, CINII = 44.44%, CINIII = 70%, & SCC = 90%), whereas survivin expressions were positive for both nuclear as well as cytoplasm staining(CINI = 28.57%, CINII = 22.22%,CINIII = 40%, SCC = 75%). Moreover, p16^{INK4A} protein was associated with CIN grade & in cervical cancer; survivin protein was also related with clinical stages, CIN grade; the p16^{INK4A} & survivin expressions were positively correlated with cervical cancer, & associated with poor prognosis of cervical cancer.

Conclusion: Briefly, p16^{INK4A} & survivin expressions were positively correlated with the clinico-pathological & prognosis of cervical cancer.P16^{INK4A} linearly correlates well with increasing grade of CIN & SCC, whereas as survivin does not shows linear correlation with increasing grades of CIN . But it was the useful marker for differentiating CIN & SCC, especially in diagnostically difficult cases.

Keywords: P16^{INK4A}, Survivin ,Carcinoma in-situ , Squamous cell carcinoma, cervix

INTRODUCTION

P16^{INK4A} & survivin are promising biological marker for early diagnosis & prognostic evaluation in cervical cancer. The p16^{INK4A} & survivin expression were detected in more than 50% of cervical cancer. The cervix is target for viruses and other carcinogens, which may lead to invasive carcinoma. Worldwide cervical cancer is the second most cancer in women.[1] There are 1.7 million cases in the developing world and as many as 5-13 millions women have precancerous lesion[2,3]. The development of cancer is a multifactorial process. The association of cervical cancer with high risk human papilloma virus (HPV) infection has long been established. However, in a substantial number of females, HPV produces transient infection which gets cleared off by host immune responses.

One of the consequences of this viral cellular protein interplay is the up-regulation of a tumor suppressor gene p16 INK4A which is a cyclin dependent kinase (CDK4) inhibitor[4,5]. P16^{INK4A} serves as a surrogative marker for the oncogenic activities of HPV in replication-competent cells of cervical epithelia and its overexpression is well established in cervical intraepithelial neoplasia[6,7].

Survivin seems to be an early marker of cervical carcinogenesis. Apoptosis is important because it removes unwanted or potentially dangerous damage cells through out life. Survivin is an inhibitor of apoptosis which exerts its effect by binding to the caspases[5].

MATERIALS & METHODS

The present study was conducted in MGM Hospital,Warangal for a period of three years. All the cervix biopsies and hysterectomy specimens received in the pathology department were considered.

Cervix biopsies and hysterectomy specimens of females in the age groups 30-70 years , premalignant and malignant lesions of cervix were included. Only samples with definite histopathological diagnosis were considered. Representative areas in the biopsies are only included. Cervix biopsies and hysterectomy specimens of females of <30yrs were excluded. Unusual tumour types and inadequate samples were excluded.

Cervical biopsies and the hysterectomy specimen were fixed in 10%formalin and were sent for routine histopathological processing. After a histopathological diagnosis of the lesion was made,the paraffin blocks of the

samples which had met the criteria of inclusion were collected. Then carried further for p16^{INK4A} and survivin staining. The results were recorded for individual case.

Positive control: Rectal carcinoma tissue for p16INK4A and normal pancreatic tissue for survivin

Negative control: Involved the omission of primary antibody.

RESULTS

In 3 years we had reported 46 cases in MGM Hospital,Warangal. Out of 46 cases studied majority of the cases (36.95%) were between 61-70 years, least age groups in our study were between 31-40 yrs (Table 1). It is also observed that with increasing age there is increase in the percentage of malignant cells. Age distribution of CIN and SCC are shown in Table 2 and Figure 1. Figures 2,3,4& 5 showing results of P16 ^{INK4A} and Survivin.

Table 1 : Age distribution of cases studied

Age	No of cases	Percentage of cases
31-40	4	8.69 %
41-50	10	21.73 %
51-60	15	32.60%
61-70	17	36.95 %

Table 2 : Age distribution of CIN

Age (yrs)	CIN 1	CIN 2	CIN 3
31-40	1	0	1
41-50	1	2	2
51-60	2	4	3
61-70	3	3	4

Figure 1- Age distribution of Squamous cell carcinoma

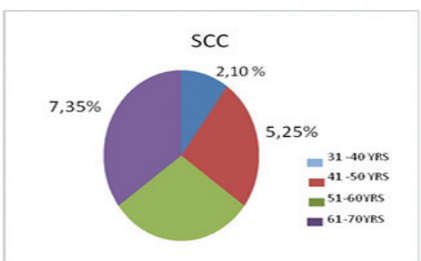


Figure 2: Demonstration of P16 and Survivin in CIN I

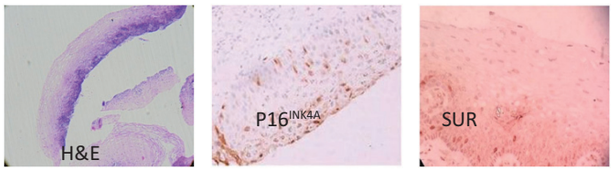


Figure 3: Demonstration of P16 and Survivin in CIN II

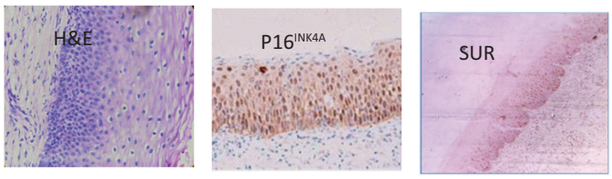


Figure 4: Demonstration of P16 and Survivin in CIN III

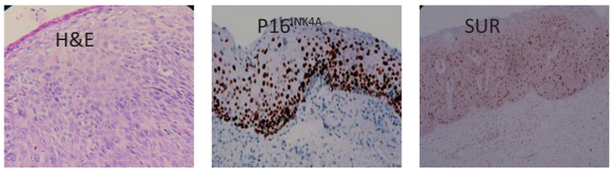
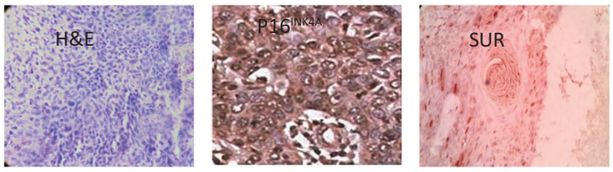


Figure 5: Demonstration of P16 and Survivin in Squamous cell carcinoma



Results of p16 immunostaining in lesions of uterine cervix

p16^{INK4A} immunostaining was carried out on 46 lesions of uterine cervix out of which 31 cases showed positive staining which included 90% (18/20) cases of squamous cell carcinoma cervix, 28.57% %(2/7)cases of CIN1, 44.44% (4/9) cases of CIN2 ,70% (7/10) of CIN3.p16 expression increased with increasing grade of CIN and also in SCC. Most of the SCC showed p16 ^{INK4A} expression. We also noticed p16 ^{INK4A} staining confined to the lower 1/3 of epithelium in CIN1.In CIN 2, p16 ^{INK4A} staining was confined to the lower 2/3 of the epithelium, and in CIN 3,the dysplastic epithelium showed full thickness p16 staining. Diffuse and intense p16^{INK4A} staining was observed in SCC.

Out of 20 cases of squamous carcinomas 2 cases were negative for p16 ^{INK4A} expression and 18 cases exhibited variable degree of expression for p16 INK4A. 2 cases of CIN 1, 4 cases of CIN 2 and 7 cases of CIN 3 exhibited variable degree of expression for p16 ^{INK4A}.

Results of survivin immunestaining in lesions of uterine cervix;

Survivin immunostaining was carried out on 46 lesions of uterine cervix out of which 23 cases showed positive staining.

The survivin expression in various grades of CIN was CIN 1(28.57%),CIN 2 (22.22%),CIN 3(40%) and SCC (75%).In CIN and SCC, survivin staining showed both nuclear as well as cytoplasmic positivity.

DISCUSSION

Out of 20 cases of squamous carcinomas 5 cases are negative for survivin expression and 15 cases exhibited variable degree of expression for survivin. Out of 7 case of CIN 1 studied 2 cases, out of 9 cases of CIN 2 studied,2 cases and out of 10 cases of CIN 3 studied 4 cases exhibited variable degree of expression for survivin. Cervical cancer has well recognized precancerous state, morphologically well recognized as cervical intra epithelial neoplasia, which is graded from CIN 1 to 3. Thus, the alterations of cellular proteins at different levels can be analysed at every step of progression to cancer. It is now recognized that the higher grade CIN (2 and 3) is related to the persistence of HPV infection with its integration into the cellular genomic DNA.[8] This results in disturbances in the expression of several cellular proteins, one of which is p16 ^{INK4A}, an important cell cycle regulatory molecule. The up regulation of p16 ^{INK4A} gene product has been shown to occur following HPV infection which has been very well documented in various studies in literature. Our study is broadly in accordance with results of the previous publications.[9] A minor difference was the p16 ^{INK4A} expression in 44.5-70 % of CIN2/3 in our study as compared to 75-100% expression reported in most of the earlier studies.

We have excluded technical reasons for negativity as many of these cases showed positive internal controls and further, these cases were immunostained at least twice with consistent results.The explanation for the same could be attributed to the use of a different clones of antibodies used in different studies. The other possible explanation could be the difference in HPV types causing CIN.[10] p16 INK4A is considered a surrogate marker for high risk HPV infection according to several reports which have mainly examined HPV16 and 18 sub types. A third reason for p16 ^{INK4A} negativity in CIN2/3 could be the epigenetic modification of the p16 ^{INK4A} gene promoter.

Although there were several previous reports on the role of p16 in cervical carcinogenesis there is a paucity of them in Indian literature in spite of the fact that cervical cancer is one of most common cancers among females in India.

Ruchi Gupta *et al*[11] first studied the p16 ^{INK4A} expression in CIN and invasive squamous cell carcinoma of the uterine cervix in our population. The youngest patient in our study was 32 and the oldest was 70 years old. In this study, most of the CIN 1 cases were p16 ^{INK4A} negative(71.43%).The high percentage of negativity of p16 ^{INK4A} in CIN1may be due to latent or subclinical HPV infection with low viral load that may be insufficient for p16 ^{INK4A} expression.

Ishikawa et *al*[12] found that overexpression of p16 ^{INK4A} in CIN1was more common in cases with HPV 16 and HPV 52 infection. The other possible reason for lower expression of p16 ^{INK4A} in low grade lesions may be because a certain percentage is thought to be caused by low risk HPV types. Previous studies indicated that viral oncoprotein of low- risk HPV such as HPV-6 have no effect on p16 ^{INK4A} because the affinity of HPV-6 E7 protein for cellular p16 is ten-fold lower than that of HPV-16 E7 for pRb[13].

Kang et *al*[14] study concluded that p16 ^{INK4A} gene silencing during CIN was not a rare event and also does not correspond with either HPV status or CIN grading. Thus a proportion of high grades CIN are p16 ^{INK4A} negative as most recent reports indicate.

Table 3 - p16 ^{INK4A} expression in cervical squamous neoplasm- various studies

Author	CIN1%	CIN2%	CIN3%	SSC%
	No of + ve/no of analysed	No of +ve/ no of analysed	No of +ve/no of analysed	No of +ve/no of analysed
Focchi et al[15]	91%80/88	100%65/65	Not done	100%47/47
Lorenzato et al[16]	69%20/29	93%40/43	Not done	Not done
Wang et al[17]	72%54/75	89%17/19	100%19/19	Not done
Agoff et al[18]	57% 43/76	75%60/80	91%103/113	92%42/46
kleating et al[19]	87.5%21/24	91.8%34/37	Not done	Not done
Tringler et al[20]	72%13/18	100%46/46	Not done	100%19/19
Ruchi gupta et al[11]	50%10/20	60%12/20	70%14/20	95%19/20
Present study	28.57%(2/7)	44.44%(4/9	70%(7/10)	90%(18/20)

Our study shows that most of the(90%) SCC lesions show p16 ^{INK4A} over expression, this further emphasizes the important causal relationship between HPV and cervical cancer. However, a few patients with cervical cancer had p16 ^{INK4A} negativity.

Nieh et *al*[21] showed that a proportion of their cervical cases had neither HPV infection nor p16 ^{INK4A} expression. The possible explanation for their absence of p16 ^{INK4A} expression in these high grade lesions could be methylation of the p16 ^{INK4A} promoter resulting in silencing of the p16 ^{INK4A} gene. Our finding are similar to those of Branca et *al*[22],Ozgul et al and Geok chin tan et *al*[23]. which also found that p16 ^{INK4A} expression was directly related to the increasing grade of CIN. Our study showed that the intensity increased with increasing grade of CIN. The presence of survivin staining in normal epithelium

was also seen in studies of other human malignant neoplasm and normal tissues[24]. Branca et *al*[22] and Geok chin et *al*[23]. studies were similar to our study;normal squamous epithelium was in the negative –weak category.The authors also described that survivin staining of normal squamous epithelium was only demonstrated in the cells of parabasal layer.In contrast ,CIN and SCC showed both nuclear as well as cytoplasmic survivin staining.Frost et *al*[25]. suggest that the shift in intracellular distrubition of survivin could be due to nuclear translocation mechanism or a result of artificial disruption caused by HPV infection which leads to survivin expression in the nucleus and cytoplasm.

Branca et *al*.[22] discuss the value of survivin as independent predictor of high-risk HPV where 84.6%of high risk HPV type shows moderate and intense survivin expression.They found that survivin expression was a specific marker of CIN as it was consistently negative in biopsy with out CIN.This is in accordance to our study where survivin expression was moderately to intensely express in CIN.

However in contrast to their study where survivin expression was directly related to the grade of CIN, our study shows no such correlation. The expression was found to be even higher in CIN1 compared to CIN 2. Our study was similar to Geok Chin Tan et *al*[23]. The survivin expression was found to be higher in CIN I as compared to CIN 2 in Geok Tan et *al*.[23] study.

CONCLUSION

This retrospective and prospective study was undertaken over a period of 3years from September 2012 to August 2015 in the Department of Pathology, MGM Hospital Warangal. Present study shows that $p16^{INK4A}$ expression correlates well with the increasing grade of CIN. Survivin does not correlate well with the increasing grade of CIN,it could be useful in differentiating CIN 3 from SCC. Our data support the use of $p16^{INK4A}$ and survivin IHC determining the various grades in CIN as well as between CIN 3 and SCC, especially in diagnostically difficult situations

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