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Original Article

Comparative analysis of p53 and p21 proteins in normal cervix and HPV associated precancerous and cancerous lesions of cervix

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ARTICLE INFO

Article history:
Received 28 April 2015
Accepted 9 May 2015
Available online 3 June 2015

Keywords:
Cervical cancer
HPV-E6
p53
p21
p-p53-ser46

ABSTRACT

Introduction: HPV-E6 oncoprotein of High-Risk HPV plays critical role in the degradation of p53 protein for acquisition of malignant phenotype in cervix cells. The present study, was sought to analyze the expression of HPV-E6, p53 and p21 in cervical cancer to explore possible impact of HPV-E6 in the modulation of these proteins.

Methods: The expression of HPV16/18-E6, p53 and p21 proteins in cervix tissues [normal (n = 100), cervical intraepithelial neoplasia (CIN; n = 67), squamous cell carcinoma (SCCs; n = 153)] was analyzed by immunohistochemistry and revalidated by Western Blotting. The p53 protein functionality was determined by using phosphospecific p-p53-ser46 antibody.

Results: The negative expression of HPV-E6 and p53 but, mild immunoreactivity of p21 was noticed in normal stratified squamous epithelium. In CIN and SCCs, the HPV-E6 was found to be predominantly over-expressed in 80.0% and 84.9% of cases respectively. Significant nuclear accumulation of p53 was observed in 77.6% of precancerous and 83.0% of cancerous tissues whereas, 74.6% of CIN and 73.8% of SCCs represented loss of nuclear p21 expression as compared to normal ($p = 0.0001$, $p = 0.0001$). Pearson's correlation test revealed significant inverse association between p53 and p21 proteins both in CIN ($p = 0.0001$) and SCCs ($p = 0.0001$). Interestingly, significant positive association of HPV-E6 with p53 and negative association with p21 were also detected in CIN ($p = 0.0001$) and SCCs ($p = 0.0001$). No positive expression of p-p53-ser46 was detected in any of the cases.

Discussion: The present study provides the evidences of inhibition of p53 and p21 trans-activity by HPV-E6 resulting alteration of cell growth inhibitory signals in cervical cancer.

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http://dx.doi.org/10.1016/j.jasi.2015.05.001

1. Introduction

Cervical cancer is one of the leading cancers among women in India that contributes more than 1/5th of global burden.[1] The multistep process of cervical carcinogenesis is always been associated with high risk human papillomavirus (HPV) −16 and 18 genotypes infection.[2,3] Obstinate HPV infection and the continuous overexpression of their oncogenes E6 and E7 are The prime steps required for the malignant transformation of cervix cell, as these proteins bring the alteration in p53 and pRB genes.[4,5] Therefore, identification of certain molecular agents that are actually contributed either individually or synergistically with HPV has become chief facet of cervical cancer research.

The p53 gene mediates major tumor suppressor pathways.[6] This protein is critically involved in regulating the cell-cycle,[7] apoptosis[8] and DNA repair.[9] In the normal cell, the activity of p53 protein is controlled by MDM2 through either ubiquitin dependent cytoplasmic p53 degradation or transcriptional repression of p53 in the nucleus.[10] The activity of p53 is effectively enhanced during cellular stresses leading to its phosphorylation and translocation to the nucleus. In the nucleus, it specifically binds to the DNA sequences and triggers the transcription of target genes. p53 also contributes to tumor suppression through cell cycle arrest[11] and apoptosis.[12] Point mutation in p53 gene is reported in many human cancers but is quite uncommon in cervical cancer. In contrast, inactivation of p53 is believed to play a major role in the carcinogenesis of the uterine cervix.[13–15] It is suggested that, the loss of p53 function in cervical cancer is mainly associated with enhanced protein degradation promoted by the HPV-16/18 E6 oncoprotein.[13] The expression of p53 was observed to be increased in cervical high grade lesion (HSIL) and carcinoma[16,17] but the clinical impact of HPV mediated p53 inactivation is still barely understood in cervical cancer due to several contradictory findings. P21 is another tumor suppressor, which get transcriptionally regulated by activated p53.[18] In the normal cell, the level of p21 remains dormant and the protein gets activated only with response to the cellular stress occurred by DNA damage. The expression of this protein has found to be elevated in human cervical cancer.[19,20] Although, the studies have also shown the loss of p21 protein during the HPV driven cervical transformation,[4] but the exact mechanism still not fully understood.

Therefore, in the present study the expression of p53 and p21 proteins was analyzed in normal, precancerous and invasive cancer cervix tissues to determine their biological role in cervical pathogenesis. Furthermore the association of HPV-16/18 oncoprotein E6 with p53 and p21 was also examined in order to delineate its possible role in the deregulation of p53 and p21 transactivity in cervical cancer.

2. Materials & methods

2.1. Tissue specimens

The institutional ethics committee of Vardhman Mahavir Medical Collage & Safdarjung Hospital, New Delhi, India has approved this study. The cervical punch biopsy tissue samples of cervical intraepithelial neoplasia (CIN) and carcinoma patients were collected from Department of Obstetrics and Gynecology, Safdarjung Hospital. A written informed consent was obtained from the subjects before sample collection. Total 320 patients, including 67 histopathologically confirmed CIN; 153 clinically proven, untreated invasive squamous cell carcinoma (SCCs) and 100 normal controls (patients undergoing hysterectomy for utero-vaginal prolapse, with histologically normal cervix) were enrolled in the study. The clinical staging of tumors was confirmed by International Federation of Gynecology and Obstetrics (FIGO) criterion.

2.2. Hematoxylin and eosin staining

Each sample was fixed in 10% buffered formalin and embedded in paraffin. The slides containing 5 μm thickness sections were then processed for routine H&E staining. Subsquentially the stained slides were examined under a light microscope to confirm tissue architecture and then processed for immunohistochemical analysis (IHC).

2.3. Immunohistochemistry

The immunoexpression of HPV-E6 oncoprotein, p53 and p21 was identified by: (a) mouse monoclonal HPV-16/HPV-18 E6 antibody (ab 70, Abcam, Inc., Cambridge, UK) (b) mouse monoclonal p53 antibody (M7001, Dako Cytomation, Glostrup, Denmark) and (c) rabbit polyclonal p21 antibody (ab 18209, Abcam, Inc., Cambridge, UK) respectively. The IHC was carried out as described by us previously.[21] In brief, the sections were first deparaffinised in xylene and dehydrated through graded alcohols. Endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide (H2O2) in 50 ml methanol for 45 min. Antigen retrieval for all the proteins was done in citrate buffer (10 mM) pH-6.0 by heating the sections at 900 W for 10 min and 360 W for 5 min in household microwave oven. Later, the serial sections were incubated overnight in humid chamber, at 4 °C with primary antibodies for HPV-E6 (1:100); p53 (1:50) and p21 (1:100). On the next day, The slides were thoroughly washed with TBS and incubated with polymer based EnVisionTM (Dako Cytomation, Glostrup, Denmark) for 1 h at room temperature. The chromogenic visualization reaction was done by using 3,3-diaminobenzidine hydrochloride (DAB). The stained slides were then examined under light microscope (Olympus BX-51, Japan). In the negative control; primary antibody was replaced by isotype-specific immunoglobulin G. Apart from this the expression of active form of p53 (p53-Ser46) was also evaluated in all the tissues by using phospho-specific, rabbit polyclonal p-p53-ser46 antibody (ab76242; Abcam, Inc., Cambridge, UK; dilution 1:50) to determine p53 functionality. Immunohistochemistry results were evaluated on the basis of staining intensity and percentage positivity as described by us previously.[21] The intensity of protein expression was scored on the scale of 0–3 positivity ('0', negative staining; '1', weak; '2', moderate and '3', intense positive staining); while percentage positivity of stain cells was ranged from 1 to 4+ ('0', complete absence; '1', <5%; '2', 5–20 %; '3', 21–50%; '+4', >50% positive stained cells).

Resulting scoring was calculated by taking the product of both intensity as well as percentage positivity value.

2.4. *Immunoblotting (in representative samples)*

Western blotting was carried out for the revalidation of immunohistochemistry results. In this respect, total cellular protein was extracted from normal as well as precancerous and cancerous (n = 10) frozen cervix tissues by using Ready PrepTM total protein extraction kit (cat. no. 163-2086, Bio-Rad Laboratories, Inc., USA) as per manufacturer's instructions. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Inc., USA). Proteins (80 μg of/lane) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Polyvinlydenedifluoride membrane (PVDF) followed by blocking with 5% non-fat skim milk 4 °C. The membranes were incubated with anti-HPV-E6 (1:1000 dilution), anti-p53 (1:1000 dilution), anti-p21 (1:500 dilution) primary antibodies at 4 °C for overnight and then with with HRP conjugated Rabbit/Mouse anti-IgG secondary antibody (Abcam antibodies) for 2 h at room temperature. Protein bands were visualized on X-ray film by using enhanced chemiluminescence system (ECL, Santa Cruz Biotechnology, CA, USA).

2.5. *Statistical analysis*

Statistical analysis was performed by SPSS (18.0) statistical software (SPSS Inc., Chicago, IL, USA). $\chi 2$ test (2-sided) was performed to determine the variation in proteins expression among study groups as well as association of proteins with clinicopathological parameters. The protein–protein inter-correlation was determined by Pearson's correlation test (2-sided). The results were considered statistically significant when p was <0.05 ($\chi 2$ analyses) or <0.01 (Pearson's correlation test).

3. Results

3.1. *Histological findings*

The morphological features of cervix cells were studied by H & E staining which displayed following changes in cervical epithelium with respect to disease progression.

3.1.1. *Ectocervix (stratified squamous epithelium)*

All the normal ectocervix tissues showed presence of non-keratinizing stratified squamous epithelium with intact basal, parabasal and superficial zone. In the basal zone, mitotically active single layer of columnar germinal cells were observed overlying the cervical stroma. In the parabasal zone cells were highly adherent to each other as well as irregular in size with centrally places nuclei. In the superficial zone, cells were loosely attached having abundant cytoplasm with small dark pyknotic nuclei (Fig. 1A).

3.1.2. *Lesions of squamous epithelium of cervix*

The cervical lesions were categorized in CIN-I, CIN-II and CIN-III on the basis of morphological changes occurred in squamous epithelium. In CIN-I, mild dysplasia of adjacent layers of basal zone was noticed. The cells were either single or scattered in small groups. Cytoplasmic vacuolization with hyperchromasia of nuclear chromatin was also present

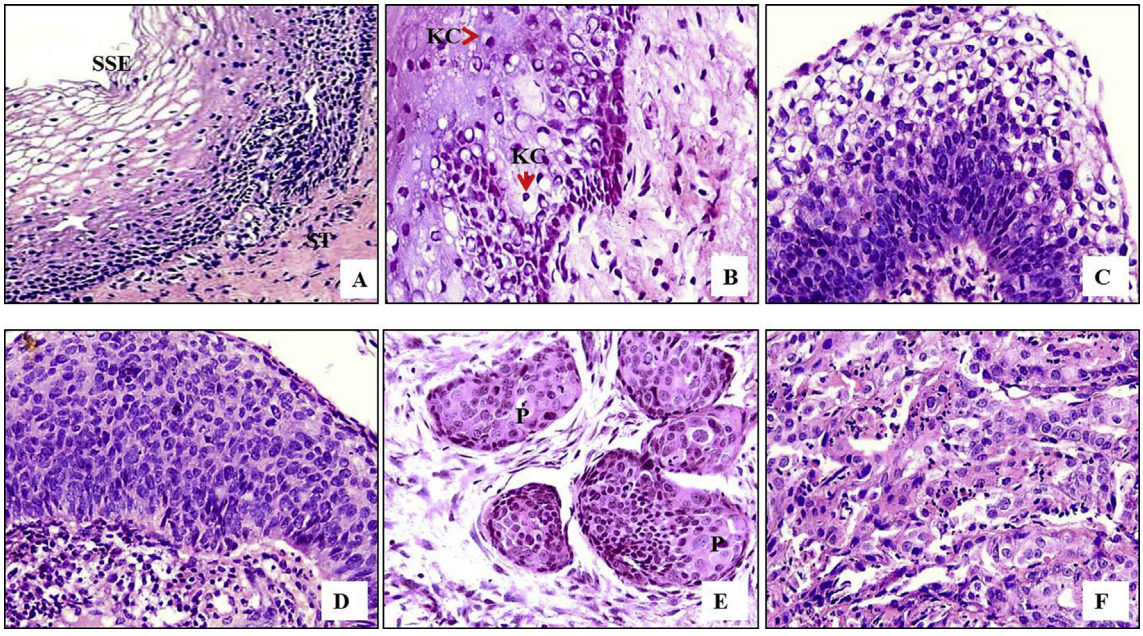

Fig. 1 – H&E staining in normal, CIN and invasive SCCs cervix tissues. A. Normal cervix showing stratified squamous epithelium (SSE) with underlined stroma (ST, 100X) B. CIN-I showing vacuolated cells and koilocytotic changes (KC; arrow) in cells (400X) C. CIN-II depicting severe dysplasia of middle third layer of epithelium D. CIN-III illustrating severe dysplasia involving all the layers of epithelium E. Early stage SCCs showing epithelial pearl (P) formation F. Late stage SCCs showing large, rounded, multinucleated tumor cells and complete invasion of epithelium into stroma. B–F: magnification X200.

Table 1 – Total expression of p53 and p21 proteins in normal, premalignant and malignant cervix tissues.

Cases (N)	HPV-E6 (cyto/Nucle) n (%) Mean ± S.E		*p* Value	p53 (Nucle) n (%) Mean ± S.E		*p* Value	p21 (Nucle) n (%) Mean ± S.E		*p* Value
	+	–		+	–		+	–	
Normal (N = 100)	00 (0) 0.00 ± 0.00	100 (100)	0.0001[a] (p < 0.001)	00 (0) 0.00 ± 0.00	100 (100)	0.0001[d] (p < 0.001)	39 (39.0) 1.1 ± 0.12	61 (61.0)	0.001[g]
CIN (N = 67)	55 (82.0) 3.61 ± 0.36	12 (17.9)	0.0001[b] (p < 0.001)	52 (77.6) 2.88 ± 0.31	15 (22.3)	0.0001[e] (p < 0.001)	17 (25.3) 0.76 ± 0.22	50 (74.6)	0.01[h]
SCCs (N = 153)	130 (84.9) 4.67 ± 0.26	23 (15.0)	0.0001[c] (p < 0.001)	127 (83.0) 4.37 ± 0.31	26 (16.9)	0.0001[f] (p < 0.001)	40 (20.1) 0.31 ± 0.04	113 (73.8)	0.0001[i] (p < 0.001)
Significance (N Vs C Vs S)	**p=0.0001***			**p=0.0001****			**p=0.0001*****		

p-values are calculated by using the chi-square test. $p \leq 0.05$ was considered significant.
For HPV-E6: a, Normal Vs CIN; b, CIN Vs SCCs; c, Normal Vs SCCs. For p53: d, Normal Vs CIN; e, CIN Vs SCCs; f, Normal Vs SCCs. For p21: f, Normal Vs CIN; g, CIN Vs SCCs; h, Normal Vs SCCs Cyto, cytoplasm; Nucle, Nuclear. * HPV-E6: Normal Vs CIN Vs SCCs, **p53: Normal Vs CIN Vs SCCs, p = 0.0001, ***p21: Normal Vs CIN Vs SCCs.

(koilocytotic changes) (Fig. 1B). In CIN-II significant variations in cell and nuclear size were noticed as compared to CIN-I. Furthermore, heterogenecity of nuclear chromatin and mitotic activity was also observed above the basal layer extending in to middle one third of epithelium (Fig. 1C). In CIN-III, The undifferentiated neoplastic cells were observed in more than two third of total thickness of epithelium. Moreover, involvement of all the layers of epithelium was also noticed in CIN-III. The cells were arranged disorderly having both normal and abnormal mitotic features with significant variability in cell and nuclear size (Fig. 1D).

3.1.3. *Invasive squamous cell carcinoma (SCCs)*

The squamous epithelium has invaded into the stroma of cervix. The epithelium was not continuous and part of the epithelium is surrounded by stroma presenting epithelial

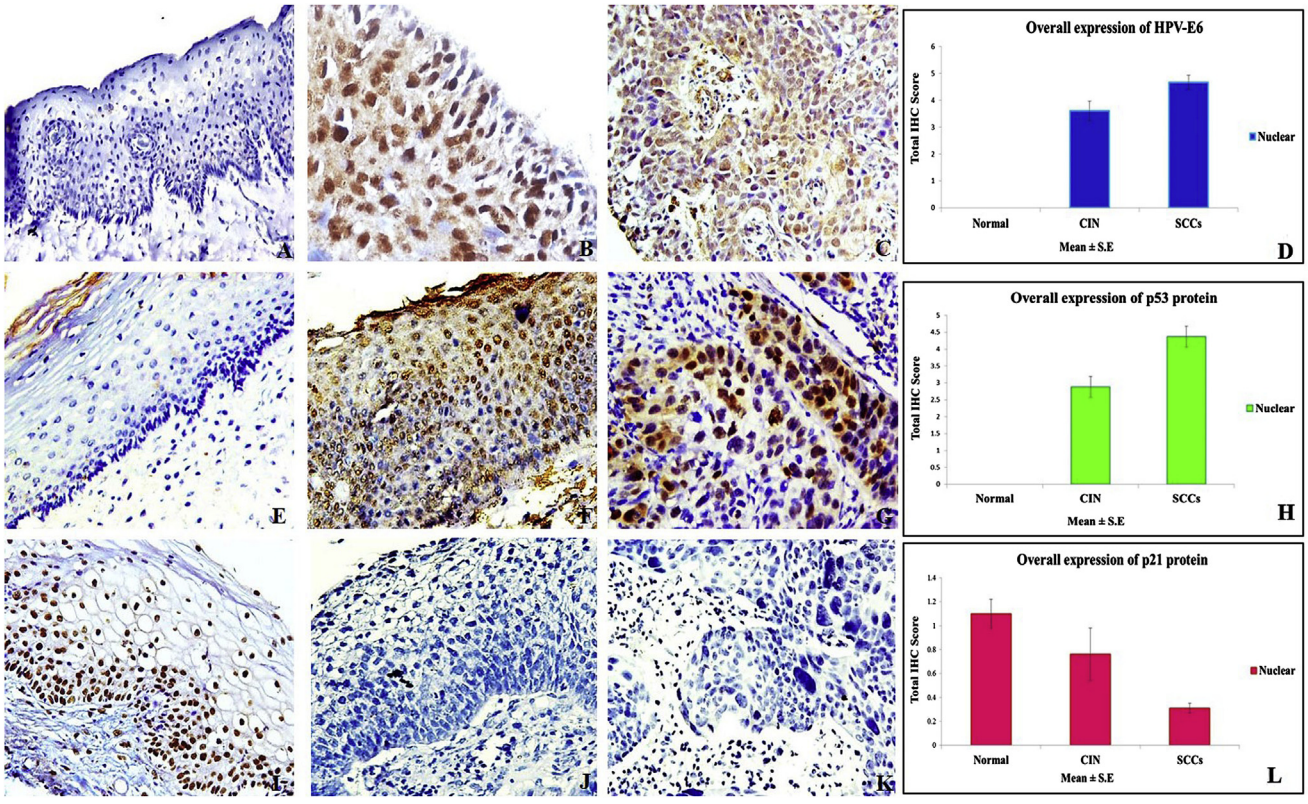

Fig. 2 – Immunohistochemical localization of HPV-E6, p53 and p21 in cervical cancer. A. Normal cervix epithelium without HPV-E6 positivity (200X) B. Cytoplasmic and nuclear expression HPV-E6 in CIN (400X) C. Cytoplasmic and nuclear over-expression HPV-E6 in SCCs (200X) D. Bar graph depicting overall expression of HPV-E6 in cervix tissues E. Normal cervix showing absence of p53 immunoexpression (200X) F. Nuclear accumulation of p53 in CIN (200X) G. Nuclear accumulation and overexpression of p53 SCCs (200X) H. Bar graph showing total score of p53 in cervix tissues I. Mild immunoreactivity of p21 in normal cervix (200X) J. Loss of nuclear expression of p21 in CIN (200X) K. p21 loss in SCCs (200X) L. Bar graph illustrating total immunohistochemical score of p21 in cervix tissues.

pearl formation in early stage cancer (Fig. 1E). In late stage cancer tissues, complete evasion of epithelium into the stroma was noticed. Majority of cells were scattered singly and they were large, rounded, multinucleated with pyknotic nuclei (Fig. 1F).

3.2. *Immunoexpression of HPV-E6, p53 and p21 in cervix tissues*

All the proteins showed differential expression in various cervix tissues and the results of IHC analysis are summarized in Table 1.

3.2.1. *HPV-E6*

The expression of HPV-E6 protein was completely absent in normal cervix tissues (Fig. 2A) while, extensive cytoplasmic/nuclear staining of HPV-E6 was observed in precancerous and cancerous tissues (Fig. 2B &C). Out of 67 CIN, 55 (82.0%) cases showed intense HPV-E6 expression as compared to normal epithelia ($p = 0.0001$). Similarly, in SCCs, HPV-E6 positivity was detected in 130/153 (84.9%) cases denoting high prevalence of HPV infection in these cases as compared to normal and CIN ($p = 0.0001$, $p = 0.0001$).The HPV positivity was significantly up-regulated with respect to the progression of disease from normal to CIN to SCCs (0.00 ± 0.00, 3.61 ± 0.36, 4.67 ± 0.26; $p = 0.0001$; Fig. 2D)

3.2.2. *p53*

p53 nuclear expression was absent in stratified squamous epithelium of normal cervix (Fig. 2E). In contrast, strong nuclear immunopositivity of p53 was noticed both in CIN and SCCs (Fig. 1F&G). Of the 67 CIN analyzed, 52 (77.6%) cases expressed with significant nuclear accumulation of p53 in comparison to normal ($p = 0.0001$). In SCCs, out of 153, 127 (83.0%) tumors revealed nuclear expression of p53 and the frequency of accumulation of p53 in SCCs was quite higher as compared to normal and CIN ($p = 0.0001$, $p = 0.0001$). Furthermore, it was noticeable that the endogenous nuclear expression of p53 was also increased with respect to progression of disease from normal to CIN to SCCs (0.00 ± 0.00, 2.88 ± 0.31, 4.37 ± 0.31; $p = 0.0001$; Fig. 3H).

3.2.3. *p21*

The mild immunoreactivity of p21 was noticed in the nucleus of benign cervix epithelium (Fig. 1I), whereas, majority of CIN and SCCs showed loss of this protein in nucleus (Fig. 1J&K). In the CIN group, loss of p21 protein was noticed in 50 (74.6%) out of 67 cases, while in SCCs, 113/153 (73.8%) presented with nuclear loss of this protein in tumor cells. The p21 protein was significantly down-regulated both in CIN and SCCs as compared to normal ($p = 0.0001$; $p = 0.0001$). The p21 expression was also gradually decreased as disease progressed from normal to CIN to SCCs (1.1 ± 0.12, 0.76 ± 0.22; 0.31 ± 0.04; $p = 0.0001$; Fig. 2L). Moreover, the revalidation of immunohistochemical expression was carried out by Western blotting and IHC results were corroborates with immunoblotting.

3.3. *Association of proteins expression with clinicopathological parameters of SCCs*

The association of all the proteins with clinicopathological parameters are depicted in Table 2. The overexpression of HPV-E6 and p53 proteins was found to be associated with age of patients ($p = 0.003$; $p = 0.04$), gravida ($p = 0.006$; $p = 0.005$),

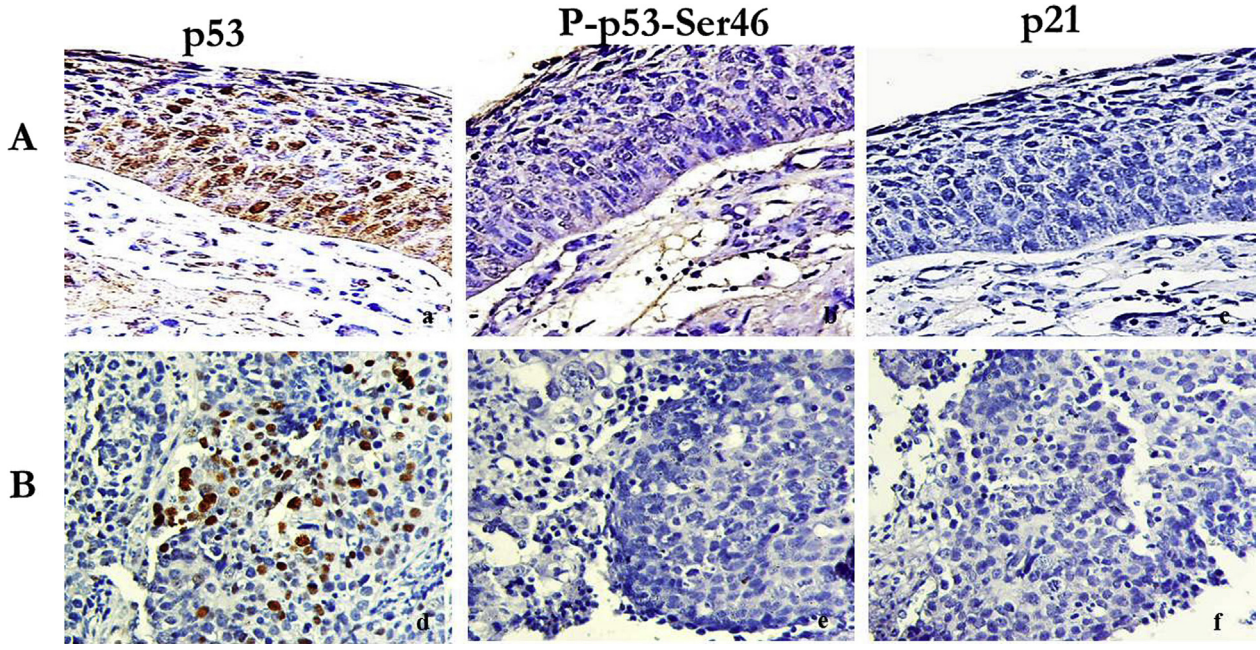

Fig. 3 – Expression of P-p53-Ser46 in HPV associated cervical cancer. A. Precancerous tissue with nuclear accumulation of wild type p53 (a) also showing loss of P-p53-Ser46 (b) and p21 (c) expression. B. Invasive tumors harboring nuclear overexpression of wild type p53 (d) simultaneously depicting loss of P-p53-Ser46 (e) and p21 proteins (f). a–f, magnification X200.

Table 2 – Association of HPV-E6, p53 and p21 proteins expression with clinicopathological parameters of SCCs patients.

Clinicopathological parameters	Patients (N = 153)	HPV-E6 (Nucle) n (%)			P53 (Nucle) n (%)			P21 (Nucle) n (%)		
		+ 130 (84.9)	– 23 (15.0)	*p value*	+ 127 (83.0)	– 26 (16.9)	*p* value	+ 40 (20.1)	– 113 (73.8)	*p* value
Age (median 55yrs) (23–85yrs)										
≤50	35	27 (77.1)	08 (22.8)		32 (91.4)	03 (8.5)		08 (22.8)	27 (77.1)	
>50	118	103 (87.2)	15 (12.7)	**0.003**	95 (80.5)	23 (19.4)	**0.04**	32 (27.1)	86 (72.8)	
Gravida (median 4) (1–11)										
<4	36	32 (88.8)	04 (11.1)		30 (83.3)	06 (16.6)		08 (22.2)	28 (77.7)	
≥4	117	98 (83.7)	19 (16.2)	**0.006**	97 (82.9)	20 (17.0)	**0.005**	32 (27.3)	85 (72.6)	
Parity (median 4) (1–9)										
<4	78	68 (87.1)	10 (12.8)		65 (83.3)	13 (16.6)		20 (25.6)	58 (74.3)	
≥4	75	62 (82.6)	13 (17.7)		62 (82.6)	13 (17.3)		20 (26.6)	55 (73.3)	
Contraception[a]										
Yes	21	18 (85.7)	03 (14.2)		17 (80.9)	04 (19.0)		03 (14.2)	18 (85.7)	
No	132	112 (84.8)	20 (15.5)		110 (81.8)	24 (18.1)		37 (28.0)	95 (71.9)	**0.05**
Habits[b]										
Yes	40	37 (92.5)	03 (7.5)		35 (87.5)	05 (12.5)		10 (25.0)	30 (75.0)	
No	113	93 (82.3)	20 (17.6)		92 (81.4)	21 (18.5)		30 (26.5)	83 (73.4)	
Tumor size (cm)										
≤4	68	50 (73.5)	18 (26.4)		54 (79.4)	14 (20.5)		19 (27.9)	49 (72.0)	
>4	85	80 (94.1)	05 (5.8)	**0.002**	73 (85.8)	12 (14.1)		21 (24.7)	64 (75.2)	
Histopathological grade										
G1	64	57 (89.0)	07 (10.9)		55 (85.9)	09 (14.0)		15 (23.4)	49 (76.5)	
G2	44	43 (97.7)	01 (2.2)		36 (81.8)	08 (18.8)		12 (27.2)	32 (72.7)	
G3	45	30 (66.6)	15 (33.3)		36 (80.0)	09 (20.0)	**0.05**	13 (28.8)	32 (71.1)	**0.05**
Lymphatic involvement										
Yes	84	75 (89.2)	09 (10.7)		70 (83.3)	14 (16.6)		21 (25.0)	63 (75.0)	
No	69	55 (79.7)	14 (20.2)	**0.02**	57 (82.6)	12 (17.3)	**0.04**	19 (27.5)	50 (72.4)	**0.05**
FIGO stage										
I + II	100	78 (78.0)	22 (22.0)		76 (76.0)	24 (24.0)		31 (31.0)	69 (69.0)	
III + IV	53	52 (98.1)	01 (1.8)	**0.001**	51 (96.2)	02 (3.7)	**0.001**	09 (16.9)	44 (83.1)	**0.01**

p values were calculated using chi square and Fisher exact test, $p \leq 0.05$ is considered as significant.
[a] Contraception includes only the use of oral contraceptive pills.
[b] Habits includes tobacco chewing, smoking and/or alcohol consumption.

lymphatic involvement ($p = 0.02$; $p = 0.04$) and stage of cancer ($p = 0.001$; $p = 0.0001$) respectively. Furthermore, HPV-E6 also showed significant association with tumor size ($p = 0.002$) and p53 with histological grades ($p = 0.05$). In addition, the significant association of p21 loss was observed with use of oral contraception ($p = 0.05$), histological grades ($p = 0.05$), lymph node positivity and progressed tumor stage ($p = 0.01$) respectively.

3.4. *Correlation between p53 and p21 in CIN and SCCs*

The functional inactivation or transcriptional suppression of p53 protein was reported to be the prime cause for the loss of cellular activity of its downstream target, p21. Therefore, in present study the expressional relationship between these two proteins was analyzed by Pearson's correlation test in order to determine their biological role in cervical cancer. On comparing the expression of p53 and p21 in CIN and SCCs, it was noticed that majority of cases showing ectopic nuclear accumulation of p53 and down-regulation of p21. In CIN, p53 was found to be accumulated in 52/67 cases, out of 52 cases, 43 tissues showed loss of p21 protein in the nucleus. Hence, significant inverse association was observed between p53 and p21 proteins in precancerous tissues ($r = -0.793$, $p = 0.0001$; Table 3). Similarly, in invasive cancer group, out of 127 nuclear p53 overexpressing cases, 104 tumors also showed nuclear loss of p21 protein, signifying inverse association between them ($r = -0.902$, $p = 0.0001$; Table 3).

3.5. *Impact of HPV-E6 on p53 and p21 expression in CIN and SCCs*

Analysis of association of HPV-E6 oncoprotein with p53 and p21 was carried out by Pearson's correlation test and results

Table 3 – Correlation between p53 and p21 proteins in CIN and SCCs.

Proteins	CIN (N = 67)		SCCs (N = 153)	
	P53 (Nucle+)	P21 (Nucle–)	P53 (Nucle+)	P21 (Nucle–)
P53 (Nucle+)	52	43 r = –0.793 **p=0.0001**	127	104 r = –902 **p=0.0001**
P21 (Nucle-)	43	50	104	113

p-values are calculated by using Pearson's correlation test. $p \leq 0.01$ was considered significant. r = Pearson's correlation coefficient; -ve, r-values, inverse association; +ve, r-values, positive association; Nucle, Nuclear.

Table 4 – Association of HPV-E6 with p53 and p21 proteins in CIN and SCCs.

Cervical tissues	HPV-E6 expression		p53 (Nucle+)		*p* value	p21(Nucle+)		*P* Value
			+	–		+	–	
CIN (n = 67)	+ve	55	39	16	r = 0.589	21	34	r = −0.426
	−ve	12	04	08	**p = 0.0001**	06	06	**p = 0.0001**
SCCs (n = 153)	+ve	130	111	19	r = 0.670	25	105	r = −0.602
	−ve	23	08	15	**p=0.0001**	16	07	**p=0.0001**

p-values are calculated by using Pearson's correlation test. $p \leq 0.01$ was considered significant. r = Pearson's correlation coefficient; -ve, r-values, inverse association; +ve, r-values, positive association; Nucle, Nuclear.

are summarized in Table 4. In precancerous group, 39/55 HPV-E6 overexpressing cases also showed distinct nuclear accumulation of p53 and significant direct association was observed between these proteins (r = 0.589, p = 0.0001). In contrast, HPV-E6 exhibited significant inverse association with p21 (r = −0.426, p = 0.0001), as 34 out of 55 HPV-E6 positive cases showed significant down-regulation of p21 expression . In invasive squamous cell carcinoma, ectopic nuclear accumulation of p53 was detected in 111 out of 130 HPV-E6 infected cases and values were statistically significant (r = 0.670, p = 0.0001). Furthermore, significant inverse association was also noticed between HPV-E6 and p21 proteins (r = −0.602, p = 0.0001).

3.6. *Expression of p-p53-ser46 (p53 functionality) in HPV associated CIN & SCC*

The expression of p-p53-ser46 (active form of p53 protein) was evaluated in HPV-E6 and wild type p53 positive cases to determine the functionality of this protein. All the HPV-E6 associated cases showing aberrant accumulation of wild type p53 concomitantly showed loss of p-p53-ser46 expression. Furthermore, the p21 protein was also down regulated in p-p53-ser46 cases (Fig. 3).

4. Discussion

The long term infection of HR-HPV with unremitting overexpression of its oncoproteins E6 and E7 is thought to be the important step in the development of cervical cancer.[22,23] during carcinogenesis, HPVs interact with plethora of cellular proteins to drive transformation process and establishment of viral infection in cervix environment (see Fig. 4). In this respect, the current study was designed to identify the interaction of HPV-E6 oncoprotein with p53 and p21 proteins and further to delineate their clinical as well as biological role in the pathogenesis of cervical cancer.

Our previous findings on multiplex PCR genotyping showed considerable positivity for HPV-16/18 infection in cervical precancerous (89.5%) and cancerous (90.1%) cases studied with highest prevalence of HPV-16 genotype.[24] In the present study, we observed significant overexpression of HPV16/18-E6 protein in precancerous and cancerous tissues (Table 1). Furthermore, we also observed significant association of HPV-E6 oncoprotein with age (p = 0.003), gravida (p = 0.006), tumor size (p = 0.002), lymphatic involvement (p = 0.02) and increased FIGO stage of cancer (p = 0.001) respectively. This indicates that sustained expression of E6 is required for the maintenance of transformation process as well as progression of cervical cancer. Our results showed concordance with various studies highlighting importance of HPV infection in development of cervical cancer.[17,25]

The oncoproteins of HPV, E6 and E7 alter cell cycle by binding to tumor suppressor's p53 and pRb, thereby deregulating important cell cycle check points.[25,26] p53 is called as a "guardian of the genome" due to its critical role in the coordination of diverse signaling pathways in response to DNA damage.[27] Mutation or the functional inactivation of p53 is the universal feature of various progressive human cancers and its altered expression is correlated with resistance to chemotherapy and poor prognosis.[28,29] Though, p53 mutation is quite uncommon in cervical cancer but its inactivation plays important role in HPV associated cervical carcinogenesis.13,[15] In the present study, we analyzed the endogenous expression p53 protein in CIN and SCC by using DO-7 antibody. However, analysis of p53 expression by DO-7 has been considered most sensitive method due its specificity for detection of both wild as well as mutant type of p53 in cells.[30] The immunohistochemical analysis revealed unusual nuclear accumulation of p53 in 77.6% of precancerous and 83.0% of cancerous tissues. Our findings concur with the studies demonstrating nuclear over expression of p53 in cervical cancer.[17,23,31] It has been suggested that, although p53 gets quickly degraded in normal cell, but its half-life can be extended from several minutes to hours in transformed cells.[32] This prompted us to speculate that the accumulated p53 might get stabilized in cervical tumor cells and cannot be degraded by normal ubiquitination pathways as a signature of transformed phenotype of cervix cells. In addition, p53 was also found to be associated with various clinicopathological parameters of SCCs patients including histological grades (p = 0.05), lymph node positivity (p = 0.04) and progressed tumor stage (p = 0.001), suggesting its role in the tumor expansion through interfering with tumor variables.

Furthermore, the noteworthy loss of p21 protein (target of p53) and its significant inverse association with p53 was also observed in CIN (r = −0.793, p = 0.0001) and SCCs (r = −0.902, p = 0.0001) cases. These results proposed that, the synthesis of p21 is inhibited during generation of cervical cancer and the sequestration of its expression may effectively regulated by abnormal p53 accumulation. Therefore, it can also be hypothesized that post-transcriptional modification of wild type

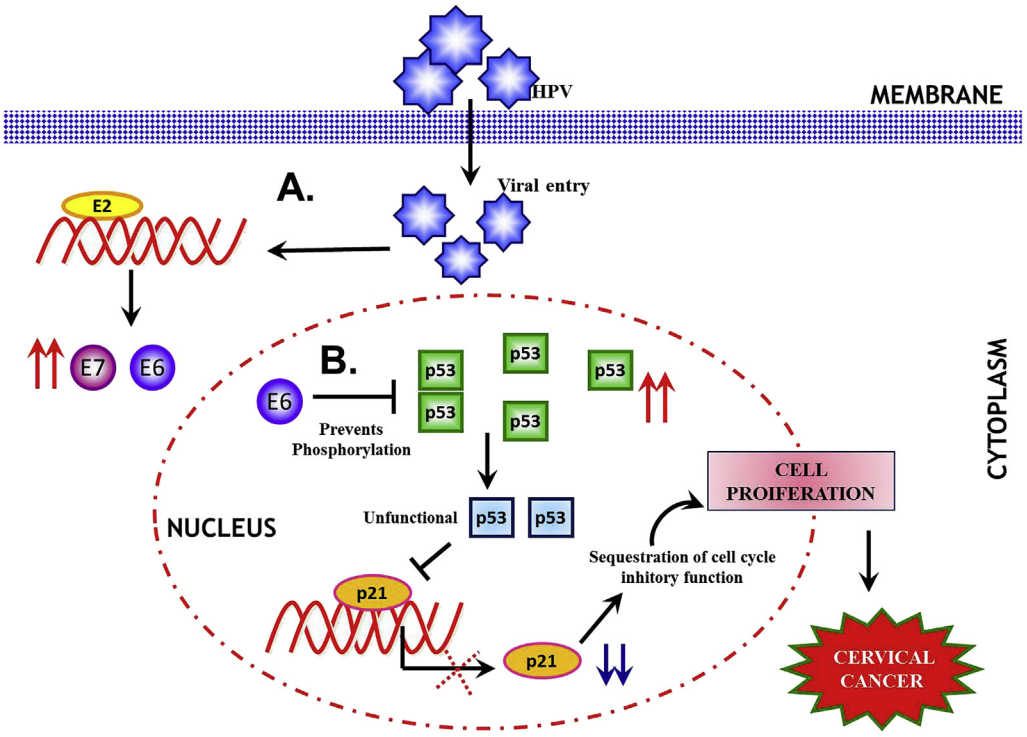

Fig. 4 – Schematic representation of interaction of HPV-E6 with p53 and p21 in cervical cancer: A. HPV viruses infect host cervix cell and transcriptionally activate E6 and E7 oncoproteins B. HPV-E6 oncoprotein then prevents phosphorylation of p53 protein to inhibit its normal function towards the transactivation of p21. This results in sequestration of cell cycle inhibitory function of p53, abnormal cell proliferation and initiation as well as progression of cervical cancer.

p53 may be one of the reasons for the abrogation of its transcriptional activity towards p21 in cervical cancer. Our finding is partially supported by the other studies, which also showed the abrogated function of accumulated p53 towards transcriptional suppression of p21 in malignant cervical and glioma cancer cells.[33,34]

Large number of reports available that highlights the E6 mediated abrogation of p53 activity through various cellular proteins. Heck et al, 1992, reported that E6 degrades p53 by E3 ubiquitin dependent and independent proteasomal degradation mechanism.[25] While, other studies have showed that p53 transactivity was also repressed by E6 via direct binding with p300 and inhibiting its acetylation.[35,36] In contrast to these studies, current report by Ajay et al, 2012 speculated another phenomenon involved in the E6 mediated inactivation of p53. The study suggests that E6 deactivates p53 by inhibiting its phosphorylation and preventing its binding to p21 promoter, thereby restraining cell-growth inhibitory functions.[34] in concordance with this important finding, our study also showed the expressional loss of p-p53-ser46 (active form of p53) in HPV and accumulated p53 CIN and SCC cases suggesting that the p53 that accumulated in cell may be the functionally inactive thereby sequestrating its function towards p21 regulation. Furthermore, on the statistical analysis we observed significant positive association between HPV-E6 with p53 and p21 in CIN and SCCs (Table 4). Therefore, in support with above study and from our observations, it can be hypothesized that, overexpression of p53 in E6 positive cells may be due inactivation of this protein. E6 may inhibit the phosphorylation of p53 instead of degrading this protein thereby, affecting its activity towards transcription of p21. This ultimately results in the disruption of p53-p21 downstream signaling and loss of cell growth inhibitory signals during the progression of cervical cancer (Fig. 4). However, this is our preferred interpretation and further studies are underway to confirm this finding.

5. Conclusion

Our study provides the evidence of p53 nuclear accumulation and p21 down-regulation in HPV16/18 positive cervical cancer highlighting the involvement of some other degradation independent mechanism in E6 mediated inhibition of p53 transactivity during initiation and progression of cervical cancer. Moreover, the significant association of these proteins with aggressive tumor variables provides the new insight for designing the therapeutic modalities for HPV associated cervical cancer preferably by reactivating p53 protein in these cases.

Conflicts of interest

All authors have none to declare.

Acknowledgments

This study was supported by grant from Indian Council of Medical Research (ICMR), New Delhi, India (Grant no: 5/13/71/2008-NCD-III).

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