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Peptidyl-prolyl isomerase Pin1-mediated abrogation of APC–β-catenin interaction in squamous cell carcinoma of cervix

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Abstract

Objective: The present study was aimed to investigate the importance of Pin1 expression in Squamous Cell Carcinoma (SCC) of cervix and to assess its level with β-catenin and APC to understand the possible involvement of Pin1 in the regulation of these proteins and subsequent activation of Wnt/β-catenin signaling. *Materials and Methods*: Expression of Pin1, β-catenin and APC was examined in 153 SCC patients by immunohistochemistry and revalidated by western blotting. *Results*: Of the 153 SCC analyzed, Pin1 was overexpressed in 73 (47.71%) cases. Loss of membranous β-catenin was noticed in 117 (76.47%) SCCs, whereas 66/153 (43.13%) and 93/153 (60.78%) cases showed its distinct cytoplasmic as well as nuclear accumulation respectively. Down regulation/loss of APC was observed in 69 (45.09%) cases, suggesting the activation of Wnt/β-catenin pathway in SCCs. Pin1 showed the significant association with nuclear β-catenin (r=.349, p<0.0001) and cytoplasmic loss of APC (r=-.287, p<0.0001). Both Pin1 as well as nuclear β-catenin were found to be associated with tumor stage (p=0.004, p=0.031) and tumor size (p=0.022, p=0.003). The Pin1 overexpression showed the significant association with disease free survival (p=0.002) but not with overall survival (p=0.421) of SCC patients. *Conclusion*: Current results explore the expressional relationship between Pin1, β-catenin and APC suggesting that Pin1 regulates the activation of Wnt/β-catenin pathway in SCCs *via* modulating the interaction between β-catenin and APC. Furthermore, the significant association of Pin1 and β-catenin with tumor variables underscores the clinical utility of these proteins in cervical cancer.

Keywords: Pin1, β-catenin, APC, Wnt/β-catenin pathway, squamous cell carcinoma, immunohistochemistry.

Introduction

In India, cervical cancer continues to be one of the major causes of cancer-related death in female, reporting 134 000 cases per year [1]. Squamous Cell Carcinoma (SCC) accounts for about 80–90% of all cervical tumors. High-risk HPV infection is crucial [2], but not the only cause for development of cervical carcinoma [3]. Majority of HPV infections are sub clinical and very few persist for the development of cervical cancer [4]. Therefore, the most important aspect of the cervical cancer research is to identify the other factors, like alteration in signaling pathways that are involved in the pathogenesis of cervical cancer to predict the final outcome of this disease.

Canonical Wnt/β-catenin pathway is fundamentally involved in the embryonic organogenesis and stem cell self-renewal in adults [5] that has been recently explored in cervical cancer. Several reports have demonstrated aberrant activation of this signaling in subset of human malignancies, including breast, colorectal and gastric [6–9]. HPV immortalized human keratinocytes transformation requires activation of canonical Wnt/β-catenin pathway in cervical cancer [10]. β-Catenin, the hallmark of Wnt/β-catenin signaling pathway is a bifunctional protein that actively involved both in cadherin dependent cell–cell adhesion and in signal transduction [11]. Anomalous cytoplasmic as well as nuclear expression of β-catenin is reported in 73–75% of cervical tumors analyzed [10, 12] but the evidences that linking to the up-regulation of β-catenin and subsequent activation of this signaling in this cancer model is still missing. In unstimulated cell, the level of β-catenin is controlled by degradation complex, which is composed of Glycogen Synthase Kinase 3β (GSK3β), Adenomatosis Polyposis Coli (APC), Casein Kinase-1α (CK1α) and Axin [13]. APC, the key component of destruction complex negatively regulates the Wnt signaling by antagonizing the function of β-catenin [14]. The functional relationship between APC and β-catenin was found to disrupt in many cancers due to the mutation in these proteins [15, 16] but such mutation is rarely observed in cervical cancer [17], whereas APC found to be hypermethylated in this cancer [18].

Pin1, a peptidyl-prolyl *cis-trans* isomerase specifically catalyzes the Serine/Threonin-Pro phosphorylation sites of certain proteins and affects their activity or protein–protein interaction [19–21]. Pin1 is vigorously over-expressed in diverse of human malignancies including cervical cancer [22–30]. Pin1 thought to be critical catalyst for multiple oncogenic pathways [31, 32] as it interacts with various signaling pathways including Wnt signaling. Pin1 regulates the β-catenin turnover and sub cellular localization in breast cancer by interfering its interaction

with APC [21], but this mechanism is barely understood in the cervical carcinoma.

Hence, the purpose of the current study was to assess the significance of Pin1 expression in invasive SCCs of cervix and further to correlate its level with Wnt/β-catenin pathway components, β-catenin and APC to determine its contribution in the regulation of these proteins in cervical cancer.

Materials and Methods

Tissue specimens

Total 153 patients with proven cervical squamous cell carcinoma, including 111 cases of non-keratinizing SCC and 42 cases of keratinizing SCC subtypes were enrolled in this study between June 2009–August 2010. The cervical punch biopsy sample were collected after obtaining the clearance from institutional ethical committee (VMMC, Safdarjung Hospital, New Delhi, India) and written consent from each patient. In addition, the normal cervix tissues (n=50) were also collected from hysterectomies performed for adnexal masses (utero-vaginal prolapse), in which the final diagnoses were non-neoplastic. All the patients included in our study had no family history of cervical cancer, no sign of distance metastasis and were not chemo- or radio-therapeutically treated before tumor removal. On the other hand, those patients who have diagnosed with other inflammatory diseases and sexually transmitted infection along with malignancy were excluded from the study. The age of the patients ranged from 23–85 years with median age of 55 years. The histopathological diagnosis of patients were studied by following *World Health Organization* criteria as described by Tavassoli and Devilee (2003) [33] and cancer staging was done according to the guidelines of *International Federation of Gynecology and Obstetrics* (FIGO). The clinicopathological features of patients are summarized in Table 1.

Table 1 – *Clinicopathological characteristics of SCCs patients (n=153)*

Characteristics	Values
Age [years]	55.12±8.60 (23–85)
Gravida	4.32±2.066 (1–11)
Parity	3.71±1.61 (1–9)
FIGO stage:	
▪ I	32 (20.91)
▪ II	68 (44.44)
▪ III	49 (32.02)
▪ IV	4 (2.6)
Histological type:	
▪ Squamous cell carcinoma	153 (100)
▪ Non keratinizing	111
▪ Keratinizing	42
▪ Adenocarcinoma	0 (0)
▪ Squamo-adenocarcinoma	0 (0)
Histo grade:	
▪ WDSCC	64 (41.83)
▪ MDSCC	44 (28.75)
▪ PDSCC	45 (29.41)
Tumor size (clinical) [cm]:	
▪ ≤4	68 (44.44)
▪ >4	85 (55.55)
Lymph node involvement:	
▪ Positive	84 (54.9)
▪ Negative	69 (45.09)

FIGO – International Federation of Gynecology and Obstetrics; WDSCC – Well Differentiated Squamous Cell Carcinoma; MDSCC – Moderately Differentiated Squamous Cell Carcinoma; PDSCC – Poorly Differentiated Squamous Cell Carcinoma. Values are presented as mean ± SD (range) or number (%).

Patient follow-up

Among the 153 enrolled patients, only 96 could be accessible for follow up at regular periodic intervals in VMMC & Safdarjung Hospital during the period of December 2009–December 2012. The time from date of diagnosis of cancer to the recurrence/metastasis was considered for disease free survival (DFS), whereas the time from date of cancer diagnosis to the death due to cancer was recorded for overall survival of patients.

Immunohistochemistry

The 5 μm thick sections of formalin-fixed, paraffin-embedded tissues of cervical cancer specimens are mounted on poly-L-Lysine coated slides and processed for conventional histological assessment by Hematoxylin–Eosin (HE) staining and then by immunohistochemistry. The expression of Pin1, β-catenin and APC was detected by: (a) mouse anti-human monoclonal Pin1 antibody (ab63623; Abcam, Inc., Cambridge, UK), (b) mouse anti-human monoclonal β-catenin antibody (M3539, Dako Cytomation, Glostrup, Denmark), and (c) rabbit anti-human polyclonal APC antibody (Sc-896; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). In brief, the sections were processed for immunohistochemistry by deparaffinization in xylene, dehydration through graded alcohols. Endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide (H_2O_2) in 50 mL methanol for 45 minutes. Antigen retrieval for all the proteins were done in citrate buffer (10 mM) pH 6 by heating the sections at 800 W for 20 minutes (APC and Pin1) and 900 W for 15 minutes (β-catenin) in household microwave oven. Later, the serial sections were incubated overnight in humid chamber, at 4^0C with primary antibodies for Pin1 (1:50), β-catenin (1:100) and APC (1:100). The slides were then briefly washed with PBS and incubated with polymer based EnVision™ (dextran coupled with peroxidase and goat secondary antibody molecules against rabbit and mouse immunoglobulins in buffered solutions, Dako Cytomation, Glostrup, Denmark) for one hour at room temperature. The chromogenic visualization reaction was done by using 3,3'-diaminobenzidine hydrochloride (DAB), counterstained with Mayer's Hematoxylin, mounted and then examined by light microscope (Olympus BX-51, Japan). The confirmed human breast cancer tissues expressing Pin1, β-catenin and APC were used as a positive control, while in the negative control; primary antibody was replaced by isotype-specific immunoglobulin G.

Scoring of Immunohistochemical staining

Evaluation of immunohistochemistry results for all the proteins was independently studied by two observers (P.J. & I.D.) and followed the scoring criterion given by Brabletz *et al.* (2000) [34]. Protein expression was first

semi-quantified by the intensity of staining: 0–3+ ('0', negative staining if there is total absence; '1', weak; '2', moderate and '3', intense positive staining), and then by the percentage of positive stain cells ranging from 1 to 4+ ('0', no positive stain cells; '1', <5%; '2', 5 to 20 %; '3', 21 to 50%; '+4', >50% positive stained cells). Resulting scoring was calculated by multiplying intensity and percentage value and the final score of IHC was ranged from 0 to 12. Based on multiplication score, the immunoreactivity then again classified on: 0 (negative; IHC score 0), 1 (mild; IHC score 1, 2), 2 (moderate; IHC score 3, 4, 6), or 3 (intense; IHC score 8, 9, 12). Cytoplasmic and nuclear staining for Pin1, cytoplasmic and/or nuclear immunostaining for APC and β-catenin were considered as positive [6, 7, 22].

Protein extraction and Western blotting (in representative samples)

The selected cases expressing all the proteins were then processed for Western blotting analysis for the revalidation and confirmation of immunohistochemistry results. In this respect, total cellular protein was extracted from normal (n=10) as well as cancerous (n=10) frozen cervix tissues by using Ready Prep™ 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). Samples containing 80 µg of protein/lane were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to Polyvinlydene-difluoride membrane (PVDF). The membranes were then incubated in a blocking solution containing 5% non-fat skim milk and 0.1% Tween 20 in Tris-buffered saline at 4^0C. On the next day, the membranes were incubated with anti-Pin1 (1:1000 dilution), anti-β-catenin (1:500 dilution) and anti-APC (1:1000 dilution) primary antibodies at 4^0C for overnight, which is then followed by incubation with HRP conjugated Rabbit/Mouse anti-IgG secondary antibody (Abcam antibodies) for two hours at room temperature. Protein bands were visualized on X-ray film by using enhanced chemiluminescence system (ECL, Santa Cruz Biotechnology, CA, USA).

Statistical analysis

Statistical analysis was performed by SPSS (18.0) statistical software (SPSS Inc., Chicago, IL, USA). The χ^2 test and Fischer's exact test (2-sided) were performed wherever applicable for determining the association between protein expression and clinicopathological parameters, while Pearson's correlation test (2-sided) was used to determine the protein–protein correlation. Survival curves were constructed by the Kaplan–Meier method and compared using the *Log-rank* test. The results were considered statistically significant when p was <0.05 (for χ^2 test and Fischer's exact and *Log-rank* tests) and <0.01 (for Pearson's correlation test) within all of statistical analyses.

Results

Immunohistochemical analysis of Pin1, β-catenin and APC in SCCs of the cervix

The staining pattern of all the proteins in cervical carcinomas varied greatly with IHC score ranging from 0–12. Hardly any Pin1 immunopositivity was noticed in normal cervix epithelium (Figure 1A). While extensive cytoplasmic as well as nuclear expression of Pin1 was observed in SCCs (Figure 1B). Of the 153 SCCs examined, 73 (47.71%) displayed increased Pin1 immunostaining in tumor cells. Of these 73 cervical carcinomas, 38 had intense (IHC score 8, 9, 12), 20 (IHC score 3, 4, 6) had moderate and 15 (IHC score 1, 2) had mild expression of Pin1. However, Pin1 expression was found to be significantly associated with FIGO stage (p=0.004), tumor size (p=0.022) and frequency of lymph node involvement (p=0.006) (Table 2).

Table 2 – ***Association of Pin1, β-catenin and APC with the clinicopathological parameters of SCCs patients***

Parameters	Patients	Pin1 (C+N$^+$)			β-Catenin (C$^+$)			β-Catenin (N$^+$)			β-Catenin (M$^-$)			APC (C$^-$)		
	N	-	+	*P*	-	+	*P*	-	+	*P*	-	+	*P*	-	+	*P*
Total	153	80	73		87	66		60	93		117	36		69	84	
Age [years]:																
▪ ≤50	35	22	13	.162	20	15	.841	10	25	.844	21	14	.316	3	31	.076
▪ >50	118	58	60		67	51		50	68		96	22		66	53	
Tumor size [cm]:																
▪ ≤4	68	43	25	.022	38	30	.870	34	34	.003	46	22	.014	8	60	.810
▪ >4	85	37	48		49	36		26	59		71	14		61	24	
Histo grade:																
▪ G1	64	33	31	.747	38	26	.757	21	43	.173	49	15	.608	20	44	.612
▪ G2	44	25	19		23	21		19	25		39	5		29	15	
▪ G3	45	22	23		26	19		20	25		29	16		20	25	
Lymphatic involvement:																
▪ Yes	84	35	49	.006	48	36	.938	29	55	.256	77	7	.102	30	54	.158
▪ No	69	45	24		39	30		19	50		40	29		39	30	
FIGO grade:																
▪ I + II	100	61	39	.004	52	48	.123	46	54	.031	75	25	.052	49	51	.578
▪ III + IV	53	19	34		35	18		14	39		42	11		20	33	

Fisher's exact test (2-sided) and p≤0.05 was considered significant. C – Cytoplasmic; N – Nuclear; M – Membrane; +ve – Positive expression; -ve – Negative expression.

β-Catenin was immunolocalized on the plasma membrane of epithelial cells in benign cervix tissues (Figure 1D). In contrast, cancer cells demonstrated anomalous cytoplasmic and nuclear staining of β-catenin. Reduced (IHC score 1, 2)/or loss (IHC score 0) of membranous β-catenin was observed in 117/153 (76.47%) cervical carcinomas while, 66/153 (43.13%) of SCCs showed cytoplasmic stabilization of β-catenin (Figure 1E). Distinct nuclear accumulation of β-catenin was noted in 93/153 (60.78%) of tumors, whereas 30/153 (19.60%) cases showed both cytoplasmic as well as nuclear expression of the protein. Out of 93 nuclear β-catenin expressing cases, 30 cases revealed mild nuclear with fraction of cytoplasmic staining (IHC score 1, 2), 21 were moderate (IHC score 3, 4, 6) and 42 cases showed intense nuclear immunoreactivity for β-catenin (IHC score 8, 9, 12) with no evidences of membranous staining (Figure 1F). Loss of membranous as well as nuclear translocation of β-catenin were revealed significant association with increased tumor stage (p=0.052 and p=0.031) and tumor size (p=0.014 and p=0.003) in SCCs (Table 2).

Reduced/or loss of cytoplasmic expression of APC protein was noticed in 69/153 (45.09%) cases. Among these cases, 39/69 (56.52%) SCCs appeared with mild (IHC score 1, 2) but detectable staining of APC, while 30/69 (43.47%) cases did not reveal any expression of APC, suggesting the complete loss of this protein in these cases (Figure 1, H and I). Paired normal cervical epithelium gave distinct cytoplasmic staining (Figure 1G).

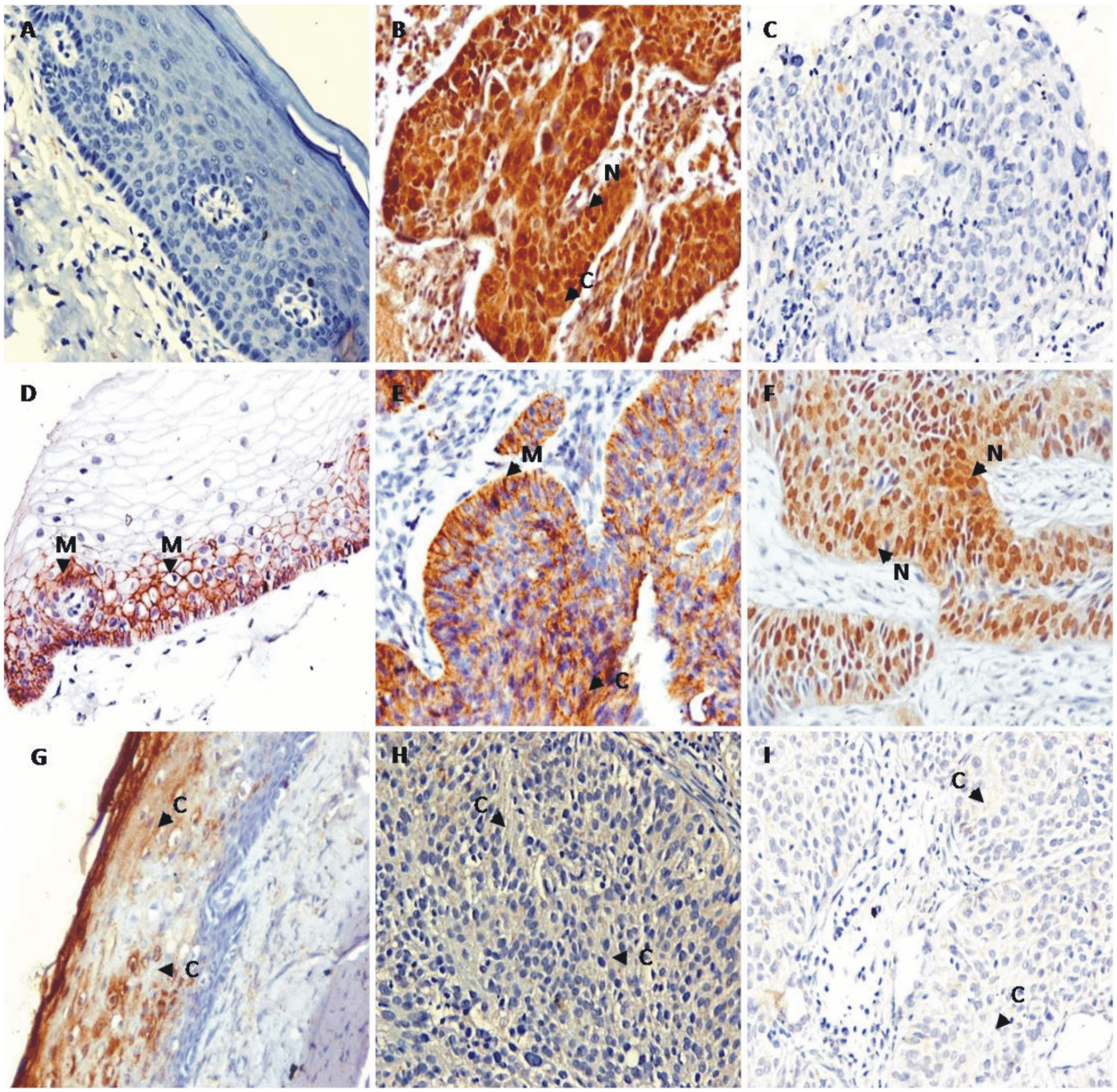

Figure 1 – ***Immunohistochemical localization of Pin1, β-catenin and APC in normal and Squamous Cell Carcinoma (SCC) of cervix tissues: (A) No expression of Pin1 in normal cervix tissue; (B) Cytoplasmic (C) and nuclear (N) overexpression of Pin1 in SCC; (C) Negative control with isotype-specific immunoglobulin G; (D) Membranous β-catenin in normal cervix; (E) Reduced membranous but increased cytoplasmic level of β-catenin in SCCs; (F) Nuclear accumulation of β-catenin in SCCs; (G) Cytoplasmic expression of APC in normal cervix epithelium; (H) Reduced cytoplasmic expression of APC in SCCs; (I) Complete loss of cytoplasmic APC in SCC. (A–I) Original magnification, ×200. Arrow heads show Cytoplasmic (C), nuclear (N) and membranous (M) localization of proteins.***

Interestingly, we also found nuclear immunostaining of APC in some invasive SCCs (data not shown). There was no significant correlation was observed between loss of APC expression and any of the clinicopathological characters (Table 2).

Western blotting

The immunoblots of Pin1, β-catenin and APC and β-actin (loading control) proteins revealed the major bands of 18 kDa, 88 kDa, 110 kDa and 41 kDa respectively. The Western blotting analysis also concordats the results of immunohistochemistry and confirms the significant up-regulation of Pin1 and β-catenin as well as down-regulation of APC in SCCs (C1, C2, C3) as compared to normal (N1, N2, N3) (Figure 2).

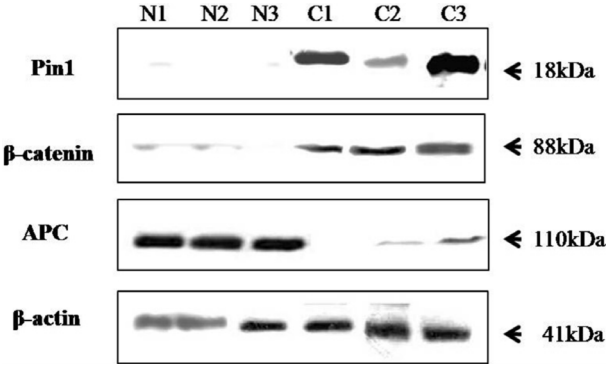

Figure 2 – ***Western blotting analysis of Pin1, β-catenin and APC in normal and SCCs. 80 µg/lane of total soluble protein was immunoblotted with anti-Pin1, anti-β-catenin and anti-APC primary antibodies. β-Actin was used as a loading control within analysis. N1–N3: Protein bands of Pin1, β-catenin and APC in normal cervix. C1–C3: Protein bands of Pin1, β-catenin and APC in SCCs.***

Relationship between Pin1, β-catenin and APC expression

Inter-protein correlations have been calculated with the help of Pearson's correlation test and are depicted in Table 3.

Table 3 – ***Correlation between Pin1, β-catenin and APC in SCCs***

Protein expression (*n*=153)	Pin1 ($C+N^{+ve}$)	β-Catenin (M^{-ve})	β-Catenin (C^{+ve})	β-Catenin (N^{+ve})	APC (C^{-ve})
Pin1 ($C+N^{+ve}$)	73	34	31 (r=-0.230) p=0.004	49 (r=0.349) p<0.0001	40 (r=-0.280) p<0.0001
β-Catenin (M^{-ve})	34	117	54 (r=-0.398) p<0.0001	81 (r=-0.840) p<0.0001	48 (r=0.484) p<0.0001
β-Catenin (C^{+ve})	31	54	66	35 (r=0.526) p<0.0001	34 (r=-0.198) p=0.014
β-Catenin (N^{+ve})	49	81	35	93	45 (r=-0.382) p<0.0001
APC (C^{-ve})	40	48	34	45	69

Pearson's correlation coefficient test and significance was considered at $p \leq 0.01$; C – Cytoplasmic; N – Nuclear; M – Membrane, +ve – Positive expression; -ve – Negative expression.

The nuclear expression of β-catenin was observed in 49/73 (67.12%) of Pin1-positive cases. Thus, there was significant positive association between Pin1 over-expression and nuclear localization of β-catenin (r=.349, p<0.0001). Pin1 expression also showed the significant inverse association with reduced/loss of APC in SCCs (r=-.287, p<0.0001), as 40/73 (54.79%) as 40/73 Pin1 positive cases showed loss of APC protein. Pin1 over-expressing cases also showed significant mild expression of cytoplasmic β-catenin (r=-.230, p=0.004). Furthermore, nuclear as well as cytoplasmic β-catenin expression revealed negative correlation with cytoplasmic APC (r=-.382, p<0.0001 and r=-.198, p=0.014) and membranous β-catenin (r=-.840, p<0.0001 and r=-.398, p<0.0001).

Survival analysis

Of the 96 regularly followed up (for limited period of 36 months) patients, 19 cases showed recurrence of disease. Five patients showed metastasis, five patients die from cancer and 67 patients were disease free. The overexpression of Pin1 was observed in 49/96 cases, among these cases recurrence was observed in 18 cases and 29 cases were with disease free and two were showed metastasis. Significant association was observed between Pin1 positive and negative group of SCC patients with respect to disease free survival (p=0.004, *Log-rank* test) but not with the overall survival (p=0.421, *Log-rank* test) as out of five only three patients showed Pin1 over-expression (Figure 3, A and B).

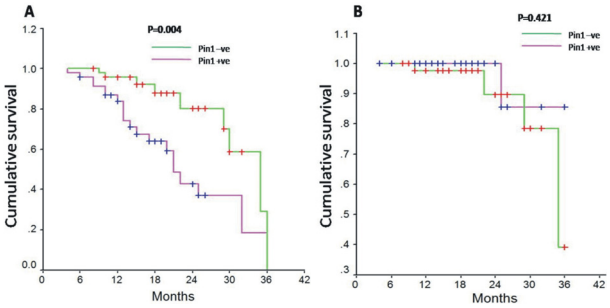

Figure 3 – ***Kaplan–Meier analysis of the probability of disease free survival and overall survival of SCC patients: (A) Disease free survival curve; (B) Overall survival curve comparing SCCs patient with respect to Pin1 positive and negative expression (+ indicates censored observations).***

Discussion

Cervical cancer remains as the major cause of death in Indian women and second most common cancer worldwide [1]. Although, HPV is central causative agent but many molecular alterations have been characterized in the development of this malignancy, which are not directly associated with HPV.

In recent years, it has become clear that Pin1 is strictly overexpressed in cancer and its elevated level significantly correlates with the up-regulation of cyclin D1 [22, 27]. It also modulates the expression of cyclin D1 through the activation of oncogenic pathways like c-Jun/Ap-1 and β-catenin/TCF transcription factor in breast cancer [25]. Its expression also found to be associated with the up-regulation of β-catenin in colorectal cancer [23]. These

findings indicate that Pin1 might contribute to the oncogenesis by transactivating β-catenin signaling and its target genes, like Cyclin D1. In the current study, overexpression of Pin1 was noticed in 47.71% (73/153) of SCCs, which is in concordance with the finding of Li *et al.* (2006), who also reported the overexpression of Pin1 in cervical cancer. They also observed an increase in Pin1 positivity along with the progression of disease from normal-CIN-invasive cancer. However, Pin1 immunopositivity was higher in adenocarcinomas than that in SCCs, but author did not find association of Pin1 with any of the clinicopathological parameters [28]. Although, we have not analyzed the expression of Pin1 in pre-cancerous lesion and in other histological subtypes of cervical cancer, but significant association of Pin1 with increased FIGO stage (p=0.004), tumor size (p=0.02) and lymph node involvement (p=0.006) in SCCs was observed in present study. This signifies that Pin1 expression is associated with aggressive behavior of cervical tumors. Apart from that, efforts have also been made in our study to determine the impact of Pin1 overexpression on the disease outcome of the patients. On the survival analysis, patients with Pin1 positive tumors had shown significant shorter disease-free survival as compared to patients that did not show overexpression of this protein (p=0.004). Whereas, the significant difference was not observed between Pin1 positive and negative group with respect to the overall survival of patients (p=0.421). However, the follow up of patients is being continued to determine the prognostic utility of Pin1 as well as β-catenin up-regulation and APC loss in SCCs.

In normal cell, the level of β-catenin is controlled by APC dependent proteosomal degradation complex, composed of GSK3β, Axin, CKα and APC [13, 35] and activation of Wnt signaling is still evident from the nuclear mislocation of β-catenin [16, 22, 36]. Inactivation or mutation of APC gene leads to cytoplasmic stabilization and nuclear accumulation of β-catenin followed by stimulation of TCF/LEF resulting abnormal transcription of oncogenes, such as c-Myc and cyclin D1 and finally subsequent activation of Wnt/β-catenin pathway leading to carcinogenesis. Hence, misbalancing in APC-arbitrated regulation of β-catenin is the fundamental feature for the deregulation of this pathway. In our immunohistochemical analysis, strong nuclear and cytoplasmic accumulation of β-catenin as well as cytoplasmic diminution/loss of APC protein was detected in 60.78%, 43.13% and 45.09% of SCCs respectively. The significant inverse association between nuclear as well as cytoplasmic β-catenin with loss of APC (r=-.382, p<0.0001; r=-.198, p=0.014) conforms, the disruption in the functional relationship between them and it is presumed that there is the possibility of activation of Wnt/β-catenin pathway in squamous cell carcinoma of cervix. To best of our knowledge, for the first time our study demonstrated association between the expression profile of two major components of Wnt/β-catenin pathway, *i.e.*, β-catenin and APC in SCCs. Apart from that, it has been proposed that APC also acts in nuclei in β-catenin regulation exporting it for inactivation [37–39]. While another study reported that APC physically associated with Wnt target genes and represses them through the binding with C-terminal binding protein (ctBP) [40]. In our study, nuclear expression of APC was also observed in some cases (data not shown). Hence, in line with previous reports and from our findings it has been speculated that, APC plays an antagonistic role in the Wnt pathway irrespective to its cellular localization.

Furthermore, Pearson's correlation test revealed the significant positive association of Pin1 with nuclear β-catenin (r=0.349, p<0.0001) as well as inverse association with APC (r=-.287, p<0.0001), which supports the probability of the involvement of Pin1 in the activation of Wnt/β-catenin pathway in cervical cancer. As Pin1, affects the protein stability, protein conformation and protein–protein interaction by isomerizing the Ser/Thr-Pro motifs of its target proteins, it is suggested that β-catenin is the important substrate for Pin1 as it contains three Ser/Thr-Pro motifs. Pin1 binds to the $pSer^{246}$-Pro motif of β-catenin located at the centre of Armadillo repeats [21, 41]. According to Ryo *et al.* (2001), Pin1 interferes with the physiological behavior of β-catenin and APC and blocks the interaction between them. It also binds and isomerizes the $pSer^{246}$-Pro peptide bond in β-catenin, which affects its ability to bind APC, thereby regulates the turnover of β-catenin [21]. This may be the possible mechanism behind the Pin1 modulated activation of β-catenin signaling. Ryo *et al.* (2001) also observed decreased cytoplasmic and increased nuclear fraction of β-catenin in Pin1 over-expressing vector-transfected breast cancer cells [21]. Similarly, we also noticed the significant reduced cytoplasmic expression of β-catenin in Pin1 positive cases (r=-0.230, p=0.004). Both the findings further strengthened the involvement of Pin1 in the regulation of sub cellular localization and nuclear translocation of β-catenin, which is the hallmark feature of activation of Wnt/β-catenin pathway. However, a significant down-regulation of APC in Pin1 positive cases (r=-0.287, p<0.0001) was also observed in our study. This findings advocate that Pin1 may lead the down-regulation of APC in those cancer where mutation in the gene encoding APC is quite uncommon. Hence taken together, our results revealed the possibility of involvement of Pin1 in the activation of Wnt/β-catenin signaling pathway through amending the interaction between β-catenin and APC. However, we intended to investigate the expression profile of various Wnt/β-catenin pathway inhibiters like GSK3β, transducers and downstream targets and their relationship with Pin1 to logistically confirm its role in the activation of this pathway in cervical squamous cell carcinoma.

Conclusions

Our data demonstrated the overexpression of Pin1 and its association with β-catenin and APC in squamous cell carcinoma of cervix, which delineates its contribution in the regulation of these proteins and subsequent activation of Wnt/β-catenin signaling pathway in this cancer. Furthermore, association of Pin1 and β-catenin

with various tumor variables underscores the clinical efficacy of these proteins in cervical cancer.

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