

Downregulated Expression of WWOX in Cervical Carcinoma: A Case-Control Study

Shikha Srivastava¹, Uday Pratap Shahi², Arti Divya³, Sadhana Gupta⁴, Indu Singh⁵, Jagat Kumar Roy^{1*}

1. Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi, India.

2. Department of Radiotherapy and Radiation Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

3. Indian Railways Cancer Institute and Research Centre, Varanasi, India.

4. Department of Obstetrics and Gynaecology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

5. Apollo Clinic, Mehmoorganj, Varanasi, India.

Submitted 11 June 2020; Accepted 29 November 2020; Published 27 January 2021

Integration of human papilloma virus (HPV) in human genome is a random event, and fragile sites are one of the most susceptible sites for viral integrations. *WWOX* (WW-domain containing oxidoreductase) gene harbours the second most common fragile site, FRA16D, and can be an important candidate for HPV integration and cervical carcinogenesis. Our aim was to evaluate the potential role of *WWOX* in cervical carcinogenesis. Presence of HPV and its genotype was detected by PCR in normal cervix tissues and human cervical carcinoma. The expression of *WWOX* transcript and its protein was examined by RT-PCR, RNA *in situ* hybridization, and immunoblotting. Southern blotting and sequencing were used to determine the alternative transcripts of *WWOX*. Statistical analysis were performed by Mann Whitney U-test, Pearson correlation coefficient test at significance level of P value ≤ 0.05 . Prevalence of HPV was observed in cervicitis (40%), cervical intraepithelial neoplasia patients (50%), and invasive cervical carcinoma patients (89.6%). Clinicopathological findings suggested a correlation of reduced level of *WWOX* protein and progression of cervical carcinoma deciphering its role in tumorigenesis. Furthermore, we observed aberrant *WWOX* transcript having deleted exon 6-8 region in invasive cervical cancer tissues as well as normal cervix samples. More than 60% of cervical carcinoma samples showed reduced protein level with an increase in wild type transcript level suggesting the involvement of a negative regulator, pAck1 (activated Cdc42- associated kinase) which might ubiquitinate *WWOX* protein leading to its degradation. Also, nuclear retention of *WWOX* transcript in invasive cervical carcinoma tissues suggests its regulation at post-transcriptional level. Our findings suggest that *WWOX* acts as a tumor suppressor in cervical carcinoma and could act as a potential therapeutic target for the disease.

*Corresponding author: Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, India. E-mail: jkroy@bhu.ac.in

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Key words: Cervical cancer, clinical- pathological parameters, genotypes, cervical carcinoma patients, *WWOX* variants

Mutation or inactivation of any of the DNA repair genes, tumor suppressor genes or genes of regulatory pathways leads to accumulation of mutated cells causing cancer. Cervical cancer is the most common gynecologic malignancy ranking first in developing countries affecting almost all the age groups (1). Since it is asymptomatic in early stages, its detection is difficult, and therefore leads to high mortality. Human papilloma virus (HPV) is the most important etiological factor found to be associated with 99.7% of the cases (2). Although not all individuals infected with HPV develop cancer, and HPV negative cervical carcinoma patients were also reported, hence identification of host cell factor(s) contributing to the development of cervical cancer is essential.

Earlier, comparative genomic hybridization and loss of heterozygosity (LOH) studies have shown genomic alterations in 16q region in multiple tumor types including lung, breast, hepatocellular, prostate, etc. (3-6). Also, chromosomal region 16q23.2 contains FRA16D, the second most active common human fragile site, thus assuming the presence of a tumor suppressor gene at this site. Later WW domain-containing oxidoreductase (*WWOX*) or fragile-site FRA16D oxidoreductase (*FOR II*) gene was mapped (7, 8) at 16q23.3-24.1, a region spanning FRA16D. It was explored as a tumor suppressor by induction of ectopic *WWOX* expression leading to suppression of anchorage independent growth in breast carcinoma cell lines (9). Subsequent studies demonstrated that *WWOX* expression was altered in different human malignancies, e.g., oral, gastric, breast, prostate, esophageal, lung, osteosarcoma, hepatocellular carcinomas, etc. (10-15), where partial or total loss in its activity was observed thus establishing it as a putative tumor suppressor gene.

The gene is more than 1.1 Mb encoding a 46.6 kDa protein having two N-terminal WW domains, a short-chain dehydrogenase (SDR) domain, and a nuclear localization signal (NLS) domain. Eight spliced variants of human *WWOX* transcript have been reported in various human cancers and cell lines (15-17).

WWOX interacts with different partners by its first WW domain (18). SDR domain has sequence homology with a group of hydroxysteroid dehydrogenases involved in metabolism of broad range of substrates including steroids (19). *WWOX* plays a proapoptotic role (20) and functions as a suppressor of transcriptional activity as well as it has a role in DNA repair (21).

Since progression of cervical cancer is associated with HPV infection, chromosome 16 may be an important target for HPV integration due to the presence of second most common fragile site on it. Fragile histidine triad diadenosine triphosphatase (*FHIT*) gene spanning FRA3B, the most common fragile site was reported to be significantly inactivated in cervical carcinomas (22). *WWOX* share clear similarities in genomic structures and functions with *FHIT* and hence might have an involvement in cervical carcinogenesis. In this study we sought to find out whether there is altered expression of *WWOX* in different histological types of uterine cervix cancer and to correlate it with clinicopathological characteristics thus understanding the role of *WWOX* in cervical carcinogenesis.

Materials and methods

Sample and data collection

A total of 69 fresh cervical tissue biopsies (58 invasive cervical cancer, 5 cervicitis and 6 cervical intraepithelial neoplasia (CIN)) and 48 normal

cervix tissues from hysterectomy patients were obtained along with histopathological reports from Sir Sunderlal Hospital, Apollo Clinic and Indian Railways Cancer Institute and Research Centre, Varanasi, India. The samples obtained were immediately stored at -80 °C for further use. All the cervical carcinoma samples were classified according to FIGO classification by pathologists. Non-cancerous tissue samples (controls) were obtained from women undergoing hysterectomy with some ailment other than cancer. The patients undergone chemo or radiotherapy were not included in the study. Ethical clearance for the present study was taken from the Institutional ethical committee. Written consent was taken from each of the participants or guardian/relative of the patient included in the study. Age range for the patients was 25-75 years with the mean age of 52.35±11.02 years.

Human cervical carcinoma cell lines, SiHa and HeLa, were obtained as gift from Dr. G. Narayan (BHU, Varanasi).

DNA isolation, HPV detection and genotyping

Genomic DNA was isolated from normal, precancerous and invasive cervical cancer tissues by standard phenol-chloroform protocol as described previously (23). Presence of HPV infection was detected by using MY09/11 (24) and

GP5+/6+ (25) primer sets. The samples positive for HPV were further genotyped using type specific PCR (HPV types 16, 18, 31, 33 and 35), and those samples negative for the above mentioned HPV types were sequenced and analyzed as described previously (26).

WWOX mRNA expression analysis

Total RNA was extracted from normal cervix, invasive cervical carcinoma tissue samples and cervical cancer cell lines (HeLa, SiHa) using Tri-Reagent (Sigma-Aldrich, USA) and first strand cDNA was synthesized from 2.5 µg of total RNA using reverse transcriptase (MBI Fermentas, USA) as per manufacturer's protocol. Expression analysis was done by semi-quantitative RT-PCR using specific primers from the region of exon 8 and exon 9 (Table 1) since amplification of this region (product size 491 bp) in the mRNA indicates the presence of only full length WWOX transcript (27).

Actin (a housekeeping gene) served as a loading control. RT-PCR was carried out using 0.5 µL of cDNA in a 25 µL reaction volume containing 10 pmol of each primer, 4 µL of dNTP mix (containing 200 µM each of dATP, dTTP, dCTP and dGTP), 1x PCR buffer (10 mM Tris-Cl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂) and 1 U Taq Polymerase (Bangalore Genie, India). PCR product was electrophoresed on 1.5% agarose gel, analyzed

Table 1. List of oligonucleotide sequences

Type	Oligonucleotide sequence	Amplicon size
WWOX Exon 8 FP	5'-AAAACGACTATTGGGCGATG-3'	491 bp
WWOX Exon 9 RP (RT-PCR)	5'-GTGTTGGAGGGACATTTGGA-3'	
WWOX Exon 4 FP	5'-AACCACCCGGCAAAGATAC-3'	1076 bp
WWOX Exon 9 RP (RT-PCR)	5'-GTGTTGGAGGGACATTTGGA-3'	
RNA-RNA in situ hybridization probe	5'-AAAACGACTATTGGGCGATG-3' 5'-GTGTTGGAGGGACATTTGGA-3'	491 bp
WWOX Exon 4 FP	5'-CTGGCGTTTACTGTGGATGA-3'	236 bp
WWOX Exon 5 RP (Southern hybridization probe)	5'-ATGCGTGACACTGCTTCACT-3'	

FP: Forward primer, RP: Reverse primer

under UV transilluminator, and image was captured using gel documentation system (G:Box, Syngene, Europe). Densitometric measurement was done and each sample was normalized with the value of actin.

Analysis of WWOX alternative transcript by Southern blotting

WWOX variants were analyzed by using specific primers from the region of exon 4 and exon 9 (Table 1) since amplifying this region will take account of all the variants of WWOX. The amplification of full length transcript will give the product size of 1076 bp while lower size amplicons will be generated for other variants since they have smaller length as compared to wild type transcript. PCR was carried out and products were resolved on 1% agarose gel along with an appropriate DNA ladder. In all the experiments, samples from controls along with cervical cancer patients were loaded on the same gel. After electrophoresis, the gel was depurinated in 0.25N HCl, denatured in denaturing solution (1.5M NaCl and 0.5N NaOH), transferred onto the nylon membrane and UV crosslinked. The membrane was pre-hybridization in buffer (6X SSC, 0.5% SDS, 5X Denhardt's solution, 50% formamide, 100 µg/mL sonicated single stranded salmon sperm DNA) for 2 h at 42 °C. A digoxigenin labeled cDNA probe was synthesized from the region of exon 4 and exon 5 (Table 1) of WWOX since probe from this region will be able to detect all the variants. The probe was hybridized onto the membrane at 42°C overnight for detecting the alternative transcripts. Blot was incubated with anti-DIG alkaline phosphatase (ALP) conjugated secondary antibody (Roche; USA, 1:4000) and detected by colorimetry (NBT/BCIP, Sigma-Aldrich, USA). The bands on the agarose gel corresponding to the bands on the southern blot were excised, eluted using gel extraction kit (MBI Fermentas, USA) and sequenced directly (Applied Biosystems 3130 four capillary Genetic Analyser; ABI, USA) using both

forward (exon 4) and reverse primers (exon 9).

Western blot analysis

Total cell lysate was prepared from normal cervix, cervicitis, CIN, invasive cervical carcinoma tissues, and cervical cancer cell lines (HeLa, SiHa) using RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM DTT, 1 mM PMSF, 1% Nonidet NP40, 0.1% SDS) containing protease (Roche, USA) and phosphatase inhibitors at 4 °C, and then centrifuged at 10,000 rpm for 10 min to remove cell debris. For Western blotting, 50 µg of protein sample was heat denatured, separated by 12% SDS-PAGE, and transferred onto PVDF membrane (Millipore, USA). Immuno-detection was done for WWOX and activated Cdc42-associated kinase (pAck1) using anti-WWOX rabbit polyclonal primary antibody (1:750, Abcam, UK) and Anti-pAck1 rabbit polyclonal primary antibody (1:100, Abcam, UK) along with ALP conjugated goat anti-rabbit secondary antibody (1:3500, Bangalore Genie, India). The same blot was reprobed with actin used as a protein loading control and detected by mouse monoclonal anti-actin primary antibody (1:1000, Sigma, USA) and ALP conjugated goat anti-mouse secondary antibody (1:2000, Bangalore Genie, India). Detection was done by colorimetric method with NBT/BCIP. For the detection of pAck1 protein, 150 µg of total protein lysate was loaded onto 10% SDS gel. Densitometric measurement was performed for each group of samples by normalizing each sample with the value of actin using AlphaImager 2200 Software.

RNA *in situ* hybridization

Total RNA was isolated from normal cervix tissue and cDNA was synthesized as described above. Semi-quantitative RT-PCR was done using primers from the region of exon 8 and exon 9 (Table 1). The amplicon (491 bp) was purified and cloned in pGEM T-Easy Vector. One µg of clone having the desired insert was transcribed *in vitro*

using T7/SP6 RNA polymerase for sense and antisense probe, respectively. The reaction was carried out with 2 µL 10X transcription buffer, 2 µL NTP labeling mix with DIG-UTP (Roche, USA), 5 U RNase inhibitor, and 30 U T7/SP6 RNA polymerase as per manufacturer's protocol. Sense riboprobe was used as a negative control.

For RNA-RNA *in situ* hybridization paraffin embedded tumor biopsies were sectioned (5 µm thickness, Rotary microtome, Leica, Germany), deparaffinized in xylene, and rehydrated through grades of ethanol. Sections were fixed in 4% paraformaldehyde in 1X PBS (phosphate buffer saline) and digested with proteinase K (10 µg/mL) at 37°C for 20 min. Washing was done using chilled glycine (2 mg/mL) and prehybridized in hybridization buffer for 2 h at 60°C. Appropriate dilutions of antisense and sense riboprobes were added and slides were incubated at 60°C overnight in a moist chamber. The slides were then washed in 5X SSC, 2X SSC and 0.2X SSC 10 min each at 60°C and incubated with anti-DIG antibody (1:2000) and chromogenic detection was carried out in NBT/BCIP solution till the color developed. The reaction was stopped after the adequate signal and slides were dehydrated in grades of ethanol and mounted in DPX.

Statistical analysis

Non-parametric Mann Whitney U-test was applied to compare normal and tumor tissues. For correlation analysis, Pearson correlation coefficient test were done. SPSS statistical software (version 16) and GraphPad prism5 were used for statistical

analysis. The tests were considered significant if P value was ≤ 0.05 .

Results

HPV status in pre-cancerous and cervical carcinoma tissues

All the pre-cancerous and cervical carcinoma samples as well as controls were first checked for the presence of HPV infection. We observed that the prevalence of HPV was different at different histological grades of cervical tissues. It was 40% (2/5) in cervicitis, 50% (3/6) in CIN patients, and 89.6% (52/58) in invasive cervical carcinoma (although sample size is small in case of cervicitis and CIN patients to make any conclusive remarks). In all the stages HPV16 was observed to be the most prevalent type followed by HPV18 and 31 (samples include single as well as multiple HPV type infections) (Table 2). A total of 9 cervical tissue samples (3 cervicitis, 3 CINs, and 3 invasive cervical carcinomas) were negative for HPV infection. None of the normal cervical tissue (control) was found to be infected with HPV.

Increased expression of WWOX mRNA transcript in cervical carcinoma

Out of the total invasive cervical carcinoma tissues, 42 carcinoma tissues and 13 normal cervical tissue samples were analyzed for the presence of full length WWOX mRNA. Interestingly, a ≥ 2 fold increase in relative expression level of WWOX mRNA was observed in 54.7% (23/42) of invasive cervical carcinoma samples while 21.4% (9/42) showed lower expression level as compared to

Table 2. Prevalence of HPV in different cervical histological types

HPV	Cervicitis N=5 (%)	CIN N=6 (%)	SCC N=58* (%)	Total
HPV -ve	3 (60)	3 (50)	3 (5.1)	9
HPV +ve	2 (40)	3 (50)	52 (89.6)	57
HPV16	1 (50)	3 (100)	39 (75)	43
HPV18	1 (50)	-	18 (34.6)	19
HPV31	1 (50)	1 (33.3)	10 (19.2)	12

* HPV positivity for 3 SCC samples were not known

normal cervical tissues. In case of cervical carcinoma cell lines, a 2.5 fold increase in *WWOX* mRNA expression level was observed in SiHa cells while lower expression was observed in HeLa cell line (Figure 1a-b). Statistical analysis of mean relative expression (mean \pm SD) was done for normal (0.47 \pm 0.07) and invasive cervical carcinoma tissues (0.95 \pm 0.07) which showed a significant increase in *WWOX* transcript level (Figure 1c, $P = 0.001$) in cervical carcinoma tissues. A few of the normal cervix tissues and cervical cancer samples showed very high level of *WWOX* mRNA expression, though, even after including those samples not much difference in statistical significance was observed (Figure 1d).

Loss of *WWOX* protein expression in cervical carcinoma patients

WWOX is a tumor suppressor gene as reported in various cancers, but our results showed a higher transcript level of *WWOX* in cervical carcinoma samples. Thus, next we examined the expression level of *WWOX* protein to determine whether it correlates with the increased mRNA level in 53 invasive cervical cancer, 48 normal cervix, 5 cervicitis, and 6 CIN tissue samples. We observed that >80% of the invasive cervical carcinoma samples showed a reduced expression level of full length *WWOX* protein as compared to normal cervix tissue out of which 34% (18 out of 53) of tissues showed a ~60% reduction in *WWOX* protein level while only 20% of cervicitis and 33.3% of CIN samples showed reduced expression (although sample size is small) (Figure 2a-b). None of the tissues showed complete absence of

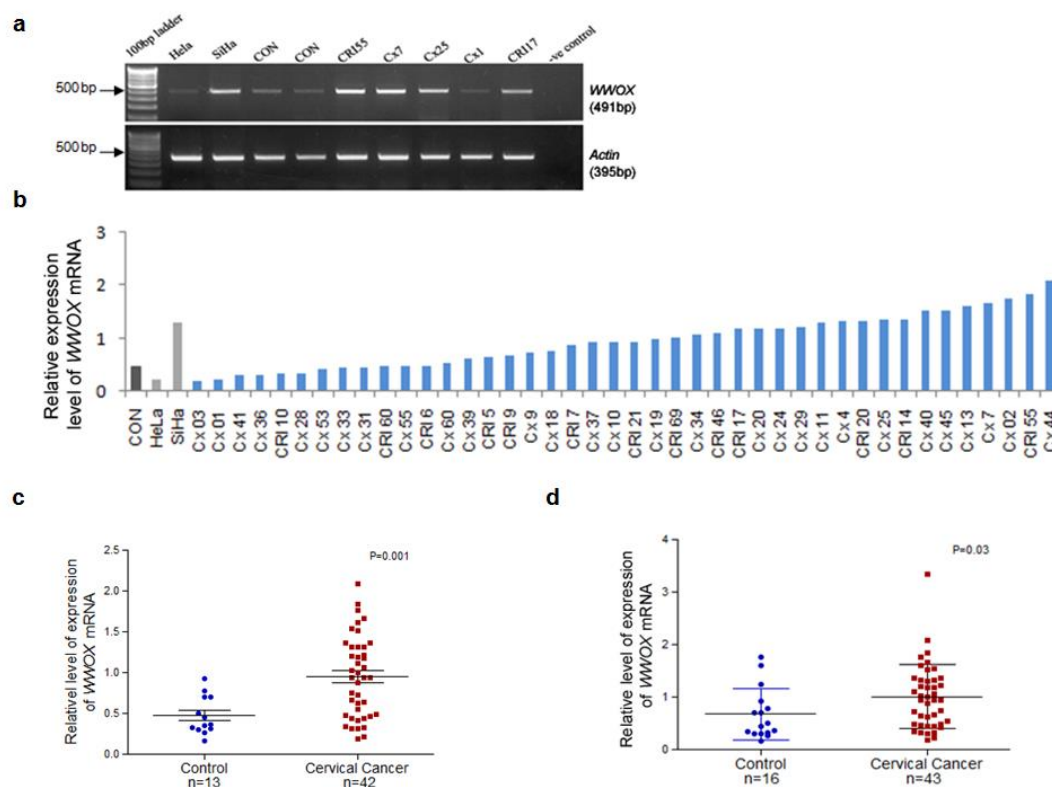


Fig. 1. Analysis of expression level of *WWOX* mRNA in invasive cervical carcinoma tissue samples and control tissues. a) A semi-quantitative RT-PCR amplifying the region of exon 8 to 9 of *WWOX* transcript (491 bp) along with actin as an internal control (395 bp) in normal cervical tissues and invasive cervical carcinoma samples; b) Densitometric analysis of *WWOX* transcript along with actin was done to calculate relative expression level in control (normal cervix tissues, n=13), cervical carcinoma cell lines (HeLa and SiHa), and invasive cervical carcinoma samples (n=42); c) Mean \pm SD of *WWOX* transcript showed relative level of expression of transcript in invasive cervical carcinoma samples ($P = 0.001$) (Mann-Whitney U test); and (d) in total number of invasive cervical carcinoma samples (including those showing high expression level) as compared to normal cervix. CON: normal cervix tissues; Cx and CRI: cervical cancer patient samples.

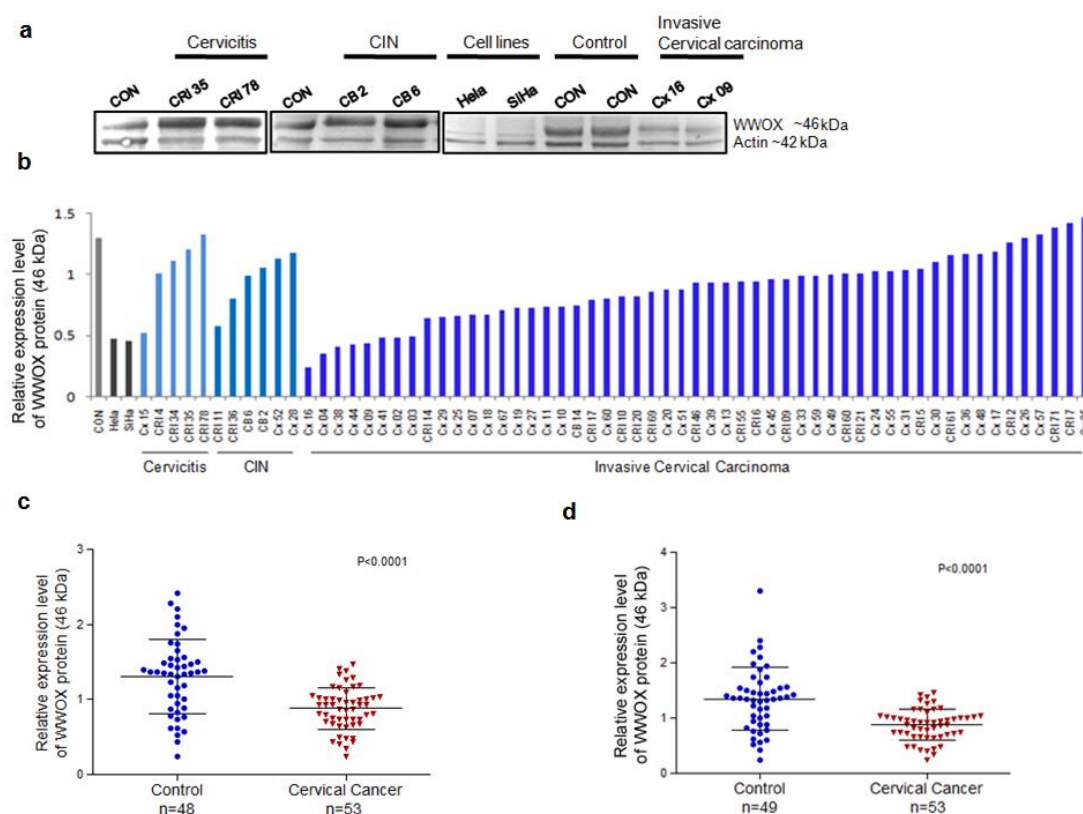


Fig. 2. Determination of WWOX protein expression. WWOX protein levels were assessed in control (n=48) as well as cervicitis (n=5), CIN (preinvasive cervical cancer tissues, n=6) and invasive cervical carcinoma samples (n=53). a) total protein lysate was prepared from cervix tissues of normal, CIN, cervicitis and cancerous samples and Western blot analysis was done. ~50 µg of protein sample was resolved on SDS-PAGE and immunoblotted with respective primary and secondary antibodies; b) Densitometric analysis was done for each sample and normalized with actin to correct for loading differences. Bar graph was plotted for each tissue sample showing its relative expression level as compared to control; c) Mean ± SD of normal cervical tissues with invasive cervical carcinoma samples (P < 0.0001) (Mann-Whitney U test) and (d) total number of invasive cervical carcinoma samples including those showing high WWOX protein expression level. CON: normal cervix tissues; Cx, CB and CRI: cervical cancer patient samples.

WWOX protein, and no other WWOX isoform other than wild type was detected in any of the samples. A reduced expression (< 50%) of WWOX protein was also observed in both HeLa and SiHa cervical carcinoma cell lines (Figure 2a-b). Statistical analysis of mean relative expression showed 1.34 ± 0.5 (mean±SD) for controls and 0.89 ± 0.29 for invasive cervical carcinoma samples (Figure 2c), thus suggesting a very significant (P= 0.0001) decrease in relative expression level of WWOX. A few of normal cervix tissue samples showed a high expression level of WWOX protein although, even after including those samples not much change in statistical significance was observed (Figure 2d).

Correlation of WWOX protein with clinico-pathological parameters

Next, we analyzed the expression pattern of WWOX protein in different clinical stages (FIGO) and grades of pre- and cervical carcinoma tissues. Low relative level of WWOX expression was observed in all the histological types, i.e., 60% of cervicitis cases (3/5), 83% of CIN (5/6), and 100% in stage I (5/5), 82% in stage II (23/28), 94.7% in stage III (18/19) of cervical carcinoma cases. We observed a statistically significant gradual decrease in WWOX expression level in CIN (P= 0.05; sample size of CIN is small to give any conclusive remarks) and cervical carcinoma (P= 0.0003) as compared to control samples (Figure 3a) thus

suggesting the involvement of WWOX with the advancement of the disease. However, we did not observe any significant correlation between the level of WWOX protein expression with tumor grade (differentiation) and age of the patients (Figure 3b-c). The samples showing very high WWOX protein expression level (1 normal cervix sample in case of histological types and 1 of grade I cervical tumor sample) were also added and data was further analyzed statistically but no significant difference was observed (data not shown).

In order to determine if WWOX mRNA (full length) level correlates with the level of WWOX protein, correlation analysis was performed. We could not observe a significant correlation between relative expression level of WWOX transcript and

its protein (Pearson correlation, $r = -0.28$, $P = 0.09$) thus suggesting that the low level of WWOX protein was not dependent on reduction in the level of WWOX transcript (Figure 3d-e) but might be due to the involvement of some other factors contributing towards down regulation of WWOX protein.

Presence of alternate WWOX transcripts

Furthermore, we tried to investigate the possible mechanism of regulation which might be involved in the down-regulation of WWOX protein. Investigation for the presence of alternatively spliced transcripts of WWOX was performed in 44 invasive cervical tumor samples and 16 normal cervical tissues. Although, multiple bands were observed after RT-PCR using specific primers from

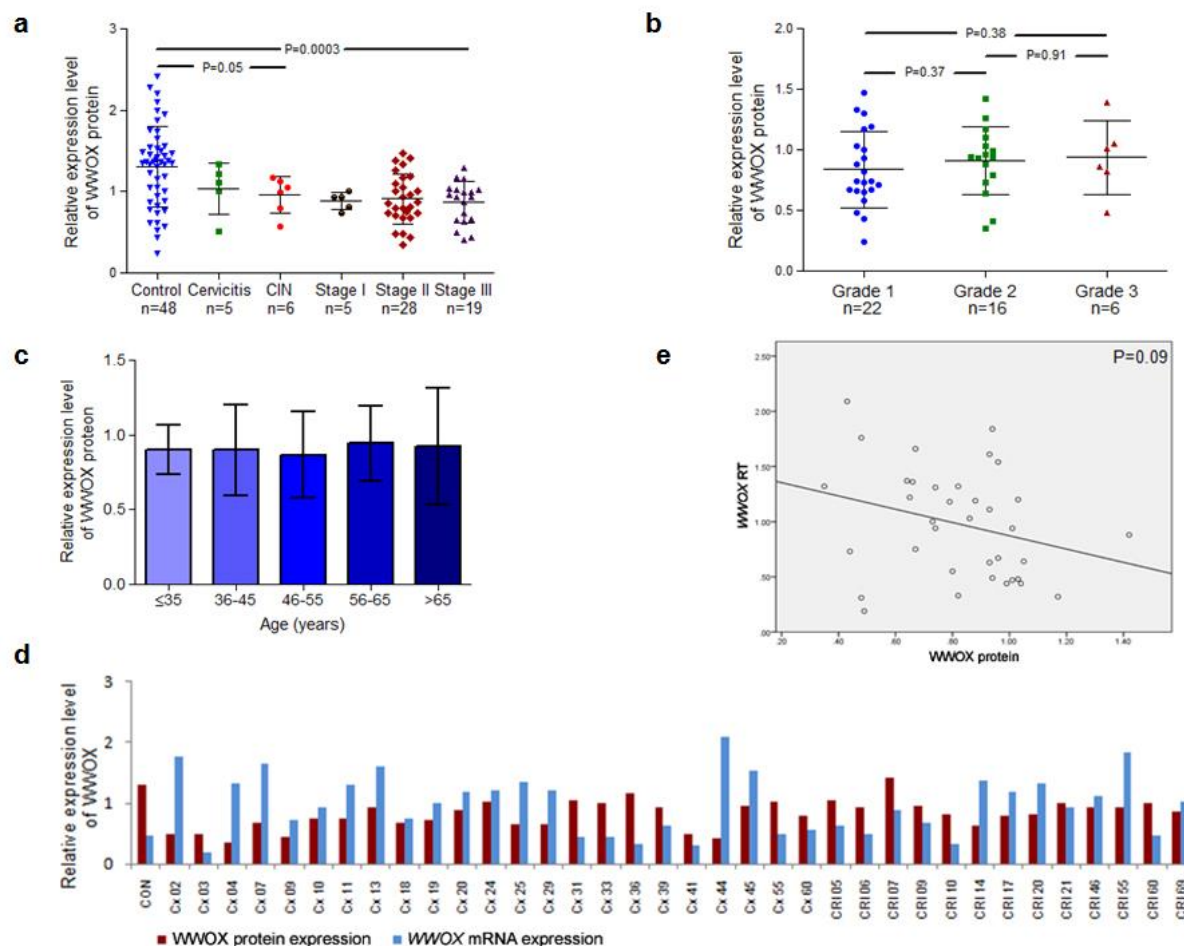


Fig. 3. Association of WWOX protein level with clinicopathological parameters. a) Significant association ($P = 0.0003$) was observed between WWOX protein level with increasing severity of the disease; no significant association was found with grade of tumor (b) and patient's age (c); d) Relative level of expression of WWOX mRNA and protein in each cervical cancer tissue along with normal cervical tissue sample was analyzed; e) no significant correlation was observed between WWOX protein and mRNA level (Pearson correlation, $r = -0.28$, $P = 0.09$).

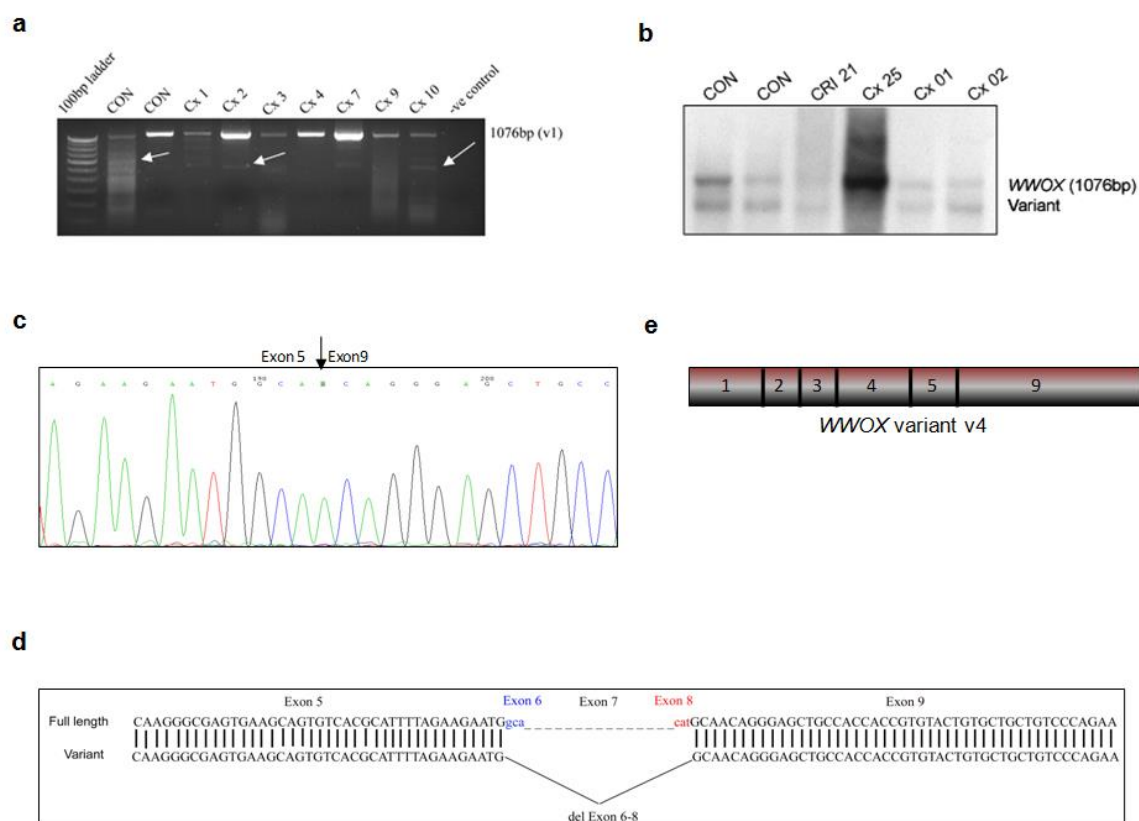


Fig. 4. Analysis of WWOX transcript and its variants. a) RT-PCR was done to amplify the region from exon 4 to exon 9 in control and cervical carcinoma samples. An amplicon of 1076 bp represents the full length transcript present in all the samples. Some aberrant transcripts of small size (arrow) were also observed; b) Southern blotting was performed by using a cDNA probe from the region of exon 4 and exon 5 of WWOX mRNA. A variant along with normal full length transcript (1076 bp) was observed in cervical cancerous tissue samples as well as control tissues; c) sequence analysis of variant shows the region of abnormal fusion of exon 5 and 9 (arrow); d) alignment of variant and full length transcript shows deleted region of exon 6 to exon 8; e: alignment shows the spliced variant corresponds to WWOX transcript variant 4 (v4).

WWOX exon 4 and exon 9 (Figure 4a), Southern blot showed presence of only two bands, one at 1076 bp position (indicating the presence of full length transcript) and another smaller band detected at 518 bp position (Figure 4b). Sequencing 1076 bp band in various samples confirmed the presence of full length transcript without any sequence alteration while small sized band when aligned with the full length transcript sequence (1076 bp) showed a deletion of exon 6 to exon 8 region (Figure 4c-d). This short transcript corresponds to WWOX mRNA transcript variant 4 (Figure 4e) which was detected in 32% (14/44) of invasive tumor samples and 25% (4/16) of normal cervical tissues. Since alternative spliced form (variant 4) occurred in tumor samples as well as in normal cervix tissues, it is very unlikely that they alter

WWOX protein function and have any significant role in cervical carcinogenesis.

Correlation between expression level of WWOX and its negative regulator pAck1

To investigate the possible factors involved in negative regulation of WWOX protein, the expression level of activated tyrosine phosphorylated Ack1 (pAck1), a negative regulator of WWOX protein, was observed by immunoblotting. A 117 kDa band of pAck1 was detected (Figure 5a) in a total of 22 cervical cancer samples and 19 normal cervical tissues investigated in this study. In 40.9% (9/22) of cervical carcinoma tissue samples, high relative level of expression of pAck1 was observed as compared to normal cervix samples while equal or reduced level of expression

was detected in 59% (13/22) of samples (Figure 5b).

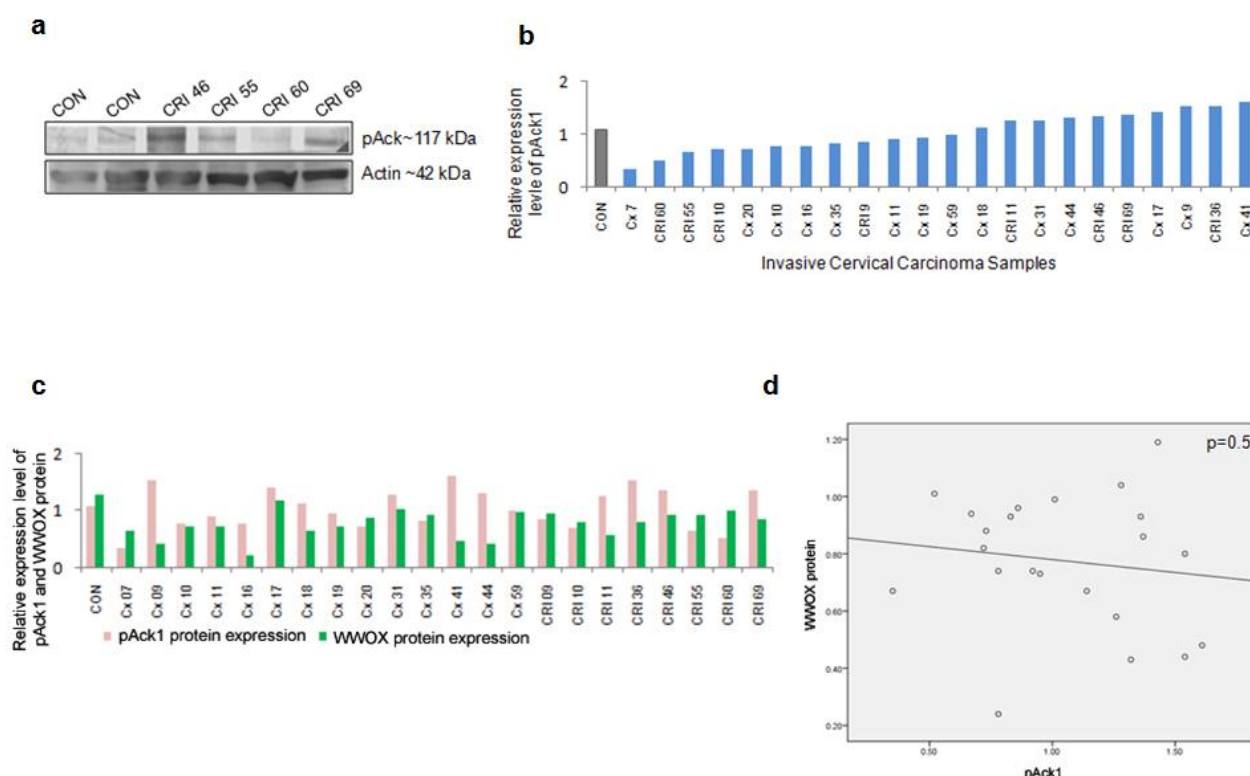


Fig. 5. Correlation between WWOX protein expression level and its negative regulator pAck1. a) Immunoblot analysis was done for pAck1 (active form of ACK1) in control cervix tissues (n=19) and cervical cancer tissue samples (n=22). Protein lysate was prepared, loaded onto SDS-PAGE and immunoblotted with pAck1 antibody. Actin was used as loading control for immunoblotting; b) Densitometric analysis was done for each sample and normalized with actin. Bar graph was plotted showing the relative expression level of controls and invasive cervical cancer tissues; c) Relative expression level of WWOX protein and pAck1 was plotted and analyzed with 50% samples showing pAck1 mediated downregulation of WWOX protein; d) No significant correlation was observed between WWOX and pAck1 protein level (Pearson correlation, $r = -0.139$, $P = 0.5$). CON: normal cervix tissues; Cx and CRI: cervical cancer samples.

A correlation analysis was performed to investigate whether the low level of expression of WWOX correlates with the high level of expression of pAck1. No significant correlation was observed between WWOX and pAck1 protein level (Pearson correlation, $r = -0.139$, $P = 0.5$) (Fig. 5c, d). Thus, the result proposed that the reduced expression level of WWOX protein might involve ubiquitin mediated degradation by pAck1 in some of cervical carcinoma cases suggesting the involvement of some other mechanism apart from this.

Nuclear localization of mRNA transcript in cervical carcinoma tissues

To validate the increased expression level of full length WWOX transcript, its localization was studied through RNA-RNA *in situ* hybridization.

64.2% of the cervical carcinoma samples showed a reduced WWOX protein expression level with high expression level of WWOX transcript hence the localization of WWOX full length transcript was detected in these tissue sections. Invasive cervical carcinoma tissue sections showed intense nuclear along with weak cytoplasmic staining with antisense RNA probe (Figure 6d-e, g-h) while in normal cervical tissue samples the staining observed was mainly cytoplasmic localization (Fig. 6; a-b). Sense probe acting as negative control did not show any signal thus confirming the specificity of antisense probe (Fig. 6; c,f,i). Nuclear localization of WWOX mRNA suggests nuclear retention of full length WWOX transcript in cervical carcinoma samples which

was not observed in normal cervical tissue samples.

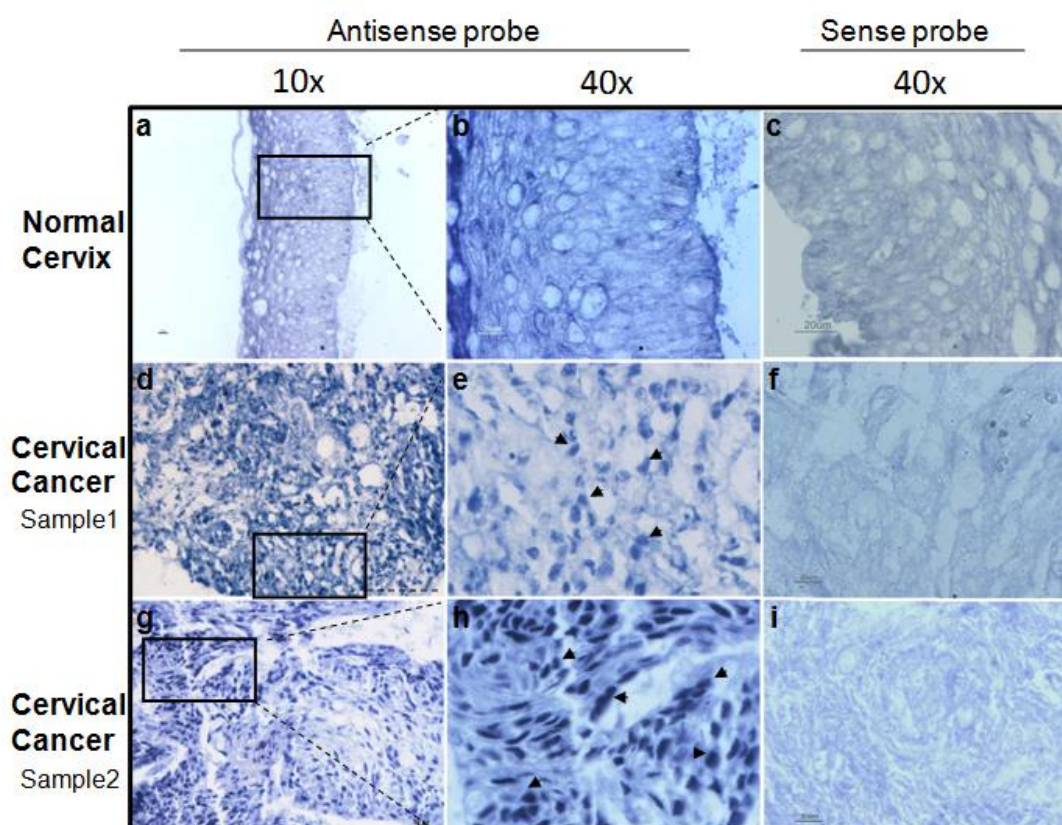


Fig. 6. Localization of WWOX transcript in normal cervix tissues and invasive cervical carcinoma samples. RNA-RNA *in situ* hybridization was done using RNA probe from the region of exon 8 to 9. 5 µm tissue sections were de-paraffinized, rehydrated in grades of ethanol and fixed in 4% paraformaldehyde. Sections were treated with proteinase K and incubated with appropriate antisense and sense probes. After washing, slides were incubated with anti-DIG antibody and chromogenic detection was carried out. Cytoplasmic staining was observed in normal cervix (a, b) while intense nuclear staining was observed in invasive cervical cancer sample with antisense probe (d, e, g, h). Sense probe acts as negative control for all the samples (c, f, i).

Discussion

Integration of HPV in human genome is a random event, and it is known that fragile sites are susceptible to viral integrations. *WWOX* gene harbours the second most common fragile site, FRA16D, and can be an important candidate gene involved in cervical tumorigenesis.

In the present study, we examined different histological types of cervical biopsy samples for the presence of HPV infection. 40% cervicitis (inflammatory cervical cases), 50% CINs and 89.6% invasive cervical carcinoma samples were found to be infected with HPV. Also, HPV16 was observed to be the most prevalent type followed by HPV18 and 31 (Table 2) as shown in our previous study (28). This result was in agreement with the

other reports on HPV type infection in different populations (HPV16 being 87% in Odisha (29), 68% in Andhra Pradesh (30), 98.7% in Venezuela (31) and 92.5% in Morocco (32) in invasive cervical carcinoma).

Further, our results demonstrated a significantly increased level of expression of *WWOX* full length transcript ($P = 0.001$) in cervical carcinoma tissues and SiHa cell line as compared to control (normal cervical tissue) samples. This data was in contrast with the previous reports in other cancers where a reduction in *WWOX* transcript was observed (15, 33).

Moreover, to validate our findings we examined *WWOX* protein level in all the cervical histological cases. Interestingly, a very significant

decrease in relative expression level of full length WWOX protein in invasive cervical carcinoma samples ($P = 0.0001$) as well as HeLa and SiHa cervical carcinoma cell lines was observed which was in agreement with previous studies where reduced or absence of WWOX protein was reported in paraffin sections of CIN and invasive cervical carcinoma thus validating our observation (34, 35).

Further, association of WWOX protein expression level with various clinicopathological parameters showed its significant down-regulation in case of CIN ($P = 0.05$) and cervical carcinoma cases ($P = 0.0003$) but the reduction was not significant in cervicitis ($P = 0.12$) thus suggesting the involvement of WWOX with the progression of cervical carcinoma. No significant correlation between the level of WWOX protein expression with tumor grade (36) and age of the patients was observed.

In the present study, multiple HPV genotypes were found in cervical carcinoma samples but HPV16 was found to be predominant (~90% cases). To make any conclusion of correlation of expression of WWOX with a particular HPV genotype, a comparable number of other genotypes were also required. For detailed analysis and better understanding of the association between WWOX with particular HPV genotypes, further studies with large number of samples of other HPV genotypes will be required.

In the present study, we could not get any correlation between the relative expression levels of WWOX protein and its full length transcript thus suggesting that the downregulation of WWOX protein was not due to reduced expression of WWOX transcript. These results are in agreement with a previous study in cutaneous squamous cell carcinoma where down-regulation of WWOX protein level was reported though no alteration in mRNA transcript level was observed thus suggesting the involvement of translational blockade in cancer cells (27).

Furthermore, we investigated the possible pathway(s) which might be involved in down-regulation of WWOX protein. Examination of alternatively spliced transcripts of WWOX in all the samples, including normal cervical tissues showed the presence of a variant lacking exons 6-8 corresponding to WWOX mRNA transcript variant 4. The variant 4 was observed in 32% of the invasive cervical carcinoma tissues along with the full length WWOX transcript. This variant was also reported in various tumors e.g., gastric (16), breast (12), esophageal carcinomas (14), etc. where it was reported to be involved in tumorigenesis. In the present study we observed this variant in 25% of the normal cervical tissue samples also along with cervical carcinoma tissues which shows that this variant might not have a significant role in the context of initiation/progression of cervical cancer.

Next, we studied the pathway involved in degradation of WWOX protein which might be responsible for its reduced expression in cervical cancer tissues. We examined pAck1, a negative regulator of WWOX, present down-stream in the pathway. A previous study showed an up-regulation of activated tyrosine phosphorylated Ack1 (pAck1) in prostate cancer where it phosphorylates WWOX at Tyr287, resulting in its polyubiquitination and degradation (13). In cervical carcinoma tissues we observed high relative level of expression of pAck1 in 40.9% of the samples as compared to normal cervical tissues though no significant correlation was observed between pAck1 and WWOX protein suggesting that pAck1 was not involved in cervical carcinogenesis in all the cases and predicting the involvement of some other pathway apart from this.

To check for the high transcript level of full length WWOX, its localization was studied through RNA-RNA *in situ* hybridization. An intense nuclear staining along with weak cytoplasmic staining with antisense RNA probe in invasive cervical carcinoma tissues was observed showing nuclear mRNA retention of WWOX while no such nuclear

retention was observed in normal cervix tissue samples. This indicates that full length *WWOX* transcript is relatively stable. The same phenomenon may be speculated in case of SiHa cell line where we observed low level of protein expression with high mRNA transcript level. This suggests that there may be some defect in the mechanism of export of mRNA from the nucleus to the cytoplasm leading to reduced level of expression of *WWOX* protein. Nuclear mRNA retention has now been observed as one of the important mechanisms in regulating transcription-related proteins (37).

Similar findings were reported in previous studies on doppel protein expressed in human astrocytic tumor cells (38). This phenomenon was explained on the basis of unsuccessful capping process, improper packaging or unspliced mRNAs though the detailed mechanism was unknown. We can also explain it on the basis of some defect in pre-mRNA processing, e.g. capping process due to which export of mRNA from the nucleus to cytoplasm was getting affected consequently resulting in lower *WWOX* translation. Also, involvement of miRNA in translationally repressing target mRNA is another mechanism involved in regulating gene expression (39) which has been observed in several cancers, e.g., lung (40), ovarian (41), osteosarcoma (42), oral (43), etc. where it inhibits the translation or causes the degradation of targeted mRNA molecules, leading to carcinogenesis. Thus, the mechanism involved in nuclear mRNA retention remains to be understood.

In conclusion, the present study demonstrates a novel mode of regulation which provides a new paradigm for understanding the regulatory mechanism of *WWOX* gene expression in cervical cancer. Several mechanisms for the regulation of *WWOX* have been reported in different tumors including epigenetic regulation (promoter methylation) (44), translational blockade (27), ubiquitin mediated degradation (13), etc. We

observed a significant reduction of *WWOX* protein expression with the increase in severity of the disease thus indicating its involvement in tumor progression. This suggests that *WWOX* might be playing a role as a tumor suppressor in cervical cancer and can be explored as a potential candidate in cancer therapeutics.

Acknowledgements

We thank Dr. Sathees C. Raghavan (IISc) and Dr. Manoj Kumar (Banasthali Vidyapith) for their comments and suggestions on the manuscript. We thank Dr. Saroj Gupta (MD Pathology), SG Lab for providing the histopathological reports of cervical cancer patients. We thank doctors of the Department of Radiotherapy and Radiation Medicine and the hospital staff of PP-ODP for their help. The equipment facilities generated through UGC-CAS, DST-FIST, ISLS, IoE-BHU, and CSIR are acknowledged. We also thank Dr. Attila Lorincz for providing us clone of HPV-31.

Conflict of interest

The authors declare that there are no conflicts of interest.

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