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Regular paper

Abnormal level of paxillin in cervical cancer cells is involved in tumor progression and invasion

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Human papillomavirus (HPV) is the primary causative agent for the uterine cervical cancer. The expression of oncoproteins E6/E7 promotes apoptosis inhibition and increases the risk of cervical cancer progression. Some research reported that elevated expression of paxillin (PXN) stimulated cancer growth and invasion. However, the clinical significance of PXN in cervical cancer has not been well characterized so far. We found that PXN mRNA expression and protein level are significantly upregulated in cervical cancer cells compared to adjacent normal cells. Furthermore, the paxillin over-expression was correlated with potential of tumorigenesis and invasion. Cervical cancer cells with increased paxillin expression had an ability to form more tumor clones and were characterized by higher invasiveness as well. Therefore, our findings suggest that paxillin may act as an important prognostic factor for cervical cancer patients as it promotes tumor regeneration and invasion.

Keywords: cervical cancer, HPV, focal adhesion, paxillin, cancer invasion

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Abbreviations: HPV, Human papillomavirus; PXN, paxillin; pRB, protein retinoblastoma; PFA, paraformaldehyde

INTRODUCTION

Cervical cancer is the second common cancer among the gynaecological malignancies worldwide. Human papillomavirus (HPV) infection is the key etiological agent for cervical cancer development (Parkin *et al.*, 2002), which accounts for 10–15% of cancer worldwide (Zur Hausen *et al.*, 1991). The most important and frequent types of HPVs are HPV-16 and HPV-18. Both are high-risk types causing neoplastic lesions which are responsible for 80% of cervical cancer progression (Munoz *et al.*, 2006 and zur Hausen *et al.*, 2009). The incidence of deaths due to the HPV-related cancers in women is 1/10 (Scheurer *et al.*, 2005). HPV-16 contains 7900 bp long double-stranded circular DNA, which exists episomally in the nucleus of the infected cell to complete the life cycle of the virus (Stubenrauch *et al.*, 1999). There are three coding regions in the genome namely early, late and long. During the virus life cycle, the proteins coded by the early region such as E1 to E7 are expressed early, potentially involved in host cell proliferation and cell survival (Weyn *et al.*, 2011). After HPV infection, the two major viral oncoproteins E6 and E7 are able to interact with tumor suppressor p53 protein and factors of apoptotic/growth signaling pathways and thus promote malignant transformation, proliferation and immortalization (Bedel *et al.*, 1987 and Brooks *et al.*, 2005). This is achieved by binding of E6 protein to the p53 which results in the accelerated degradation of p53 through its association with E3 ubiquitin ligase. The HPV-16 infection and expression of its oncoproteins E6 (Werness *et al.*, 1990) and E7 were shown to be involved in the activation of cell cycle program by the inactivation of tumor suppressor protein retinoblastoma (pRB) (Song *et al.*, 1997). The HPV-16 infection and expression of its oncoproteins (E6 and E7) were also reported in the progression and metastasis of cervical cancer (Bosch *et al.*, 1995).

Tumor metastasis is a complicated process by which the cancer cells exit the primary site and create new tumors in different parts of the body as secondary sites. Some research reported that scaffold protein paxillin plays a major role in tumor cell adhesion and migration. Paxillin acts as an adapter protein and is involved in a wide range of functions such as cell proliferation, motility, survival, metastasis, tissue remodelling and matrix organization by co-ordinating different signals from integrins, cell surface and growth receptors (Turner *et al.*, 2000; Schaller *et al.*, 2001; Brown *et al.*, 2004). By protein-protein interactions and binding to adhesion molecules such as actin and integrins, paxillin induces cytoskeletal rearrangements and thus ultimately causes migration and metastasis of bone, prostate, lung and colorectal cancer (Hagel *et al.*, 2002; Bokobza *et al.*, 2010; Sen *et al.*, 2012; Chen *et al.*, 2013; Zheng *et al.*, 2018). Phosphorylation of PXN (Tyr118) was found to be a major factor involved in the invasiveness of AGS cells (Li *et al.*, 2009). In addition, paxillin acts as a docking site for the oncoproteins such as E6 and v-Src, which interfere with the signalling pathways crucial for cell growth and migration (Turner, 2000). Therefore, in the present study, we evaluated PXN mRNA and protein expression in five different cervical cancer samples and compared it to the adjacent control samples. Furthermore, the tumorigenic and invasion potential of paxillin-expressing cancer cells was also assessed.

MATERIALS AND METHODS

Sample collection

The cervical cancer biopsies were obtained from the patients (n=15) at the time of surgery in the Gynaecology Department at our hospital. We also collected the non-malignant cervical tissues (n=15) after getting approval from the patients. Cervical cancer patients details: High grade; well-differentiated squamous cell carcinoma

(WDSCC). Both normal and cervical cancer tissues undergone histopathological examination by haematoxylin-eosin staining. The tissues were then washed in ice-cold PBS, dried and frozen in liquid nitrogen as described previously (Pillai *et al*., 1998).

The obtained cancer and the corresponding control tissues were thoroughly washed with PBS solution containing antibiotics and incubated overnight in DMEM/F12 (GIBCO) with penicillin (500 U/mL) and streptomycin (500 µg/mL). Tissues were enzymatically digested for 1 h in PBS solution containing 1.5 mg/mL of collagenase and 20 µg/mL of hyaluronidase. Cell monolayers were prepared and serially passaged. Cell culture was performed in T-75 flasks or Corning cell culture dishes in DMEM with 10% FBS and antibiotics at 37ºC in a humidified 5% CO_2 and 95% air atmosphere.

RT-PCR analysis

By using RNAeasy kit from Qiagen, total RNA extraction was performed according to the manufacturer's protocol and RT-PCR was performed in Biorad iCycler machine. The primers sequences were used as mentioned previously (Chen *et al*., 2013; Yang *et al*., 2015). The following parameters were employed for RT-PCR: initial denaturation 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 15 s, and extension at 70°C for 15 s. The amplicons were visualized by 2% agarose gel electrophoresis and the relative mRNA expression levels were normalized to the housekeeping gene GAPDH.

Analysis of telomerase expression

We used a modified TRAP (Telomerase Repeat Amplification Protocol) method and the assay was performed as described (Yang *et al*., 2015) to detect telomerase activity.

Transient transfection

Cells were seeded into a 6-well plate with a density of $1x10^6$ cells/well. After 24 h incubation transient transfection was performed. To 200 µL of Opti-MEM (Invitrogen) 4 µL of transfection reagent Lipofectamine and 3 µg of paxillin m-GFP plasmid DNA were added and incubated at room temperature. After 15 minutes of incubation, the reaction mixture was added to the cells. After 24 h of incubation, the cells were prepared for further experiments. The paxillin m-GFP plasmid was obtained from Dr Xang laboratory, Xinjiang Medical University.

Immunostaining

Cells grown on poly-L-Lysine-coated coverslips for 24 h were fixed in 4% PFA (paraformaldehyde) in 1× PBS for 5 min at room temperature. The cells were then incubated with 0.1 M glycine for 5 min, washed with PBS and incubated with FITC-conjugated anti-paxillin antibody for 30 minutes. The unbound antibodies were removed by further wash with PBS and coverslips were mounted on the slides and sealed. Cells were analyzed using a confocal microscope under 60x objective.

Western blot analysis

Protein extraction and separation was performed as previously (Teng *et al*., 2010). Primary antibodies: rabbit anti-paxillin, 1:500 (Cell Signaling) and mouse anti-GAPDH, 1:1000 (ProteinTech) were used.

In vitro cell culture assays

Soft agar assay. Cells were cultured in 6-well plates with a range of 2×10^5. The bottom surface of the 6 well plates was covered with 2 mL of 6% agar containing: DMEM/F12 and 10% FBS. After solidification, again 2 mL of 0.3% agar mixture containing DMEM/F12 and 10% FBS was poured over it, incubated for 3 weeks at 37°C. Finally, crystal violet (0.005%) staining was performed to visualize and count the colonies.

Matrigel invasion assay was performed according to the protocol described previously (Ho *et al*., 2007).

Statistical analysis

The values presented are mean ± S.D. and Student's *t*-test was performed to compare the differences. All *P*-values mentioned are 2-sided and the *P*-value lower than 0.05 (significant) and 0.01 (highly significant) were considered as statistically significant.

RESULTS

Cervical cancer samples were positive for E6 and E7

The surgically obtained cervical cancer (S1, S2, S3, S4, S5) and normal tissue samples (designed as C) were subjected to DNA isolation and the DNA integrity was checked with β-globin primers by PCR amplification. The integrity of the entire amplified DNA sequence was good (550 bp). Further, the intact DNA samples underwent PCR amplification with HPV, HPV-16, E6 and E7 consensus primers. We observed that almost all cervical cancer DNA samples were HPV- (440 bp) and HPV-16- (210 bp) positive (Fig. 1A). Further, these DNA samples showed the expression of oncogenes E6 (514 bp) and E7 (297 bp). However, the control samples were negative for the HPV, HPV-16 and oncogenes. Out of 5 samples, 4 DNA samples showed higher E6 expression whereas the S2 sample alone showed moderate positivity for E6 (Fig. 1A). The amplicon size of all the PCR products was compared with 1 kb DNA ladder. Figure 1B presents the quantitative graph representing the number of samples with proper DNA integrity and oncogenes expression. Importantly, we also found enhanced expres-

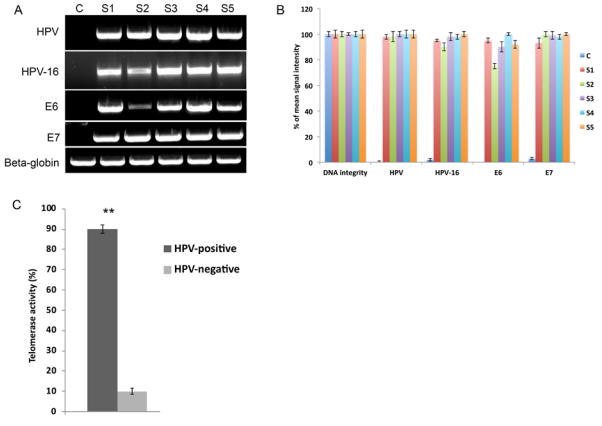

Figure 1. Assessment of human papillomavirus (HPV) presence and oncogenes expression in cervical cancer samples.
PCR amplification (**A**) and representative graph (**B**) showing the samples positivity towards HPV, HPV-16, and E6 and E7 genes. (**C**) Telomerase expression in control and cervical cancer tissues. The values plotted in the graph are the average of three independent experiments. Error bar is the value of standard deviation. ***P*<0.01.

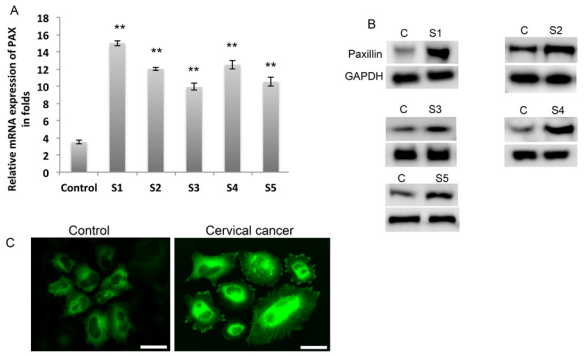

Figure 2. Analysis of *PAX* expression in control and cervical cancer cells.
RT-PCR (**A**) and Western blot (**B**) analysis showing a 4-fold increase in expression of *PAX* gene and paxillin protein in cancer cells, respectively. (**C**) Immunocytochemical paxillin staining showing increased signal in cervical cancer cells in comparison to normal cells. The scale bar represents 11 μm.

sion of telomerase in all the HPV-positive cervical cancer samples in comparison to the normal tissue (Fig. 1C, $^{**}P<0.01$). Telomerase plays a major role in cellular immortality and therefore shortening of telomeres correlates with cellular senescence. Our results confirmed that HPV infection and oncogene expression predominantly contributed to cervical cancer development.

Cervical cancer cells showed up-regulated paxillin expression

Paxillin can promote tumor cell migration and invasion (Hagel *et al.*, 2002; Li *et al.,* 2009; Bokobza *et al.*, 2010; Sen *et al.*, 2012; Zheng *et al.*, 2018). Therefore, we have examined all the isolated cervical cancer samples for paxillin expression. Using RT-PCR and western blot analysis, we found that the relative mRNA expression level for PAX gene was increased and consequently the paxillin protein level also was enhanced in all the cervical cancer cells obtained from 5 different samples (Fig. 2A and 2B). The mRNA and protein expression level of paxillin was significantly higher in comparison to the adjacent control cells. We further investigated the staining pattern of paxillin in the control and cervical cancer cells. The immunostaining showed an enhanced expression of paxillin in cervical cancer cells with much stronger signal intensity than in the control cells (Fig. 2C).

Enhanced PAX expression promotes *in vitro* tumor formation and invasion

As all the cervical cancer samples showed elevated paxillin expression, all of them were subjected to clone formation assay. Figure 3A is the representative picture showing that cervical cancer cells with higher paxillin expression formed remarkably more colonies on soft agar plate than the control cells. The relative number of clones generated by paxillin-over-expressing cervical cancer cells was significantly higher as presented in the quantification graph (Fig. 3B, $^{*}P<0.04$; $^{**}P<0.01$). We further performed a confirmatory experiment, where the control cells were transiently transfected with PAX-mGFP overexpression cassette and again clone formation efficiency was evaluated. We observed that PAX-mGFP transfected cells formed more colonies on soft agar plate (Fig. 3C & 3D), compared to the cells transfected with empty vector. In addition, our Matrigel invasion evaluation showed that paxillin over-expressing

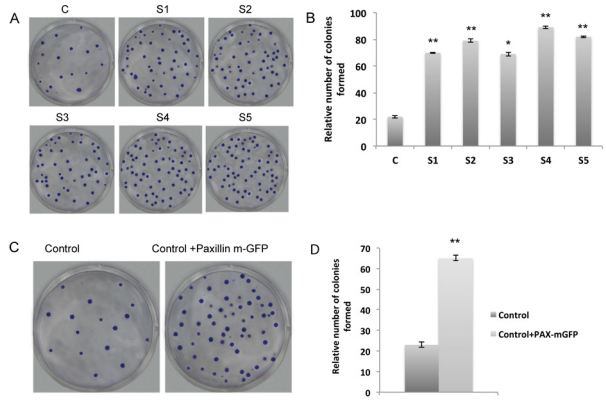

Figure 3. Cells with upregulated PAX are highly clonogenic.
Soft agar assay (**A and C**) comparing the efficiency of clone formation between control, cervical cancer cells and PAX-mGFP transfected cells. (**B and D**) are the representative quantifications showing the total number of generated colonies. The values presented in the graph are the average values from two independent experiments. Error bar represents standard deviation. $^{*}P<0.05$; $^{**}P<0.01$.

cervical cancer cells had a high capability of invading the Matrigel (Fig. 4, $^{**}P<0.01$) and thus confirmed the importance of paxillin in the tumor initiation, progression and invasion.

DISCUSSION

The malignancy of the cervix is called uterine cervical cancer and there are mainly two types of the disease: adenocarcinoma which develops from the endocervix mucus-producing gland cells and squamous cell carcinoma which originates from the squamous cells of the ectocervix. More than 90% of the cervical cancers worldwide are associated with HPV infection, which is the primary risk factor of cervical cancer (Parkin, 2002; zur Hausen, 1991; Muñoz *et al.*, 2003). The oncoproteins E6 and E7 produced by HPV subtypes 16/18 were shown to inhibit the tumor suppressor genes p53 and Rb, respectively (Yang *et al.*, 2015). As a result, the regulation of apoptosis and cell cycle events is interrupted which leads to compromised apoptosis, rapid proliferation and neoplastic transformation in the affected cells. Therefore, the prevalence of E6 and E7 are crucial for the pathogenesis of HPV-related cervical cancer, as they are involved in the immortalization and malignant transformation of the HPV-infected cells (Kaufman *et al.*, 1997; Zehbe *et al.*, 1997). It was reported that 64% of cervical cancer cases are predominantly associated with HPV-16 subtype

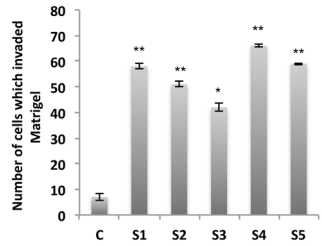

Figure 4. Matrigel invasion assay showing that *PAX* over-expressing cervical cancer cells are highly capable of invading the Matrigel.
The values presented are the averages from three independent experiments. Error bar represents standard deviation.

(Das *et al*., 1992). In our study, all the surgically obtained the cervical cancer samples contained HPV-16 subtype and almost all these biopsies were characterized by the expression of E6 and E7 oncoproteins. Therefore, the presence of these oncoproteins is more than enough to trigger an uncontrolled cell proliferation by escaping apoptosis and thus increases the risk of cervical cancer progression. The accelerated cell proliferation and compromised apoptosis are the results of the inactivation of p53 and pRb by E6 and E7, respectively (Werness *et al*., 1990; Yang *et al*., 2015). Studies reported that differential telomerase expression correlates with different grades of cancer (Das *et al*., 1992; Yang *et al*., 2015). In line with the previous findings, we observed significantly increased telomerase expression in HPV-16-infected cervical cancer biopsies. Therefore, we speculate that the presence of HPV-16 may be associated with an enhanced expression of telomerase in cancer tissues.

Paxillin is a multifunctional focal adhesion adapter protein and plays an important role in cancer growth and metastasis by integrating different signalling and growth factors involved in the cell migration (Das *et al*., 1992). Different studies in prostate, lung and bone cancer showed that paxillin was highly expressed in cancer tissues when compared to the normal tissues. This significantly overexpressed paxillin promoted tumor cell proliferation, migration and invasion (Hagel *et al*., 2002; Chen *et al*., 2013; Zheng *et al*., 2018). However, the clinical significance of paxillin in cervical cancer is still unknown. We found significantly higher mRNA expression and protein levels of paxillin in cervical cancer tissues than in normal tissues. Paxillin is a focal adhesion protein which co-ordinates the multiple signals between integrins and extracellular matrix to promote cytoskeleton remodelling for cell adhesion and migration (Turner, 2000; Li *et al*., 2009; Bokobza *et al*., 2010). The increased expression of paxillin may pave the way for increased cell adhesion, motility, proliferation and invasion. Consequently, our data showed that paxillin over-expressing cervical cancer cells have a high capability of Matrigel invasion. The *in vitro* colony formation assay further confirmed that over-expression of paxillin promotes cancer colony formation. In a control experiment, we transiently transfected normal cervical cells with paxillin-mGFP over-expression cassette. Again, the transfected cells showed higher colony formation potential when compared to the control which contained only the empty vector. Therefore, the underlying mechanism of paxillin-mediated tumorigenesis and invasion needs to be elucidated in the future. Another study revealed that phosphorylation of paxillin is in cell adhesion. The overexpression of paxillin correlated with enhanced expression of protein tyrosine phosphatase (LMWPTP), tumor recurrence and poor survival of patients (Ruela-de-Sousa *et al.,* 2016). In gastric cancer, upregulated paxillin was associated with aggressive tumor regeneration, metastasis and overall poor survival (Chen *et al*., 2013; Zheng *et al*., 2018). In addition, knockdown of *PXN* in gastric cancer tissue/cell lines inhibited cancer cells proliferation and migration, thus confirming the role of paxillin in gastric cancer progression (Chen *et al*., 2013).

In summary, we demonstrated that the presence of high-risk HPV-16 and its oncoproteins (E6/E7) is involved in cervical cancer progression and invasion. Further findings suggest that paxillin may act as an important prognostic factor for cancer patients and plays a crucial role in tumor regeneration. Hence, these findings would definitely helps to reveal the factors and other signaling pathways involved in the paxillin phosphorylation/paxillin-mediated uterine cervix tumorigenesis. Elucidating such a detailed mechanism would certainly improve the cancer treatment approaches in order to increase the survival of the patients.

Conflict of interest

All authors declare no conflict of interest.

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