Paxillin promotes tumor progression and predicts survival and relapse in oral cavity squamous cell carcinoma by microRNA-218 targeting

De-Wei Wu, Chun-Yi Chuang1,2, Wea-Long Lin1,3, Wen-Wei Sung1, Ya-Wen Cheng and Huei Lee

Graduate Institute of Cancer Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University, Taipei 115, Taiwan, Republic of China, 1School of Medicine, Chung Shan Medical University, Taichung 402, Taiwan, Republic of China and 3Department of Otolaryngology and 1Department of Pathology, Chung Shan Medical University Hospital, Taichung 402, Taiwan, Republic of China

*To whom correspondence should be addressed. Graduate Institute of Cancer Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University, Room 5, 12th Floor, No. 3 Park Street, Taipei, Taiwan, Republic of China. Tel: +886 2 27361661 ext. 7616; Fax: +886 2 22558562; Email: hl@tmu.edu.tw

High-risk human papillomavirus (HPV) 16-infected oral cavity squamous cell carcinoma (OCSCC) differs significantly from non-HPV-infected OCSCC. However, the molecular pathogenesis of HPV-infected OCSCC remains unclear. Paxillin (PXN) has been reported to promote lung tumor progression by miR-218 targeting. In addition, expression of miR-218 has been shown to be reduced by HPV16 E6 in cervical cancer. We thus asked whether PXN can promote tumor progression by E6-reduced miR-218 in OCSCC, especially in HPV-infected OCSCC. Mechanistic studies demonstrated that PXN expression increased markedly upon E6-mediated reductions in miR-218, resulting in increased colony formation and invasion capabilities in HPV-infected OCSCC cells. Among tumor specimens, HPV16/18 infection was negatively associated with miR-218 expression and positively associated with PXN expression. Kaplan–Meier and Cox regression models demonstrated that patients with low-miR-218 tumors or high-PXN tumors exhibited shorter overall survival (OS) and relapse-free survival (RFS) than those with high-miR-218 tumors or low-PXN tumors. Interestingly, HPV-infected patients with low-miR-218, high-PXN tumors and both combinations exhibited the worst OS and RFS compared with patients in their counterparts. These observations in patients were consistent with the findings from the cell model. Therefore, we suggest that PXN might be targeted to suppress tumor progression and consequently to improve outcomes in OCSCC, especially in HPV-infected OCSCC.

Introduction

Oral cavity squamous cell carcinoma (OCSCC) is the tenth most common cancer in the world. In Taiwan, OCSCC has been the fourth most common cancer in men since 2006 (1). The prognosis in OCSCC remains unsatisfactory, especially for advanced-stage tumors. Tumor metastasis is a complex process and a major cause of cancer deaths (2). Therefore, it is necessary to identify valuable molecular biomarkers to promote early diagnosis, prognosis prediction and novel therapeutic strategies for OCSCC.

Our recent report demonstrated that HPV16/18 infection was more frequently observed in Taiwanese OCSCC patients who do not exhibit cigarette smoking, alcohol drinking and betel quid chewing habits (3). Moreover, IL-10 expression has been shown to be associated with survival and relapse in HPV-infected OCSCC patients who are non-smokers, non-drinkers and non-betel quid chewers but not in those who are smokers, drinkers and betel quid chewers (3). These results support previous reports demonstrating that HPV-infected OCSCC is a distinct type of this disease compared with OCSCC without HPV infection (4). Therefore, the pathogenesis of HPV-infected OCSCC should be investigated to understand what different molecular mechanisms are distinctly involved in HPV-infected OCSCC.

Paxillin (PXN) is an adapter protein that recruits diverse cytoskeleton and signaling proteins into a complex and coordinates the transmission of downstream signaling (5). The functions of PXN seem to include the regulation of cell spreading and cell mobility (6,7). For example, PXN promotes tumor progression and metastasis in gastric, salivary adenoid cystic lung, breast and colorectal cancers (8–12). Our previous report demonstrated that PXN predicted survival and relapse in non-small cell lung cancer by miR-218 targeting. (13,14). In addition, expression of miR-218 has been shown to be reduced by HPV16 E6 in cervical cancer (15). Therefore, in the present study, we attempted to explore whether decreased miR-218 levels by HPV infection could promote PXN expression and in turn enhance tumor malignancy in HPV-infected OCSCC. We further expected that PXN expression may predict poor survival and relapse in OCSCC, especially in HPV-infected OCSCC.

Materials and methods

Study subjects

The study included 115 patients who underwent resection at the Department of Surgery at the Chung Shan Medical University Hospital in Taichung, Taiwan, between June 1998 and December 2010. The tumor type and stage of each specimen were histologically determined according to the World Health Organization’s (WHO) classification system. Fifty females (43.4%) and 65 males (56.6%), including 50 non-smokers (43.4%) and 65 smokers (56.6%), 63 non-alcohol drinkers (54.7%) and 52 patients with alcohol-drinking habits (45.5%), 57 non-betel nut chewers (49.6%) and 58 patients who habitually chew betel nuts (50.4%), were included. The tumors include 60 tongue sites (52.1%), 43 oral mucosal sites (37.3%) and 12 other sites (10.6%), as well as 61 early-stage (stage I and II; 53.0%) and 54 late-stage (stage III and IV; 47.0%) malignancies (Supplementary Table 1, available at Carcinogenesis Online). The IRB protocol CS07159 was approved by Chung Shang Medical University Hospital.

Cell lines

SAS, TW2.6, HSCC3, GN, SCC4 and SCC25 oral cancer cell lines were maintained in DMEM.Cell lines were kindly provided by Dr. T.C. Lee, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan (16). The GNM cell line was kindly provided by Dr. M.Y. Chou, Department of Dentistry, Chung Shan Medical University, Taichung, Taiwan (17). Cells were cultured and stored according to the suppliers’ instructions and used at passages 5–20. Once resuscitated, cell lines were routinely authenticated (once every 6 months; cells were last tested in December 2011) through cell morphology monitoring, growth curve analysis, species verification by isozymology and karyotyping, identity verification using short tandem repeat profiling analysis and contamination checks.

Plasmid constructs and transfection

HPV16 E6 were kindly provided by Dr J.H. Chang Tsai from the Institute of Medical and Molecular Toxicology at Chung Shan Medical University, Taichung, Taiwan. The RNA interference target sequences for HPV18 E6 (RNAi) (GACTCTGTGTATGGAGACA) have been previously verified (13). The methods were as described previously (3, 13). PXN (TRCN0000123138) RNAi were purchased from the National RNAi Core Facility, Academia Sinica, Taiwan. Different concentrations of expression plasmids were transiently transfected into lung cancer cells (1 × 10⁶) using the Turbofect reagent (Fermentas). After 48 h, the cells were harvested, and whole cell extracts were assayed in subsequent experiments.

miR-218 precursor and inhibitor transfection

The transfection of miR-218 precursor and inhibitor was described previously (13,18–20). In brief, cells were grown to confluence in six-well plates. miR-218 precursor (Pre-miR-218) (20–40nM per well) (AM17100; Ambion), miR-218 inhibitor (AM17000; 40–80nM per well) (Ambion) and their negative controls

Abbreviations:

OCSCC, oral cavity squamous cell carcinoma; OS, overall survival; PXN, paxillin; RFS, relapse-free survival.

Carcinogenesis vol.35 no.8 pp.1823–1829, 2014
doi:10.1093/carcin/bgu102
Advance Access publication April 29, 2014

© The Author 2014. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
(AM17110, AM17010; Ambion) were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s protocol. The transfection efficiency of miR-218 inhibitor and precursor was determined by mature miR-218 expression levels, which were evaluated by real-time PCR.

**Real-time quantitative RT–PCR analysis**

Total RNA was extracted by homogenization in 1-ml TRIzol reagent, followed by chloroform extraction and isopropanol precipitation. A 3-µg sample of total RNA from lung tumor tissues was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies) and oligo(dT) 12 primer. The following primer sequences were used for the amplification of the PXN gene: forward primer, 5'-ACGTCCTACAGGCTCCCATACAA-3' and reverse primer, 5'-AGCAGCGGTCACTGGTTCA-3'.

**Western blotting**

The cells were lysed with lysis buffer containing 0.5% NP-40, 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, and protease inhibitor cocktail (Roche, Indianapolis, IN). After 3 min of lysis, the cell debris was removed by centrifugation, and the protein concentration was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were separated onto SDS-PAGE gels and then transferred from the gel onto a polyvinylidenedifluoride membrane (PerkinElmer, Norwalk, CT). After blocking, the membranes were incubated with antibody at 4°C overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 1 h. The blots were observed using an enhanced chemiluminescence kit (PerkinElmer).

**Doubling time assay**

The cells were seeded in a 35-mm dish and cultured for 24, 48, 72, 96, 120 and 144 h, and then the cell number at each culture time point was counted for calculation of the doubling time.

**Boyden chamber invasion assay**

A Boyden chamber with a pore size of 8 µm was used for the in vitro cell invasion assay. Cells (1 × 10⁴) in 0.5% serum containing culture medium (HyClone, Ogden, UT) were plated in the upper chamber, and 10% fetal bovine serum was added to the culture medium in the lower chamber as a chemoattractant. The upper side of the filter was covered with 0.2% Matrigel (Collaborative Research, Boston) and diluted in RPMI-1640. After 16 h, the cells on the upper side of the filter were removed, and cells that adhered to the underside of the membrane were fixed in 95% ethanol and stained with 10% Giemsa dye. The number of invasive cells was counted. Ten contiguous fields of each sample were examined to obtain a representative number of the cells that invaded across the membrane.

** Colony formation assay**

For the colony formation assay, 500 transfected cells were plated in a 6-well plate for 10 days. Colonies were fixed with methanol/acetic (1:1) and stained with crystal violet (1 mg/ml).

**Real-time PCR for miR-218 analysis**

DNase I-treated total RNA (10 µg) was subjected to microRNA RT–PCR analysis with the TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems), miRNA Assays (Applied Biosystems), and a Real-Time Thermocycler 7500 (Applied Biosystems). RNU6B was used as the small RNA reference housekeeping gene. For The miR-218 mRNA levels in lung tumors that were higher than the median value were defined as 'high', whereas levels lower than the median value were defined as 'low'.

**Immunohistochemical analysis**

Immunohistochemical analysis was used to detect PXN expression. Specimens were formalin-fixed and paraffin-embedded. In brief, 3-µm sections were cut, mounted on glass and dried overnight at 37°C. All sections were then deparaffinized in xylene, rehydrated through alcohol and washed in phosphate-buffered saline. This buffer was used for all subsequent washes. Sections for PXN detection were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0). An anti-PXN antibody (NeoMarker, Fremont, CA) was used as the primary antibody for 60 min at room temperature, followed by a horseradish peroxidase-conjugated secondary antibody for 60 min at room temperature. The sections were then incubated with 0.05% H₂O₂ in methanol for 10 min at room temperature to block endogenous peroxidase activity. After incubation with a biotinylated goat anti-mouse secondary antibody (Avidin Biotin System, Vector Laboratories), the sections were incubated with 0.02% 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) for 5 min at room temperature. Finally, the sections were counterstained with hematoxylin, dehydrated, mounted with Permount, and examined under a microscope. Sections were scanned, and the intensity of PXN expression was scored for each tissue sample.

**Fig. 1.** PXN is deregulated by HPV E6, and cell proliferation and oncogenic potential are enhanced. (A) The expression levels of PXN were evaluated by western blotting in six oral cancer cell lines. (B) HPV18 E6 was knocked down in GNM cells by HPV18 E6 RNAi (at various doses). HPV16 E6 was overexpressed using an E6 overexpression plasmid (at various doses) in TW2.6 cells. The PXN mRNA level was determined by real-time PCR, and the levels of HPV16 E6, p53, PXN and β-actin were evaluated by western blotting. β-Actin was used as a protein loading control. NC, non-specific control. (C) The proliferation rate of TW2.6 cell with or without E6 overexpression plasmid and PXN-knockdown plasmid is shown in the top, and the doubling time is shown at the bottom. (D) Representative colony formation and the number of invading cells for TW2.6 with or without E6 overexpression or PXN RNAi treatment and for GNM cells with E6 or PXN RNAi treatment. (E) The colony formation and number of invasive cells were evaluated for TW2.6 with or without E6 overexpression or PXN RNAi treatment and for GNM cells with E6 or PXN RNAi treatment. NC, non-specific control; miR-218i, miR-218 inhibitor. In all experiments, the relative mRNA level in the NC controls was arbitrarily assigned as 1.
Paxillin promotes tumor progression in oral cancer

by a conventional streptavidin peroxidase method (DAKO, LSAB Kit K675, Denmark). Signals were developed with 3,3'-diaminobenzidine for 5 min and counterstained with hematoxylin. Negative controls were obtained by incubation without the primary antibody. The intensities of signals were evaluated independently by three observers. Immunostaining scores were defined as the cell staining intensity (0 = nil; 1 = weak; 2 = moderate; and 3 = strong) multiplied by the percentage of labeled cells (0–100%), leading to scores from 0 to 300. A score more than 150 and include 150 itself were rated as 'high' immunostaining, whereas a score less than 150 was rated as 'low'.

Statistical analysis
Statistical analysis was performed using the SPSS statistical software program (version 18.0; SPSS). The association between clinical parameters and PXN protein expression was analyzed by the chi-square test. Survival plots were generated using the Kaplan–Meier method, and differences between patient groups were determined by the log-rank test. Multivariate Cox regression analysis was performed to determine the OS and RFS. The analysis was stratified for all known variables and protein expression.

Results
E6-mediated PXN promotes colony formation and invasion in oral cancer cells
PXN has been shown to be derepressed by E6-mediated reductions in miR-218 expression in cervical cancer and lung cancer (13,15). We thus expected that PXN expression may be elevated by E6-mediated reductions in miR-218 and may consequently promote tumor growth and metastasis. HPV18 E6-positive GNM and HPV16/18 E6-negative SCC25, HSC3, TW2.6, SAS and SCC4 oral cancer cells were collected to examine whether different PXN expression levels could be observed between E6-positive and E6-negative cells. Western blotting data revealed that PXN expression in E6-positive GNM cells was higher than in E6-negative SCC25, HSC3, TW2.6 and SAS cells; however, SCC4 cells exhibited relatively high PXN expression compared with the former four E6-negative oral cancer cells (Figure 1A). We next explored whether HPV16/18 E6 could promote PXN expression. E6-negative TW2.6 cells were transfected with a HPV16 E6 cDNA plasmid, and HPV18 E6 was knocked down in HPV18 E6-positive GNM cells using RNAi transfection. Western blotting revealed that PXN expression was dose-dependently increased by ectopic E6 expression in TW2.6 cells and reduced by E6 silencing in GNM cells (Figure 1B). The changes in PXN mRNA and protein expression were consistent with E6-knockdown or -overexpression (Figure 1B). This result suggests that PXN expression is modulated by E6 predominately through the regulation of PXN transcription. We examined the possibility that E6-mediated PXN expression could promote cell proliferation. The doubling time of E6-knockdown and PXN-knockdown GNM cells was significantly increased compared with parental cells with non-specific RNAi transfection (NC) (28.6 h versus 21.5 h for E6-knockdown; 29.2 h versus 21.5 h for PXN-knockdown; Figure 1C right panel). Conversely, the doubling time was dose dependently reduced by E6 overexpression in TW2.6 cells compared with parental cells with empty vector transfection (VC) (32.5 h versus 23.5 h; Figure 1C left panel). Interestingly, the doubling time of E6-overexpressing TW2.6 cells was nearly restored by PXN-knockdown (31.5 h versus 32.5 h; Figure 1C). We further tested whether E6-modulated PXN attenuate colony formation and invasion. Representative colony formation and Boyden chamber assay results are shown in Figure 1D. Colony formation and Boyden chamber assays demonstrated that colony formation and invasion was...
Increased more than 3-fold by ectopic E6 expression in TW2.6 cells (Figure 1E). However, the increase in colony formation and invasion by ectopic E6 expression was nearly rescued by PXN-knockdown in E6-overexpressing TW2.6 cells. On the other hand, colony formation and invasion decreased markedly in E6-knockdown GNM cells compared with NC cells (Figure 1E). In addition, both capabilities were unchanged by PXN-knockdown in E6-silenced GNM cells (Figure 1E). These results suggest that E6-mediated PXN expression is responsible for cell proliferation, colony formation and invasion in HPV-infected oral cancer cells.

**HPV E6-mediated PXN expression via decreasing miR-218 is responsible for colony formation and invasion in HPV-infected oral cancer cells**

miR-218 expression was significantly reduced by HPV16/18 E6 in cervical and lung cancer cells (13,15). As mentioned above, E6-mediated PXN promotes colony formation and invasion in oral cancer (Figure 1). We therefore expected that PXN elevation by E6-mediated reductions in miR-218 could be responsible for colony formation and invasion. HPV16 E6 was ectopically expressed in TW2.6 cells, and HPV18 E6 was silenced in GNM cells (Figure 2A). miR-218 expression levels were dose-dependently decreased and increased in E6-overexpressing TW2.6 and E6-knockdown GNM cells, respectively (Figure 2A). We further examined whether PXN expression levels could be modulated by a miR-218 precursor and miR-218 inhibitor. As expected, PXN protein and mRNA expression levels were dose-dependently decreased and increased by miR-218 precursor and inhibitor treatment in TW2.6 and GNM cells, respectively (Figure 2B). To explore whether PXN expression could be upregulated by E6 via E6-mediated reductions in miR-218, GNM cells were transfected with E6 RNAi or miR-218 inhibitor or both combinations. PXN protein and mRNA level were markedly reduced by E6-knockdown in GNM cells, but slightly elevated by miR-218 inhibitor compared with NC cells (Figure 2C). However, PXN mRNA and protein levels were unchanged in GNM cells with E6 RNAi plus miR-218 inhibitor treatments (Figure 2C). These results clearly indicate that upregulation of PXN by E6 is predominantly mediated through E6-mediated reductions in miR-218. To explore whether PXN derepression by E6-mediated reductions in miR-218 could be responsible for colony formation and invasion, GNM cells were treated with E6 RNAi, miR218 inhibitor or PXN RNAi alone or with combinations of two or three of the former agents. The colony formation and invasion capabilities decreased markedly in E6-knockdown GNM cells. However, the inhibition of both behaviors by E6 silencing could be restored by further miR-218 silencing in GNM cells. Intriguingly, the restoration of both capabilities by miR-218 silencing in E6-knockdown GNM cells was nearly diminished by PXN-knockdown (Figure 2D). These results clearly indicate that E6-mediated PXN expression via decreasing miR-218 is responsible for colony formation and invasion in HPV-infected oral cancer cells.

**miR-218 expression levels were negatively correlated with HPV16/18 infection and PXN expression in OCSCC tumors**

The presence of HPV16/18 DNA in OCSCC tumors was obtained from our previous study and further confirmed by p16 immunostaining (Supplementary Table 2, available at Carcinogenesis Online (3)). miR-218 expression levels in OCSCC tumors were determined by real-time PCR analysis. Data showed that the prevalence of low miR-218 level was significantly higher in HPV16/18 DNA-positive tumors than in HPV16/18 DNA-negative tumors (62% versus 38%, \( P = 0.012, \) Table I). However, miR-218 level was not associated with clinical parameters including age, genders, smoking status, betel quid chewing, alcohol drinking, tumor site and stage (Table I). Next, tumor paraffin sections from 115 OCSCC patients were collected to measure PXN expression using immunohistochemistry analysis.

### Table I. The correlation between clinical parameters with miR-218 and PXN in OCSCC patients and correlation between HPV 16/18 infection and the expression of miR-218 and PXN protein

<table>
<thead>
<tr>
<th></th>
<th>miR-218(^a)</th>
<th></th>
<th>PXN(^b)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Low</td>
<td>High</td>
<td>( P )</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;55</td>
<td>66</td>
<td>35 (53)</td>
<td>31 (47)</td>
<td>0.518</td>
</tr>
<tr>
<td>( \geq 55 )</td>
<td>49</td>
<td>23 (47)</td>
<td>26 (53)</td>
<td>0.647</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>50</td>
<td>24 (48)</td>
<td>26 (52)</td>
<td>0.226</td>
</tr>
<tr>
<td>Male</td>
<td>65</td>
<td>34 (52)</td>
<td>31 (48)</td>
<td>0.157</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>50</td>
<td>22 (44)</td>
<td>28 (56)</td>
<td>0.514</td>
</tr>
<tr>
<td>Smokers</td>
<td>65</td>
<td>36 (55)</td>
<td>29 (45)</td>
<td>0.230</td>
</tr>
<tr>
<td>Alcohol drinking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>63</td>
<td>28 (44)</td>
<td>35 (56)</td>
<td>0.157</td>
</tr>
<tr>
<td>Yes</td>
<td>52</td>
<td>30 (58)</td>
<td>22 (42)</td>
<td>0.015</td>
</tr>
<tr>
<td>Betelnut</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>57</td>
<td>27 (47)</td>
<td>30 (53)</td>
<td>0.067</td>
</tr>
<tr>
<td>Yes</td>
<td>58</td>
<td>31 (53)</td>
<td>27 (47)</td>
<td>0.230</td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>66</td>
<td>26 (43)</td>
<td>34 (57)</td>
<td>0.012</td>
</tr>
<tr>
<td>Buccal</td>
<td>43</td>
<td>26 (61)</td>
<td>17 (39)</td>
<td>0.159</td>
</tr>
<tr>
<td>Lip, gingiva, plate</td>
<td>12</td>
<td>6 (50)</td>
<td>6 (50)</td>
<td>0.159</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I, II</td>
<td>61</td>
<td>27 (44)</td>
<td>34 (56)</td>
<td>0.159</td>
</tr>
<tr>
<td>III, IV</td>
<td>54</td>
<td>31 (57)</td>
<td>23 (43)</td>
<td>0.230</td>
</tr>
<tr>
<td>HPV 16/18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>55</td>
<td>21 (38)</td>
<td>34 (62)</td>
<td>0.012</td>
</tr>
<tr>
<td>Positive</td>
<td>60</td>
<td>37 (62)</td>
<td>23 (38)</td>
<td>0.159</td>
</tr>
<tr>
<td>miR-218</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>58</td>
<td>12 (21)</td>
<td>46 (79)</td>
<td>0.514</td>
</tr>
<tr>
<td>High</td>
<td>57</td>
<td>24 (48)</td>
<td>22 (52)</td>
<td>0.514</td>
</tr>
</tbody>
</table>

\(^{a}\)miR-218 low: 0.01–13.5; miR-218 high: 13.6–671.1.

\(^{b}\)PXN expression score: [percentage of stained cells x staining intensity (0–3)]. The score of more than 150 and include 150 itself were regarded as ‘high’ immunostaining, whereas a finding of fewer than 150 was considered ‘low’.
Paxillin promotes tumor progression in oral cancer

The representative immunostaining results are shown in Figure 3A. The association of PXN expression with the clinicopathological parameters is shown in Table I. Our data demonstrated that high PXN expression was more frequently observed in HPV16/18 DNA-positive tumors than in HPV16/18 DNA-negative tumors (63% versus 44%, P = 0.034). High PXN expression more commonly occurred in low-miR-218 tumors than in high-miR-218 tumors (79% versus 29%, P < 0.001; Table I). However, PXN expression was not associated with patient characteristics, including age, genders, cigarette smoking, alcohol drinking, betel quid chewing, tumor sites and stage (Table I). Therefore, the association of miR-218 with HPV infection and PXN expression in OCSCC tumors seems to support the findings of the cell model.

PXN expression may independently predict poor overall survival and relapse-free survival in OCSCC, especially in HPV-infected OCSCC

Kaplan–Meier analysis indicated that patients with HPV16/18 infection exhibited shorter relapse-free survival (RFS) than those not infected with HPV16/18 (P < 0.001; Figure 3B). However, the prognostic value of HPV16/18 infection for overall survival (OS) was not observed in this study population (Figure 3B). Interestingly, patients with high PXN expression exhibited shorter OS and RFS than those with low PXN expression (P < 0.001 for OS and RFS; Figure 3B). Patients with low-miR-218 tumors exhibited shorter OS and RFS periods than those with high-miR-218 tumors (P < 0.001 for OS and RFS; Figure 3B). Patients with HPV16/18 infection combined with high PXN expression exhibited shorter OS and RFS than those with no HPV16/18 infection combined with low PXN expression (P = 0.001 for OS and P < 0.001 for RFS; Figure 3B). In addition, the prognostic significance of low miR-218 combined with HPV infection, high PXN and both combinations was observed compared with their counterparts (P < 0.05 for HPV+/miR-218− versus HPV−/miR-218+; miR-218+/PXN− versus miR-218−/PXN+ and HPV+/miR-218+/PXN+ versus HPV−/miR-218−/PXN−; Figure 3C). Cox regression analysis further demonstrated that HPV16/18 infection was associated with RFS, not OS (Table II). Patients with low-miR-218 tumors exhibited poorer OS and RFS than those with high-miR-218 tumors (P = 0.005 for OS; P = 0.002 for RFS; Table II). PXN expression was also associated with OS and RFS in OCSCC (P = 0.001 for OS; P = 0.001 for RFS; Table II). To explore whether HPV-infected patients with high PXN expression exhibited the worst OS and RFS, patients were divided into four categories using the two parameters (PXN and HPV). Patients with HPV+/PXN+ tumors exhibited the worst OS and RFS among the four categories (HR, 3.53, P = 0.004 for OS; HR, 5.901, P < 0.001; Table II). In the other combinations of HPV/miR-218, miR-218/PXN and HPV/miR-218/PXN, the highest HR value of HPV+/miR-218+, miR-218+/PXN+ and HPV+/miR-218+/PXN+ was observed among the subgroups (OS: HR, 2.98, P = 0.010 for HPV/miR-218; HR, 3.76, P = 0.001 for miR-218/PXN; HR,

Fig. 3. When HPV16/18 is present, miR-218 expression is low and PXN expression is high, the clinical outcomes in OCSCC patients are poor. (A) Representative immunostained images demonstrating high PXN expression (upper) and low PXN expression. (B) Overall survival (OS) and relapse-free survival (RFS) curves for all studied patients with positive or negative HPV16/18 infection (left), high or low miR-218 expression (middle), and high or low PXN expression. (C) Combined effect of HPV16/18 infection, miR-218 and PXN expression on OS and RFS.
In addition, midkine-mediated expression of PXN may be targeted by miR-218 in bronchial epithelial cells exposed to cigarette smoking (14,15). The involvement of E6-mediated PXN expression in OCSCC tumorigenesis may support a role for HPV infection in lung cancer development as of yet not recognized (3,4). The link between HPV infection and OCSCC has been suggested previously, but the association of HPV infection with lung cancer development is as of yet not recognized (3,4). The involvement of E6-mediated PXN expression in OCSCC tumorigenesis may support a role for HPV infection in lung tumor progression and metastasis via induction of PXN expression by E6-mediated reductions in miR-218 (13). In addition, E6-mediated PXN expression was responsible for colony formation and invasion in HPV-infected OCSCC cells, revealing the possibility that PXN or HPV/miR-218/PXN may be targeted by miR-218 in bronchial epithelial cells exposed to cigarette smoking (14,15). The association between HPV and OCSCC, but few reports have explored the molecular mechanism of HPV infection in OCSCC tumorigenesis (4,22–25). In this study, we report for the first time that E6-mediated PXN is responsible for tumor progression in HPV-infected OCSCC. This finding was consistent with a previous report demonstrating that PXN overexpression promoted cell adhesion in tongue squamous cancer cells (26). In addition, midkine-mediated migration and invasion in head and neck cancer cells is predominantly mediated through the activation of PXN-induced MMP2 expression (27). The prognostic significance of PXN on OS and RFS was suggested by the Kaplan–Meier and Cox-regression model in this study population (Figure 3 and Table II). Interestingly, the prognostic value of PXN on RFS was observed in HPV-non-infected OCSCC patients (Supplementary Figure 1 and Supplementary Table 3, available at Carcinogenesis Online). However, the prognostic value of PXN on OS was not observed in HPV-non-infected OCSCC patients (Supplementary Figure 1 and Supplementary Table 3, available at Carcinogenesis Online). In addition, HPV infection was also associated with RFS in this study population (Figure 3B and Table II). These results suggest that HPV, PXN or both may promote tumor recurrence risk but not affect OS. This result was consistent with previous studies demonstrating that HPV infection may be related to an increased risk of distant metastasis and poor survival in head and neck cancer (3).

HPV infection has been shown to be involved in the development of human cancers including cervical and oropharyngeal carcinomas (28–31). PXN was shown to promote transformation and facilitate anchorage-independent cell growth through PXN interaction with HPV E6 oncoprotein, and the LD motifs of PXN served as docking sites for the binding of E6 oncoprotein (32–34). In the present study, PXN overexpression due to E6-mediated reductions in miR-218 may play a role in tumor progression and metastasis of HPV-infected OCSCC. These results are consistent with a previous report indicating that PXN overexpression due to E6-mediated miR-218 reductions promoted tumor invasion and poor outcomes.

### Discussion

We provided evidence that PXN promoted tumor invasion and in turn predicted poor survival and relapse in OCSCC, especially in HPV-infected OCSCC. These results are consistent with our previous report, which demonstrated that PXN overexpression due to E6-mediated reductions in miR-218 promoted tumor invasion and predicted survival and relapse in non-small cell lung cancer (13). The idea for our previous and present studies was prompted by the observation that miR-218 is deregulated by E6 in bronchial epithelial cells exposed to cigarette smoking (14,15). The link between HPV infection and OCSCC has been suggested previously, but the association of HPV infection with lung cancer development is as of yet not recognized (3,4). The involvement of E6-mediated PXN expression in OCSCC tumorigenesis may support a role for HPV infection in lung tumor progression and metastasis via induction of PXN expression by E6-mediated reductions in miR-218 (13). In addition, E6-mediated PXN expression was responsible for colony formation and invasion in HPV-infected OCSCC cells, revealing the possibility that PXN or miR-218 might be potentially targeted to suppress tumor progression and metastasis and consequently to improve outcomes in OCSCC patients.

In HPV18-immortalized genital epithelial cells and high-grade dysplastic and invasive cervical carcinoma, PXN may be associated with cervical tumor metastasis (21). Many studies have investigated the association between HPV and OCSCC, but few reports have explored the molecular mechanism of HPV infection in OCSCC tumorigenesis (4,22–25). In this study, we report for the first time that E6-mediated PXN is responsible for tumor progression in HPV-infected OCSCC. This finding was consistent with a previous report demonstrating that PXN overexpression promoted cell adhesion in tongue squamous cancer cells (26). In addition, midkine-mediated migration and invasion in head and neck cancer cells is predominantly mediated through the activation of PXN-induced MMP2 expression (27). The prognostic significance of PXN on OS and RFS was suggested by the Kaplan–Meier and Cox-regression model in this study population (Figure 3 and Table II). Interestingly, the prognostic value of PXN on RFS was observed in HPV-infected and HPV-non-infected patients (Supplementary Figure 1 and Supplementary Table 3, available at Carcinogenesis Online). However, the prognostic value of PXN on OS was not observed in HPV-non-infected OCSCC patients (Supplementary Figure 1 and Supplementary Table 3, available at Carcinogenesis Online). In addition, HPV infection was also associated with RFS in this study population (Figure 3B and Table II). These results suggest that HPV, PXN or both may promote tumor recurrence risk but not affect OS. This result was consistent with previous studies demonstrating that HPV infection may be related to an increased risk of distant metastasis and poor survival in head and neck cancer (3).
in non-small cell lung cancer (13). Therefore, we suggest that HPV infection-mediated tumor progression and metastasis is not only mediated through PXN interaction with E6 oncoprotein, but also promotes PXN transcription via E6-mediated reductions in miR-218. Therefore, we suggest that the E6/miR-218/PXN pathway might be partially involved in tumor progression and metastasis in HPV-infected OCSCC. However, the studies using the E6-transgenic and orthotopic metastatic animal models are needed to further verify the possibility.

In summary, we provided evidence demonstrating that E6-mediated PXN expression was responsible for colony formation and invasion in HPV-infected OCSCC cells. A consistent finding was also observed in HPV-infected OCSCC patients, indicating that HPV-infected patients with high PXN expression exhibited the worst survival and relapse among the four categories. Therefore, we suggest that PXN or miR-218 might be targeted to suppress tumor progression and metastasis and consequently to improve outcomes in OCSCC, especially in HPV-infected OCSCC.

Supplementary material
Supplementary Tables 1–3 and Figure 1 can be found at http://carcin.oxfordjournals.org/

Funding
National Health Research Institute (NHR96-TD-G-111-006, NHR97-TD-G-111-006) and National Science Council (100-2314-B-038-043-MY3), Taiwan, Republic of China.

Conflict of Interest Statement: None declared.

References

Received December 8, 2013; revised April 2, 2014; accepted April 16, 2014