Characterization of a Candidate Tumor Suppressor Gene 
Uroplakin 1A in Esophageal Squamous Cell Carcinoma

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Abstract

Esophageal squamous cell carcinoma (ESCC) is increasing in incidence, but the knowledge of the genetic underpinnings of this disease remains limited. In this study, we identified the tetraspanin cell surface receptor uroplakin 1A (UPK1A) as a candidate tumor suppressor gene (TSG), and we investigated its function and mechanism in ESCC cells. UPK1A downregulation occurred in 68% of primary ESCCs examined, where it was correlated significantly with promoter hypermethylation (P < 0.05). Ectopic expression of UPK1A in ESCC cells inhibited cell proliferation, clonogenicity, cell motility, and tumor formation in nude mice. Mechanistic investigations suggested that these effects may be mediated by inhibiting nuclear translocation of β-catenin and inactivation of its downstream targets, including cyclin-D1, c-jun, c-myc, and matrix metalloproteinase 7 (MMP7). Cell cycle arrest elicited by UPK1A at the G1-S checkpoint was associated with downregulation of cyclin D1 and cyclin-dependent kinase 4, whereas metastasis suppression was associated with reduction of MMP7. These findings were consistent with evidence derived from clinical samples, where UPK1A downregulation was correlated with lymph node metastasis (P = 0.009), stage (P = 0.015), and overall survival (P < 0.0001). Indeed, multivariate cyclooxygenase regression analysis showed that UPK1A was an independent prognostic factor for overall survival. Taken together, our findings define a function for UPK1A as an important TSG in ESCC development. Cancer Res; 70(21); 8832–41. © 2010 AACR.

Introduction

Esophageal cancer is one of the most common malignancies and has been ranked as the sixth leading cause of cancer death over the world (1). Throughout the world, the major type of esophageal cancer is esophageal squamous cell carcinoma (ESCC). The incidence ratio of ESCC in China varies widely compared with other common cancers, wherein the high- and low-risk regions can be as high as 500:1 (2). More than 50% of ESCC cases occurred in Asia, wherein Linzhou and the nearby counties in Henan province of Northern China have the highest incidence in the world (3, 4). Like other types of cancers, the development of ESCC is also believed as a multiple-step process caused by the accumulation of activation of oncogenes and inactivation of tumor suppressor genes (TSG). Studies also showed that there is a strong tendency toward familial aggregation in the Northern China, which suggests that genetic susceptibility may be involved in the etiology of ESCC (5). Hence, to have a better understanding of ESCC, it is important to identify these key genes, elucidate their roles, and discover new biomarkers for improving clinical management.

Downregulation is a common feature of a TSG, which can be caused by gene mutation, allele deletion, promoter hypermethylation, and posttranscriptional silencing by microRNA. Therefore, comparison of expressing profiles by cDNA microarray between tumor and nontumorous tissues and characterization of downregulated genes in tumor specimen is a useful strategy to identify TSGs. Recently, our group performed an Affymetrix cDNA microarray to compare differentially expressed genes between 10 pairs of ESCC tumors and their adjacent nontumor tissues. About 220 downregulated genes were identified including uroplakin 1A (UPK1A). Uroplakin family includes four transmembrane proteins, UPK1A (27 kDa), uroplakin 1B (28 kDa), uroplakin II (15 kDa), and uroplakin III (47 kDa), which are highly conserved in many organisms, such as human, pig, dog, mouse, and rabbit. Each of these integral membrane proteins has 16-nm particles forming two crystals hexagonally, which they constitute as the urothelial plates of the asymmetrical unit membrane in urothelium (6–8). UPK1A gene comprises eight exons and encodes a protein of 256 amino acids, which belongs to the
transmembrane 4 superfamily (TM4SF), also known as the tetraspanin family (9, 10). At first, the expression of **UPK1A** was found to be highly specific to urothelial normal tissues. However, EST databases revealed that **UPK1A** could also be found in normal genitourinary tract, uterus, and prostate (11). In addition, during carcinogenesis, the expression of uroplakins decreased markedly and sometimes even totally disappeared in invasive carcinomas (12, 13). In the present study, we studied the **UPK1A** expression status and its promoter methylation in primary ESCCs and ESCC cell lines. Functional assays with a **UPK1A** reexpressing ESCC cell line were performed to characterize the biological effects of **UPK1A** in esophageal tumorigenicity, both in vitro and in vivo. Tumor-suppressive mechanism of **UPK1A** and its potential to be a new biomarker in ESCC were also addressed.

**Materials and Methods**

**ESCC samples and cell lines**

Primary ESCC tumor tissues were collected immediately after surgical resection at Linzhou Cancer Hospital. All patients did not receive preoperative treatment. Samples used in this study were approved by the committees for ethical review of research involving human subjects at Zhengzhou University and University of Hong Kong. The Chinese ESCC cell line HKESC1 was kindly provided by Professor Srivastava (Department of Pathology, University of Hong Kong), and the other two Chinese ESCC cell lines (EC18 and EC109) were kindly provided by Professor Tsao (Department of Anatomy, University of Hong Kong). Six Japanese ESCC cell lines (KYSE30, KYSE140, KYSE180, KYSE410, KYSE510, and KYSE520) were obtained from DSMZ, the German Resource Centre for Biological Material (14). All ESCC cell lines used in this study were regularly authenticated by checking the morphology and were tested for the absence of *Mycoplasma* contamination (MycoAlert, Lonza). Cells were cultivated as previously described (15).

**Semi quantitative reverse transcription-PCR**

Total RNA was extracted from frozen ESCC tissues and cell lines by the TRIzol reagent (Invitrogen), and 2 μg of total RNA were reverse-transcribed using Advantage reverse transcription-PCR (RT-PCR) kit (Clontech Laboratories, Inc.) for first-strand complementary DNA synthesis. RT-PCR was carried out with primers for **UPK1A**, **cyclin D1**, **matrix metalloproteinase 7** (**MMP7**), **c-jun**, **E-cadherin**, and **c-myc** (Supplementary Table S1).

**Tissue microarrays and immunohistochemistry**

A total of 300 formalin-fixed and paraffin-embedded ESCC tumor specimens were kindly provided by Linzhou Cancer Hospital. According to the method described previously (16), tissue microarrays (TMA) containing 300 pairs of primary ESCC tumor samples and their corresponding nontumorous tissues (duplicate 0.6-mm tissue cores for each ESCC) were constructed.

**5-Aza-2′-deoxycytidine treatment**

To study whether demethylation could restore **UPK1A** expression in KYSE510 cells, 2 × 10⁷ cells were treated with 50 μmol/L 5-aza-dC (Sigma-Aldrich Corporation) for 3 days, changing the 5-aza-dC and the medium every 24 hours. Total RNA was then extracted, and **UPK1A** expression was detected by RT-PCR.

**Bisulfite modification and promoter methylation analysis**

Genomic DNA was treated by bisulfite and then studied by methylation-specific PCR (MSP) as previously described (15). MSP primers are listed in Supplementary Table S1.

**Tumor-suppressive function of **UPK1A****

To test the tumor-suppressive function, **UPK1A** was cloned into pcDNA3.1 vector (Invitrogen) and transfected into the ESCC cell lines KYSE30 and KYSE510. Stable **UPK1A**-expressing clones (**UPK1A-30 or **UPK1A-510**) were selected for further study. Empty vector–transfected cells (Vec-30 or Vec-510) were used as control. Cell proliferation assay, foci formation assay, and colony formation in soft agar were carried out as described previously (15). For in vivo tumorigenicity assay, 2 × 10⁵ **UPK1A**-expressing cells (**UPK1A-30 or **UPK1A-510**) and control cells (Vec-30 or Vec-510) were injected s.c. into the right and left dorsal flank, respectively, of 4- to 5-week-old nude mice (10 mice per group). Tumor formation in nude mice was monitored by measuring the tumor volume, which was calculated by the formula, \( V = 0.5 \times L \times W^2 \), over a 4-week period. To visualize the tumor structure, sections (5 μmol/L) of a paraaffin-embedded tumor were stained with H&E. All animal experiments were approved by the University of Hong Kong Committee on the Use of Live Animals in Teaching and Research (CULATR).

**Wound healing and invasion assays**

For wound healing assay, the cell layer was wounded using a sterile tip. The spread of wound closure was observed after 24 and 48 hours and photographed under a microscope. Invasion assay was performed with a chamber containing a polycarbonate membrane (8-μm pore size) and coated with a layer of extracellular matrix (Chemicon International) according to the manufacturer’s instructions. The number of cells that invaded through the Matrigel was counted in 10 fields under 20× objective lens. The experiments were repeated three times.
In vivo metastasis assay

All animal procedures were performed with the approval of CULATR. Briefly, 2 × 10⁵ cells (four groups, including Vec-30, UPK1A-30, Vec-510, and UPK1A-510) were injected i.v. through the tail vein into 4- to 5-week-old severe combined immunodeficient (SCID)-Beige mice (six mice per group). After 12 weeks, mice were euthanized. The number of tumor nodules formed on the liver surfaces was then counted. The livers and lungs were excised and embedded in paraffin for further study.

Flow cytometry

UPK1A-510 and Vec-510 cells (2 × 10⁵) were fixed in 70% ethanol and stained with propidium iodide, and DNA content was analyzed by Cytomics FC (Beckman Coulter). The results were analyzed by ModFit LT2.0 software.

Immunofluorescence

UPK1A-510 and Vec-510 cells were grown on gelatin-coated coverslips, fixed with 4% paraformaldehyde, permeabilized in PBS, which contain 0.1% Triton-X 100, and blocked with 1% bovine serum albumin. The cells were then treated with antibodies targeting β-catenin (Cell Signaling Technology) and UPK1A (Abcam) at 4°C overnight. After washing with PBS, cells were incubated with FITC-conjugated antimouse secondary antibody and Texas red–conjugated antirabbit secondary antibody at room temperature for 1 hour. Antifade 4′,6-diamidino-2-phenylindole (DAPI) solution was added, and images were captured.

Western blot analysis

Western blotting was done according to the standard protocol with antibodies UPK1A (Abcam), UPK1A (Santa Cruz Biotechnology), β-catenin (Cell Signaling Technology), E-cadherin (Santa Cruz Biotechnology), and cyclin-D1 (Cell Signaling Technology). β-Actin (Santa Cruz Biotechnology) was used as loading control.

Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Sciences 14.0 for Windows (SPSS, Inc.). χ² test or Fisher’s exact test was used to analyze the association of
UPK1A gene expression and clinicopathologic parameters. Student’s t test was used to analyze the results expressed as mean ± SD. The overall survival curve was plotted by using Kaplan-Meier analysis. The association of UPK1A gene expression, stage, and lymph node status was examined by univariable and multivariable Cox proportional hazard regression model (17). Differences were considered significant when P value was <0.05.

Results

UPK1A is frequently downregulated in ESCC

Semiquantitative RT-PCR was used to study the expression of UPK1A in nine ESCC cell lines and 100 pairs of primary ESCC tumors and their corresponding nontumorous tissues. The results showed that absent expression of UPK1A was detected in 68 of 100 primary ESCC tissues (Fig. 1A) and five of nine ESCC cell lines (Fig. 1B). Sequencing analysis was performed in five primary ESCCs, and no mutation was detected (data not shown). UPK1A expression in protein level was also studied by immunohistochemical staining using a TMA containing 300 pairs of primary ESCC tumor samples and their corresponding nontumorous tissues. Informative immunohistochemical results were obtained from 186 (62%) pairs of ESCCs. Noninformative samples included lost samples, unrepresentative samples, samples with too few tumor cells, and samples with inappropriate staining; such were not used in data complication. The immunohistochemical analysis showed that expression of UPK1A was detected in all nontumorous tissues. Absent expression of UPK1A was observed in 104 of 186 (56%) ESCC specimens (Fig. 1C).

UPK1A downregulation is associated with ESCC metastasis and poor prognosis

Clinical association study found that the downregulation of UPK1A was significantly associated with advanced clinical stage (P = 0.015, Fisher’s exact test) and lymph node metastasis (P = 0.009, Fisher’s exact test; Table 1). No significant association was observed between downregulation of UPK1A and other clinicopathologic parameters (Table 1). Kaplan-Meier analysis found that the downregulation of UPK1A was significantly associated with poor overall survival (P < 0.0001; Fig. 1D). By univariable analysis, downregulation of UPK1A (P < 0.0001), presence of lymph node metastasis (P < 0.0001), and advanced stage (P < 0.0001) were significant negative prognostic factors for overall survival of ESCC patients. Nevertheless, multivariable Cox proportional hazard regression analysis showed that the downregulation of UPK1A (P = 0.004) was the only independent factor for the prediction of overall survival (Supplementary Table S2).

UPK1A promoter region is frequently hypermethylated in ESCC

A 524-bp CpG island containing 28 CpG sites was found on the 5’ upstream of the UPK1A gene, which is located within a 2147-bp region reported to have the promoter activity (18). To explore the effect of promoter methylation on UPK1A downregulation in ESCC, MSP using methylation- or unmethylation-specific primers was carried out to analyze the methylation status of UPK1A. The result showed that both methylated and unmethylated alleles were detected in six of nine cell lines (Fig. 2A). In the remaining three cell lines, either methylated allele (KYSE510 and HKESC1) or unmethylated allele (KYSE410) was detected (Fig. 2A). MSP was also used to investigate the methylation frequency of UPK1A CGI in 50 primary ESCC tumors. The result showed that methylated allele was detected in 31 of 50 (62%) primary ESCC tumors. The frequency of methylation in ESCC with UPK1A downregulation (27 of 41, 65.9%) was obviously higher that that in ESCC with normal UPK1A expression (four of nine, 44.4%). To further evaluate whether the methylation of UPK1A directly mediates its repression, KYSE510 cells

Table 1. Association of UPK1A downregulation with clinicopathologic features of 186 ESCC patients

<table>
<thead>
<tr>
<th>Clinicopathologic features</th>
<th>Total case (%)</th>
<th>UPK1A expression</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Negative (%)</td>
<td>Positive (%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>44.62</td>
<td>48.19</td>
<td>51.81</td>
</tr>
<tr>
<td>Female</td>
<td>55.38</td>
<td>60.19</td>
<td>39.81</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤57</td>
<td>45.70</td>
<td>55.29</td>
<td>44.71</td>
</tr>
<tr>
<td>&gt;57</td>
<td>54.30</td>
<td>57.43</td>
<td>42.57</td>
</tr>
<tr>
<td>Clinical stage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Early stage (I–II)</td>
<td>71.50</td>
<td>51.13</td>
<td>48.87</td>
</tr>
<tr>
<td>Advanced stage (III–IV)</td>
<td>28.50</td>
<td>69.81</td>
<td>30.19</td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>60.21</td>
<td>49.11</td>
<td>50.89</td>
</tr>
<tr>
<td>N+</td>
<td>39.79</td>
<td>67.57</td>
<td>32.43</td>
</tr>
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*Significant difference.
were treated with demethylating agent 5-aza-dC. After the treatment, transcription of UPK1A was restored (Fig. 2B).

**UPK1A has tumor-suppressive ability**

To investigate whether UPK1A has tumor-suppressive ability, UPK1A was cloned into an expressing vector and stably transfected into two ESCC cell lines, KYSE30 and KYSE510. Expression of UPK1A in UPK1A-30 and UPK1A-510 cells was confirmed by RT-PCR (Fig. 2C). Protein expression of UPK1A was also tried to be confirmed by using antibodies from two companies. However, they failed to give a specific band. To confirm the protein expression of UPK1A in the clone, immunofluorescence has been done (Supplementary Fig. S1). With UPK1A-expressing clones, we showed tumor-suppressive
ability by cell proliferation assay, foci formation assay, and soft agar assay. The results showed that the efficiencies of foci formation (Fig. 2D) and colony formation in soft agar (Fig. 2E) were significantly inhibited in UPK1A-510 cells compared with Vec-510 cells ($P < 0.001$). With XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] cell growth assay, cell growth rates of UPK1A-30 and UPK1A-510 clones were also significantly suppressed compared with Vec-30 and Vec-510 cells ($P < 0.05$; Fig. 2F).

To confirm the *in vivo* tumor-suppressive ability of UPK1A, tumor formation in nude mice was performed by injecting UPK1A-30 cells ($n = 10$) or UPK1A-510 cells ($n = 10$), and Vec-30 or Vec-510 cells were used as controls. The results showed that tumor formation in nude mice was significantly inhibited in UPK1A-expressing cells ($P < 0.01$; Fig. 3A and B). With immunohistochemical staining using anti-UPK1A antibody, we confirmed that UPK1A was expressed in UPK1A-30– or UPK1A-510–derived tumors (Fig. 3C). In addition, a clear boundary between the tumor and its adjacent nontumor tissue was observed in UPK1A-30– and UPK1A-510–derived tumors (Fig. 3D). However, venous infiltration and irregular tumor invasion was observed in the Vec-30– and Vec-510–generated tumors, respectively (Fig. 3D). These data showed that UPK1A had strong tumor-suppressive ability.

**UPK1A arrests cell cycle at G1-S checkpoint by downregulating cyclin D1**

To understand how UPK1A suppresses tumor cell growth, flow cytometry was carried out to compare the DNA content between Vec-510 and UPK1A-510 cells. The result showed that the proportion of S-phase cells was significantly lower in UPK1A-510 than that in Vec-510 cells ($P < 0.05$), suggesting that UPK1A suppresses tumor cell growth by arresting cell cycle at G1-S checkpoint by downregulating cyclin D1.

![Figure 3. UPK1A inhibits tumor formation in nude mice.](image-url)
is able to arrest cells in at G1-S checkpoint (Fig. 4A and B). Further study found that G1-S checkpoint promoting factors cyclin D1 and cyclin-dependent kinase 4 (CDK4) were downregulated in UPK1A-510 cells compared with Vec-510 cells (Fig. 4C).

**UPK1A inhibits membrane-to-nucleus translocation of β-catenin**

We next studied the involvement of E-cadherin/β-catenin signaling pathway in the upregulation of cyclin D1 and CDK4. Western blotting showed that E-cadherin was upregulated in UPK1A-expressing cells compared with empty vector–transfected cells (Fig. 4C). However, no difference was detected for β-catenin. Because E-cadherin is an important component to hold β-catenin in the membrane, we examined the location of β-catenin by immunofluorescence. The result showed that β-catenin was mainly localized in nucleus of Vec-510 cells whereas mainly localized in cell membrane of UPK1A-510 cells, suggesting that UPK1A could inhibit the translocation of β-catenin from membrane to nucleus (Fig. 4D).

We next examined some downstream target genes of β-catenin, including MMP7, c-jun, and c-myc, by RT-PCR. The results showed that all tested genes were downregulated in UPK1A-510 cells compared with Vec-510 cells. However, the mRNA level of E-cadherin has no change (Fig. 4E). In addition, expression of MMP7 between tumors formed in nude mice generated from Vec-510 and UPK1A-510 cells was compared by immunohistochemistry using an anti-MMP7 antibody. Positive staining (brown) was observed in tumors derived from Vec-510 cells.

**UPK1A suppresses tumor invasion and metastasis**

To validate whether UPK1A can suppress metastasis, wound healing and cell invasion assays were performed.
Figure 5. UPK1A inhibits cancer cell invasion and metastasis. A, wound healing assay showed that cell motility was inhibited by UPK1A. Representative of images were photographed at time 0, 24, and 48 h after scratching. B, Matrigel invasion assay was used to compare invisibility between vector- and UPK1A-transfected cells. The cells that invaded through the Matrigel were fixed and stained with crystal violet (magnification, 200×). The results are expressed as mean ± SD of three independent experiments. **, P < 0.001. C, representatives of livers derived from SCID mice after tail vein injection of Vec-510 or UPK1A-510 cells. The metastatic nodules at liver surface are indicated by arrows. The summary of metastatic nodules at liver surface is mean of six SCID mice for each group. Bars, SD. **, P < 0.001. D, representatives of H&E staining show normal liver tissue (left) and liver cancer (right) observed in SCID mice injected with UPK1A-510 and Vec-510 cells, respectively. E, representatives of H&E staining show normal lung tissue (left) and lung cancer (right) observed in SCID mice injected with UPK1A-510 and Vec-510 cells, respectively.
to study cell motility. Wound healing assay showed that cell migration rate was dramatically reduced in UPK1A-expressing cells compared with empty vector–transfected cells (Fig. 5A). Cell invasion assay found that the number of invasive cells was significantly decreased in UPK1A-expressing cells compared with empty vector–transfected cells \((P < 0.01;\) Fig. 5B).

To study the metastatic effect of UPK1A in vivo, experimental metastasis assay was performed by comparing the metastatic nodules formed in the liver and lung of SCID mice. After 12 weeks, the mice were sacrificed, and metastatic nodules were counted in the surface of lung and liver. The number of nodules formed in liver was significantly higher in mice injected with Vec-510 cells \((24.2 \pm 4.4)\) than mice injected with UPK1A-510 \((3.3 \pm 1.2, P < 0.001,\) independent Student’s \(t\) test; Fig. 5C). No visible metastatic nodules were observed in the surface of lungs. H&E staining was performed on serial sections of liver and lung, and the results showed that metastatic nodules were not only detected in liver (Fig. 5D) but also in lung (Fig. 5E).

**Discussion**

**UPK1A** is an integral protein, which belongs to TM4SF family, and is thought to be specific to normal urothelium \((7, 11)\). Although several TM4SF molecules have been identified and implicated in the regulation of cell development, differentiation, proliferation, motility, and tumor cell invasion \((11)\), the biological function of **UPK1A** is largely unrevealed. In the present study, downregulation of **UPK1A** was frequently detected in ESCCs in both mRNA and protein levels. Further study found that hypermethylation played a crucial role in the inactivation of **UPK1A**, which was shown by restoring **UPK1A** expression with demethyla-
tion treatment.

The tumor-suppressive function of **UPK1A** was shown by both in vitro and in vivo assays. Ectopic expression of **UPK1A** in ESCC cell lines KYSE30 and KYSE510 could effectively suppress cell growth rate, colony formation in soft agar, migratory formation, and tumor formation in nude mice. Our studies also showed the ability of **UPK1A** to suppress cell motility and invasion. Ectopic expression of **UPK1A** in ESCC cell lines was able to inhibit cell migration and invasion. In vivo animal model showed that **UPK1A** could reduce tumor nodule formation in lung and liver, which was consistent with our TMA results, showing that the downregulation of **UPK1A** was significantly associated with lymph node metastasis in ESCC \((P = 0.009)\).

Molecular study found that the tumor-suppressive effect of **UPK1A** was closely associated with its role in cell cycle arrest at G1–S checkpoint. Ectopic expression of **UPK1A** could downregulate cyclin D1 and CDK4 expression. Activation of cyclin D–CDK4/6 complex, resulting in the cyclin D–CDK4/6–mediated Rb phosphorylation, can destruct Rb–E2F binding. The releasing E2F activates the transcription of genes necessary for S-phase entry and cell cycle progression \((19, 20)\). Further study found that **UPK1A** downregulated E-cadherin and subsequently held β-catenin in the membrane. β-Catenin maintained at low level in quiescent cells by interacting with protein kinases, adenosomatous polyposis coli and axin, casein kinase 1, and glycogen synthase kinase 3 (GSK3; ref. 21). However, when Wnt is present, it will inhibit the GSK3, resulting in the accumulation of β-catenin, which is then translocated to nucleus and causes carcinogenesis \((22)\). E-cadherin, which contains the β-catenin binding site, is another important molecule to hold β-catenin in membrane. The loss of E-cadherin will cause the nuclear translocation of β-catenin \((23)\). In the ESCC cell line KYSE510, β-catenin was mainly expressed in the nucleus. However, when **UPK1A** was transfected into the KYSE510 cells, E-cadherin expression was upregulated and β-catenin was mainly expressed in cell membrane. These data suggest that **UPK1A** is able to hold β-catenin in the membrane through the upregulation of E-cadherin. In addition, because the protein level of E-cadherin is upregulated in **UPK1A**-expressing cells, but not in mRNA level, it is possible that UPK1A may reduce E-cadherin ubiquitination. Nevertheless, more lines of evidence are required to confirm this conclusion. The influence of β-catenin translocation was further confirmed by examining the expression of its downstream targets, including cyclin-D1, c-jun, c-myc, and MMP7. In **UPK1A**-expressing cells, expressions of cyclin-D1, c-jun, c-myc, and MMP7 were all downregulated.

Another target of β-catenin is MMP7, which plays an important role in cancer invasion and metastasis \((24, 25)\). In the in vivo model, we found that **UPK1A** can inhibit the metastasis in lung and liver. In nude mice model, we also found that tumors induced by empty vector–transfected cells showed a higher level of MMP7 expression compared with tumors induced by **UPK1A**-transfected cells. In addition, a clear boundary between the tumor and its adjacent nontumor tissue was often observed in the tumors induced by **UPK1A**-30 and **UPK1A**-510 cells; however, irregular tumor invasion was frequently observed in tumors induced by Vec-30 and Vec-510 cells (Fig. 3C). The results further implied that the inhibiting effect of **UPK1A** on cancer metastasis might be through the downregulation of MMP7.

Although different treatment methods have been developed, the survival rate of ESCC patients remains unsatisfactory \((26)\). Therefore, it is necessary to find novel risk markers to guide disease management of ESCC patients for the improvement of their survival. By Kaplan-Meier analysis, downregulation of **UPK1A** in ESCC patients was significantly associated with poor survival \((P < 0.0001)\), suggesting that **UPK1A** may be a novel marker to predict overall survival. To further validate which of factors (**UPK1A** expression, lymph node metastasis, or stage) is an independent factor for predicting the overall survival, univariable and multivariable Cox proportional hazard regression analysis was performed. The result showed that **UPK1A** expression is an independent factor and the most influential factor in predicting overall survival. In conclusion, our findings indicated that **UPK1A** is a potent TSG and plays an important role in inhibiting cell proliferation and metastasis.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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