Pituitary Tumor-Transforming 1 Increases Cell Motility and Promotes Lymph Node Metastasis in Esophageal Squamous Cell Carcinoma

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Abstract

Human pituitary tumor-transforming 1 (PTTG1)/securin is a putative oncoprotein that is overexpressed in various tumor types. However, the involvement of PTTG1 in gastrointestinal cancer development and progression remains unclear. In this study, we investigated the clinical significance and biological effects of PTTG1 in esophageal squamous cell carcinoma (ESCC). Immunohistochemical studies performed on 113 primary ESCC specimens revealed a high prevalence of PTTG1 overexpression (60.2%), which was significantly associated with lymph node metastasis (regional, \(P = 0.042\); distant, \(P = 0.005\)), advanced tumor stage (\(P = 0.028\)), and poorer overall survival (\(P = 0.017\), log-rank test; \(P = 0.044\), Cox proportional hazard model). Eleven ESCC cell lines expressed PTTG1 protein at levels 2.4 to 6.6 times higher than those in normal esophageal epithelial cells (HEEpiC). PTTG1 protein expression was confined to the nucleus in HEEpiC cells but present in both the cytoplasm and nucleus in ESCC cells. Two small interfering RNAs (siRNA) inhibited PTTG1 mRNA and protein expression in three ESCC cell lines by 77% to 97%. In addition, PTTG1 down-regulation by these siRNAs significantly reduced cell motility in all three ESCC cell lines \((P < 0.01)\) in vitro, as well as popliteal lymph node metastases of ESCC cells in nude mice \((P = 0.020)\). Global gene expression profiling suggested that several members of the Ras and Rho gene families, including \(RRA\), \(RHO\), \(ARHGAPI\), and \(ARHGAD\), represented potential downstream genes in the PTTG1 pathway. Taken together, these findings suggest that PTTG1 overexpression promotes cell motility and lymph node metastasis in ESCC patients, leading to poorer survival. Thus, PTTG1 constitutes a potential biomarker and therapeutic target in ESCCs with lymph node metastases. [Cancer Res 2008;68(9):3214–24]

Introduction

Esophageal squamous cell carcinoma (ESCC) is a highly aggressive malignancy with a 5-year survival rate of 10% worldwide (1). One reason for this poor survival is the fact that ESCC frequently metastasizes to regional and distant lymph nodes, even at initial diagnosis. Lymph node metastasis is one of the most important prognostic factors in ESCC (2). Therefore, the detection and treatment of lymph node metastases are extremely important in managing this refractory cancer.

Pituitary tumor-transforming 1 (PTTG1), also known as human securin, was originally isolated from rat pituitary tumor cells (3). PTTG1 possesses multiple physiologic functions critical to normal cellular mitosis, which are exerted through the maintenance of sister chromatid separation (4). PTTG1 overexpression has been reported in a variety of cancers including ESCC (5–7). PTTG1 levels correlated with tumor invasiveness (8), and PTTG1 has been identified as a key signature gene in tumor metastasis (9). However, mechanisms underlying the involvement of PTTG1 in lymph node metastasis are poorly understood.

Recently, we discovered that PTTG1 was overexpressed in ESCC tumors versus normal organs and exhibited much higher promoter activity in ESCC cells than in normal epithelial cells (10). Based on these findings, we hypothesized that PTTG1 represented a promising potential therapeutic target in ESCC. Nevertheless, the clinical significance and biological involvement of PTTG1 in the progression of gastrointestinal cancers, including ESCCs, have not yet been elucidated. Therefore, we sought to clarify the clinical effect and the in vitro and in vivo biological functions of PTTG1 in ESCC, using 113 surgically resected primary ESCC specimens, small interfering RNAs (siRNA) directed against PTTG1, esophageal cell lines, and in vitro and in vivo metastasis assays.

Materials and Methods

Patients and surgical specimens. Paraffin-embedded sections were obtained for immunohistochemical studies from 113 patients with primary ESCC undergoing surgery at Kyoto University Hospital (Kyoto, Japan) from 1991 to 2002. The median age of these patients was 62.5 y (range, 43–82 y). Median postoperative survival was 80 mo (range, 1–159 mo). Information on gender, age, stage of disease, and histopathologic features was abstracted from medical records. All tumors were confirmed as ESCC by the Clinicalopathologic Department at Kyoto University Hospital. All cases were classified according to the sixth edition of the pathologic tumor-node-metastasis (pTNM) classification (11). Written informed consent for the research use of resected specimens was obtained from all patients before...
surgery; approval was obtained from the Kyoto University Institutional Review Board (nos. #232 and #G48).

**Table 1.** PTTG1 protein expression in 113 primary ESCC tumors determined by immunohistochemistry

(A) PTTG1 staining and clinicopathologic characteristics in 113 ESCC patients

| Variables                  | PTTG1 (− or +), n = 45 | PTTG1 (++, n = 68) | P  
|---------------------------|-------------------------|-------------------|----  
| Age, y                    |                         |                   |     0.373  
| Mean                      | 63.7                    | 62.1              |     0.679  
| SD                        | 8.6                     | 9.6               |     0.933  
| Gender                    |                         |                   |     0.042*  
| Male                      | 39                      | 57                |     0.005*  
| Female                    | 6                       | 11                |     0.028*  
| pT (primary tumor)        |                         |                   |     0.003  
| pT1                       | 15                      | 19                |     0.003  
| pT2                       | 9                       | 16                |     0.003  
| pT3                       | 16                      | 25                |     0.003  
| pT4                       | 5                       | 8                 |     0.003  
| pN (regional lymph node metastasis) |           |                   |     0.003  
| pN0                       | 21                      | 19                |     0.003  
| pN1                       | 24                      | 49                |     0.003  
| pM (distant lymph node metastasis) |           |                   |     0.003  
| pM0                       | 42                      | 49                |     0.003  
| pM1                       | 3                       | 19                |     0.003  
| pTNM stage                |                         |                   |     0.063  
| I                         | 12                      | 11                |     0.003  
| IIA                       | 9                       | 8                 |     0.003  
| IIb                       | 6                       | 15                |     0.003  
| III                       | 14                      | 16                |     0.003  
| IVa                       | 0                       | 9                 |     0.003  
| IVb                       | 3                       | 10                |     0.003  
| Histologic grade          |                         |                   |     0.003  
| 1                         | 11                      | 6                 |     0.003  
| 2                         | 24                      | 40                |     0.003  
| 3                         | 10                      | 22                |     0.003  

(B) Cox univariate analysis

| Variables                  | Risk ratio (95% CI) | P  
|---------------------------|---------------------|----  
| Age (>63 y)               | 1.01 (0.61–1.68)    | 0.957  
| Gender (male)             | 1.36 (0.72–2.57)    | 0.337  
| pT (>T1)                  | 3.65 (2.13–6.26)    | 0.0001  
| pN (1)                    | 3.48 (1.87–6.46)    | 0.0001  
| pM (1)                    | 2.28 (1.28–4.06)    | 0.0051  
| Histologic grade (>G2)    | 1.28 (0.61–2.69)    | 0.52  
| PTTG1 (++ or +++)         | 1.75 (1.01–3.00)    | 0.044  

(C) Cox multivariate analysis

| Variables                  | Risk ratio (95% CI) | P  
|---------------------------|---------------------|----  
| Age (>63 y)               | 1.08 (0.63–1.84)    | 0.784  
| Gender (male)             | 0.82 (0.43–1.66)    | 0.561  
| pT (>T1)                  | 2.91 (1.67–5.26)    | 0.0001  
| pN (1)                    | 2.23 (1.16–4.54)    | 0.016  
| pM (1)                    | 1.12 (0.58–2.12)    | 0.723  
| Histologic grade (>G2)    | 1.04 (0.49–2.50)    | 0.914  
| PTTG1 (++ or +++)         | 1.61 (0.92–2.91)    | 0.093  

* Pearson’s χ² test.
phosphate (GAPDH) antibody, clone 71.1 (1:20,000; Sigma), were used for Western blotting. Horseradish peroxidase–labeled antirabbit IgG (1:2,500; Chemicon) or antimouse IgG (1:5,000; Invitrogen) was used as a secondary antibody for Western blotting. Two siRNAs directed against PTTG1 (P1 and P2) were synthesized by Dharmacon. P1 was designed by the authors via siDirect, an siRNA design software (12), whereas P2 had previously been designed by others (13). The siRNA target sequences were as follows: P1, 5′-GTGACATAGATATTTAAAT-3′ (position 638–656, NM_004219); P2, 5′-GTCTGTAAAGACCAAGGGA-3′ (position 264–282). A nontargeting control siRNA (NTC; Dharmacon) served as a negative control.

Immunohistochemical staining. Resected esophageal specimens were fixed in 10% formaldehyde and embedded in paraffin blocks. Using 4-μm-thick sections, immunohistochemical staining was done with an Envision kit (DakoCytomation; ref. 14). As a negative control, the primary antibody was replaced with a normal mouse IgG. All slides were independently evaluated by two investigators (T.I. and Y.S.) lacking prior knowledge of each patient’s clinical information. Whenever opinions of these two evaluators differed, agreement was reached by careful discussion.

Cell cultures. Human ESCC cell lines of the KYSE series, as well as HSA/c, were established and maintained in Ham’s F12/RPMI 1640 (Invitrogen) containing 5% fetal bovine serum (FBS), as previously described (14, 15). Nonmalignant human esophageal epithelial cells (HEEpiC; ScienCell) were maintained in Epithelial Cell Medium 2 (ScienCell) according to the provider’s instructions.

Western blotting and subcellular fractionation. Cells were washed with PBS and lysed in Laemmli sample buffer (Bio-Rad) at room temperature. Protein concentration was estimated with BCA Protein Assay kit ( Pierce). Cell lysates (15 μg) were electrophoresed on a 15% polyacrylamide gel (Bio-Rad) and transferred onto polyvinylidene difluoride.
membranes (Millipore). After being blocked with TBS containing 5% skim milk and 0.1% Tween 20 for 1 h, membranes were incubated with the primary and secondary antibodies at room temperature for 2 and 1 h, respectively, and then bands were visualized with enhanced chemiluminescence reagent (GE Healthcare). All bands were quantified using ImageJ v1.34 (U.S. NIH). 5 For subcellular fractionation assays, nuclear and cytoplasmic fractions were separated with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). GAPDH and lamin A/C were used as cytoplasmic and nuclear markers, respectively, as previously described (16, 17).

**Transfections.** Three ESCC cell lines (i.e., KYSE140, KYSE410, and HSA/c) were separately and transiently transfected with three siRNAs, specifically NTC, P1, and P2, using Lipofectamine RNAiMAX (LF; Invitrogen) according to the manufacturer’s protocol. Cells were plated at $2 \times 10^5$ per well onto six-well plates (Corning) 1 d before transfection (day 0). The siRNA (200 pmol/well) was transfected into cells at day 1, and total RNA and protein were extracted at day 4. No treatment (LF+) and Lipofectamine RNAiMAX alone (LF+) were included as negative controls.

**Quantitative reverse transcription-PCR analysis.** Total RNA was extracted with Trizol reagent (Invitrogen). Quantitative reverse transcription-PCR (RT-PCR) with SYBR Green QuantiTect RT-PCR kit (Qiagen) was extracted with Trizol reagent (Invitrogen). Quantitative reverse transcription-PCR (RT-PCR) with SYBR Green QuantiTect RT-PCR kit (Qiagen) was performed using a Bio-Rad iQ5 real-time PCR machine (Bio-Rad) as previously described (16, 17).

**Immunofluorescence staining.** Immunofluorescence staining was done as previously described (18). Briefly, siRNA-transfected cells were seeded onto a coverslip at day 4 and incubated for 1 additional day. Cells were fixed in 10% formaldehyde in PBS for 15 min, treated with 0.2% Triton X-100 in PBS for 10 min, blocked with 1% bovine serum albumin in PBS, and incubated with a primary antibody and antirabbit Alexa Fluor 568 (Invitrogen) at room temperature for 1 h and 30 min, respectively. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Roche Diagnostics). The cells were mounted and viewed under a fluorescence microscope (Carl Zeiss).

**Cell migration/invasion assays.** Cell motility (chemotaxis) and invasiveness were determined with Transwell and Matrigel chamber inserts, respectively (24-well-format with 8-μm pores, BD Biosciences), as previously described (19). Cells ($5 \times 10^4$) were seeded onto the upper chamber at day 4, with 20% FBS in the lower chamber as a chemoattractant. After incubation at 37°C for 24 h, membranes were stained and all cells on the lower membrane surface were counted under a light microscope. The number of cells migrating through Matrigel inserts represented a combination of cell penetration through the Matrigel layer and cell migration through the small membrane pores (20). To distinguish between these two biological processes, we defined an invasion index (i.e., cell penetration through the Matrigel layer) as the ratio of the mean number of cells migrating through the Matrigel insert to the mean number of cells migrating through the Transwell membrane.

**In vivo lymph node metastasis assay.** This assay was done using an in vivo popliteal lymph node metastasis model (14). HSA/c cells ($3 \times 10^6$) were transfected with an siRNA in vitro, harvested 72 h after transfection, and inoculated into both footpads of 5-wk-old male athymic nude mice (Harlan) at day 0. SiRNA (20 μg) conjugated with polyethylenimine (in vivo jetPEI, Polypus Transfection) was injected intratumorally on days 7, 14, and 21. Polyethylenimine/siRNA complexes were prepared at a polyethylenimine/siRNA (N/P) ratio equal to 10, as previously described (21). For vehicle control treatments, Lipofectamine RNAiMAX (Invitrogen) and polyethylenimine (Polypus Transfection) without siRNA were used in vitro and in vivo, respectively. Ten primary footpad tumors from five mice were analyzed for each treatment condition (vehicle, NTC, P1, and P2). All mice were sacrificed at day 28, and all primary tumors and popliteal lymph nodes were enucleated and fixed in 10% formaldehyde/PBS. All lymph nodes and primary tumors were paraffin embedded, stained with H&E and PTTG1, and examined for the presence of metastases. All animal procedures were approved by the institutional animal committee and executed in accordance with institutional guidelines.

**Bead-array gene expression analyses.** HSA/c and KYSE140 cells were separately transfected with one of three siRNAs (NTC, P1, and P2) or with
Lipofectamine alone (LF+), as described above, and harvested 72 h after transfection. Following RNA quality assessment done on a Bioanalyzer 2100 (Agilent Technologies), each RNA sample (100 ng), extracted using an RNeasy kit (Qiagen), was amplified with an Illumina TotalPrep RNA Amplification kit (Ambion), hybridized to an Illumina Human RefSeq8 version 2 BeadChip containing 20,589 transcript probes composed of optimized 50-mer oligonucleotides (Illumina), washed, and stained with Cy3-streptavidin (GE Healthcare) per manufacturer’s instructions (22). Arrays were scanned with Illumina Beadarray Reader confocal scanner and data were processed using Illumina BeadStudio software. Data were subjected to intensity-dependent normalization, and differentially expressed genes associated with PTTG1 down-regulation...
were identified by significance analysis of microarrays (23). All processed and raw data are available in Minimum Information about Microarray Gene Experiment–compliant format via the Gene Expression Omnibus. Accession numbers are GSE7447, GSM180360, GSM180361, GSM180362, GSM180363, GSM180364, GSM180365, GSM180366, GSM180367, and GSM180368. Expression profiles of HSA-P1, HSA-P2, KYSE140-P1, and KYSE140-P2, which were designated gene profiles associated with PTTG1 down-regulation, were compared with expression profiles of HSA-LF+, HSA-NTC, KYSE140-LF+, and KYSE140-NTC, which were considered intact-PTTG1 cell profiles. Fold changes on bead array were calculated by dividing the average signal intensity of all down-regulated-PTTG1 profiles by the average of all intact-PTTG1 profiles. For validation of expression profiles, the mRNA expression level of each gene was analyzed by quantitative RT-PCR, as described above. Fold change for each gene assessed by quantitative RT-PCR was calculated as the ratio of average expression in all PTTG1-downregulated cells (HSA-P1, HSA-P2, KYSE140-P1, and KYSE140-P2) to average expression in all intact-PTTG1 cells (HSA-LF+, HSA-NTC, KYSE140-LF+, and KYSE140-NTC). ELISA for basic fibroblast growth factor (bFGF; immunoassay kit, BioSource) was done with cell culture medium according to the manufacturer’s protocol.

**Statistical analyses.** Survival curves were generated according to the Kaplan-Meier method, and differences in survival were analyzed by log-rank testing. Univariate and multivariate analyses were done using the Cox proportional hazards model. Correlations between PTTG1 expression and each clinicopathologic parameter were evaluated using Pearson’s χ² test. The Tukey-Kramer multiple comparison test was used to evaluate

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Figure 3. Effects of PTTG1 knockdown on cell migration in vitro and lymph node metastasis in vivo. A, cell migration/invasion assays in vitro. Columns, mean of triplicate experiments; bars, SD. Left, Transwell migration assay; numbers of cells migrating through Transwell membranes are shown. Middle, Matrigel invasion assay; numbers of cells migrating through Matrigel inserts are shown. Right, invasion index (%); ratios of cells migrating through Matrigel inserts relative to mean numbers of cells migrating through Transwell membranes are shown. **, P < 0.01, versus NTC for each group (Tukey-Kramer test). B, lymph node metastasis assay in nude mice. Left, a leg of a nude mouse at 28 d after inoculation of HSA/c cells into the footpad. The leg skin was stripped to show a swollen popliteal lymph node. The distance between the footpad tumor and the popliteal lymph node was ~1 cm. Bottom left, H&E staining of a footpad tumor (magnification, ×200). Note the “cancer pearl” in the center. Middle, PTTG1 immunostaining of a footpad tumor treated with NTC (top) and P1 (bottom; magnification, ×100). Right, representative H&E staining of popliteal lymph nodes treated with NTC (top) and P1 (bottom; magnification, ×100).
Table 2. Effect of PTTG1 knockdown on lymph node metastasis in vivo

(A) PTTG1 siRNA treatment in lymph node metastasis model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. mice</th>
<th>No. total LNs</th>
<th>No. metastatic LNs</th>
<th>Metastatic ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>NTC</td>
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<td>P1</td>
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<td>20</td>
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<tr>
<td>P2</td>
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</tr>
<tr>
<td>total</td>
<td>20</td>
<td>41</td>
<td>13</td>
<td>31.7</td>
</tr>
</tbody>
</table>

(B) Relationship between PTTG1 down-regulation and lymph node metastasis

<table>
<thead>
<tr>
<th>PTTG1 status</th>
<th>No. metastatic LNs (%)</th>
<th>No. nonmetastatic LNs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTTG1(+)</td>
<td>10 (50)</td>
<td>10 (50)</td>
</tr>
<tr>
<td>PTTG1(−)</td>
<td>3 (14.3)</td>
<td>18 (85.7)</td>
</tr>
<tr>
<td>total</td>
<td>13 (31.7)</td>
<td>28 (68.3)</td>
</tr>
</tbody>
</table>

NOTE: Metastatic ratio = number of metastatic lymph nodes / number of total lymph nodes.
Abbreviation: LNs, lymph nodes.
* Vehicle and NTC (n = 20).
† P1 and P2 (n = 21); P = 0.020, Fisher’s exact test.

Results

PTTG1 protein expression in 113 primary ESCC tumors. As shown in Table 1A, 23 patients were with stage I, 17 with stage IIa, 21 with stage IIb, 30 with stage III, 9 with stage IVa, and 13 with stage IVb ESCCs. The pattern of PTTG1 staining in ESCC was different from that observed in normal epithelium. In ESCC cells, PTTG1 staining was usually observed in the cytoplasm and only occasionally in the nucleus (Fig. 1A). In contrast, PTTG1 staining in normal esophageal epithelia was never observed in the cytoplasm, but still occasionally in the nucleus (~10% of cases; data not shown), particularly in the proliferative layer just above the basal cells. PTTG1 expression in ESCC specimens, evaluated based exclusively on cytoplasmic staining, occurred as follows: 14 tumors were negative for expression (−), 31 manifested expression in 0% to 10% of tumor cells (1+), 52 revealed expression in 10% to 30% of tumor cells (2+), and 16 were characterized by expression in >30% of tumor cells (3+). Next, we classified 2+ or 3+ PTTG1 expression as PTTG1 positive (68 of 113, 60.2%). Under this classification system, PTTG1 expression was significantly associated with regional lymph node metastasis (pN; P = 0.042), distant lymph node metastasis (pM; P = 0.005), and tumor stage (pTNM; P = 0.028), but not with depth of primary tumor invasion (pT; P = 0.933). Kaplan-Meier survival analysis showed that patients with PTTG1-positive tumors had significantly worse survivals than did those with PTTG1-negative tumors (P = 0.017, log-rank test; Fig. 1B). Furthermore, univariate analyses revealed that PTTG1 expression was a significant predictor of survival [risk ratio, 1.75; 95% confidence interval (95% CI), 1.01–3.00; P = 0.044; Table 1B]. Multivariate analyses revealed that PTTG1 expression tended to be an independent prognostic factor, although this trend did not achieve statistical significance (risk ratio, 1.61; 95% CI, 0.92–2.91; P = 0.093; Table 1C). Several tumors showed strong PTTG protein expression in cell nuclei. We also evaluated nuclear staining in all 113 ESCC tumors. Nuclear PTTG1 protein expression occurring in >10% of tumor cells was observed in only 7 of 113 (6.2%) ESCC tumors. However, nuclear PTTG1 staining did not correlate with any clinicopathologic features or survival. We also compared the no-expression group (−; n = 14) to the any-expression group (1+–3+; n = 99). In this analysis, any level of PTTG1 expression was associated with distant lymph node metastasis (pM; P = 0.049), but not with primary tumor invasion (pT; P = 0.532), regional lymph node metastasis (pN; P = 0.222), tumor stage (pTNM; P = 0.523), or histologic grade (P = 0.098). Kaplan-Meier survival analysis showed a trend toward association of any PTTG1 expression with poor survival (P = 0.164, log-rank test). Interestingly, none of the 14 PTTG1-negative tumors gave rise to distant lymph node metastases.

PTTG1 protein expression in squamous esophageal cells. We performed Western blotting to assess PTTG1 protein levels in 11 ESCC cell lines and in cultured normal esophageal epithelial cells (HEEpC; Fig. 1C). PTTG1 protein expression in all 11 ESCC cell lines was 2.4 to 6.6 times higher than in HEEpiC cells. To determine subcellular localization of PTTG1 expression, the relative nuclear versus cytoplasmic distribution of PTTG1 protein was examined in HSA/c, KYSE140, KYSE410, and HEEpiC cells by subcellular fractionation (Fig. 1D). Purity of fractions was verified by probing membranes with cytoplasmic (GAPDH) or nuclear (lamin A/C) protein-specific antibodies. PTTG1 in HEEpiC cells was expressed only in the nucleus, but not in the cytoplasm. Conversely, PTTG1 in HSA/c, KYSE140, and KYSE410 cells was expressed in both the cytoplasm and nucleus. To evaluate the effect of PTTG1 knockdown in ESCC cells, we selected HSA/c, KYSE140, and KYSE410 for further analyses because of their high native PTTG1 expression levels.

Knockdown of PTTG1 expression in ESCC cells. We transiently transfected either a PTTG1 siRNA (P1 or P2) or a...
PTTG1 into three different ESCC cell lines (HSA/c, KYSE140, and KYSE410). By quantitative RT-PCR, PTTG1 mRNA expression was inhibited by 84% to 86% in HSA/c, 88% to 93% in KYSE140, and 90% to 92% in KYSE410 cells (Fig. 2A). By Western blotting, PTTG1 protein expression was inhibited by 77% to 93% in HSA/c, 86% to 88% in KYSE140, and 92% to 97% in KYSE410 cells (Fig. 2B). By fluorescence microscopy and flow cytometry, we confirmed that transfected FAM-labeled siRNAs were taken up by >90% of cells (data not shown). A subcellular fractionation assay in PTTG1-down-regulated HSA/c cells showed that both of the PTTG1 siRNAs (P1 and P2) suppressed both nuclear and cytoplasmic PTTG1 expression (Fig. 2C). We also examined PTTG1 expression in these cells by immunofluorescence staining (Fig. 2D). Whereas PTTG1 was expressed in both the cytoplasm and nucleus of ESCC cells in negative control transfections (LF+ and NTC), the siRNAs directed against PTTG1 (P1 and P2) markedly suppressed PTTG1 expression in these cells, corroborating the results of Western blotting.

**PTTG1 knockdown reduces motility of ESCC cells.** Cell motility and invasiveness were determined by Transwell and Matrigel chamber assays, respectively (Fig. 3A). PTTG1 knockdown in HSA/c, KYSE140, and KYSE410 cells reduced the number of cells migrating through Transwell and Matrigel chambers by 94.0% to 67.8% and by 94.1% to 70.7%, respectively (P < 0.01, versus NTC). In contrast, invasion index, representing the degree of cell penetration through the Matrigel layer, was not altered by PTTG1 inhibition.

**PTTG1 knockdown suppresses lymph node metastasis in vivo.** We examined the effect of PTTG1 knockdown, using a popliteal lymph node metastasis model, in nude mice (Fig. 3B, top left). In separate experiments, highly metastatic HSA/c cells were transfected with either siRNA P1 or siRNA P2 and then inoculated into the footpads of nude mice at day 0. To maintain the knockdown effects of PTTG1, we carried out additional in vivo transfections by injecting siRNAs intratumorally with polyethyleneimine weekly from day 7 to day 21. At day 28, all mice were sacrificed and all footpad tumors (Fig. 3B, bottom left) and popliteal lymph nodes were obtained and analyzed. PTTG1 immunostaining (Fig. 3B, middle) showed that PTTG1 expression in footpad tumors was inhibited by both PTTG1 siRNAs (P1 and P2) compared with negative control transfections (vehicle only and NTC). As shown in Table 2A, ratios of metastatic to total dissected popliteal lymph nodes were lower in both PTTG1 siRNA groups than in vehicle-only or NTC groups [50.0% (5 of 10) in vehicle, 50.0% (5 of 10) in NTC, 20.0% (2 of 10) in P1, and 9.1% (1 of 11) in P2]. Moreover, this difference in metastatic ratios achieved statistical significance (P = 0.020, Fisher’s exact test; Table 2B) when metastatic ratios were compared between the PTTG1-down-regulated group (PTTG1(−); P1 and P2) and the intact-PTTG1 group (PTTG1(+); vehicle only and NTC). Representative H&E staining of a metastatic lymph node in NTC and a nonmetastatic lymph node in P1 is shown in Fig. 3B (top right and bottom right, respectively).

**Identification of potential downstream genes in the PTTG1 pathway in ESCC.** To identify target genes potentially regulated by PTTG1, we conducted bead array–based genome-wide mRNA profiling. As shown in Table 3, PTTG1 siRNA treatment resulted in the up-regulation of 12 genes and down-regulation of 8 genes in HSA/c and KYSE140 cells. Differentially expressed genes previously reported as being cell motility–related included several Ras and Rho family members, such as Rras (related-ras viral oncogene homologue), Rhogap1 (Rho GTPase activating protein 1), and Arhgdia (Rho GDP dissociation inhibitor α). We then confirmed differential expression of selected genes in these same RNA samples by quantitative RT-PCR and found that fold changes in gene expression discovered by bead array correlated strongly with fold changes found by quantitative RT-PCR (r² = 0.885; P < 0.0001, Pearson’s correlation coefficient). PTTG1 was identified as the gene most markedly down-regulated among 20,589 transcripts, closely agreeing with quantitative RT-PCR results for PTTG1 (bead-array fold change, 0.12; quantitative RT-PCR fold change, 0.14).

Additionally, we examined the expression levels of known PTTG1-related genes, including bfgf (bead-array fold change, 0.93; quantitative RT-PCR fold change, 0.96) and VEGF (vascular endothelial growth factor; bead-array fold change, 0.97; quantitative RT-PCR fold change, 1.02). This analysis revealed that PTTG1 down-regulation in HSA/c and KYSE140 cells did not alter the mRNA expression levels of bfgf and VEGF. Moreover, ELISA assays confirmed that PTTG1 knockdown treatment did not alter bfgf secretion levels in HSA/c and KYSE140 cells (HSA-LF+, 41.3 ± 4.2 pg/mL; HSA-NTC, 36.0 ± 6.4 pg/mL; HSA-P1, 41.2 ± 2.6 pg/mL; HSA-P2, 41.4 ± 3.2 pg/mL; KYSE140-LF+, 34.9 ± 7.1 pg/mL; KYSE140-NTC, 38.4 ± 4.5 pg/mL; KYSE410-P1, 37.4 ± 4.7 pg/mL; and KYSE410-P2, 37.3 ± 9.1 pg/mL; average value ± SD, not significant).

**Discussion**

Several studies have shown that PTTG1 expression in primary tumors is associated with metastasis (5–7). However, biological evidence based on animal experiments has not yet clarified the relationship between PTTG1 and cancer metastasis. In the current study, to our knowledge for the first time, we have shown that overexpression of PTTG1 in ESCC cells increases cell motility and promotes lymph node metastasis based on in vitro and in vivo functional assays.

Overexpression of PTTG1 protein was a frequent event in ESCC primary tumors and cell lines (Table 1A; Fig. 2A). This finding confirmed the observation in previous studies that PTTG1 is implicated in the development, progression, and lymph node metastasis of breast, gastric, and esophageal cancers (5–7). Our PTTG1 protein expression data in 113 tumors were compatible with these results. In the current study, protein levels of PTTG1 were associated with the presence of lymph node metastases in ESCC. In addition, PTTG1 expression was a significant survival predictor in univariate analyses, whereas PTTG1 was not an independent predictor by multivariate analysis. Because lymph node status is strongly associated with PTTG1 expression level and patient survival (Table 1A and B), PTTG1 expression may contribute to poor patient survival (Fig. 1B) by promoting lymph node metastasis.

PTTG1 protein expression was observed in both the cytoplasm and nucleus in ESCC primary tumors and cell lines. In contrast, PTTG1 was localized only to the nucleus in normal esophageal epithelial cells, particularly in the proliferative esophageal epithelial layer just above the basal cells (Fig. 1A). Western blotting using subcellular fractionated samples (Fig. 2B) confirmed this immunohistochemical finding as well as the findings in other normal cell types (24). This difference in subcellular localization of PTTG1 protein between normal and cancer cells suggests that PTTG1 localized to the nucleus may play a physiologic role in normal cell division, whereas PTTG1 localized to the cytoplasm and nucleus may contribute to poor patient survival.
### Table 3. Genes differentially expressed after anti-PTTG1 siRNA treatment in HSA/c and KYSE140 cells

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Gene symbol</th>
<th>Description; relationship with cancer</th>
<th>D-score (bead array-SAM)</th>
<th>Fold change (bead array)</th>
<th>Fold change (quantitative RT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up-regulated genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_001219</td>
<td>CALU</td>
<td>calumenin; calcium-binding protein, down-regulated in metastatic head and neck cancer cells, related to better survival in lung cancer</td>
<td>1.87</td>
<td>1.7</td>
<td>2.25</td>
</tr>
<tr>
<td>NM_019558</td>
<td>HOXD8</td>
<td>homeobox D8; possible tumor suppressor</td>
<td>1.54</td>
<td>1.17</td>
<td>1.16</td>
</tr>
<tr>
<td>NM_006931</td>
<td>SLC2A3</td>
<td>solute carrier family 2, glucose transporter/GLUT3; cancer relation unknown</td>
<td>1.27</td>
<td>2.13</td>
<td>1.4</td>
</tr>
<tr>
<td>NM_00498</td>
<td>GCLC</td>
<td>glutamate-cysteine ligase, catalytic subunit; possible suppressor in breast cancer</td>
<td>1.15</td>
<td>1.65</td>
<td>1.4</td>
</tr>
<tr>
<td>NM_001072</td>
<td>UGT1A6</td>
<td>UDP glucuronosyltransferase 1 family, polypeptide A6, transcript variant 1; its polymorphism relates to metabolism of nonsteroidal anti-inflammatory drugs and carcinogenesis</td>
<td>1.00</td>
<td>1.65</td>
<td>1.87</td>
</tr>
<tr>
<td>NM_003932</td>
<td>ST13</td>
<td>suppression of tumorigenicity 13 (heat shock protein 70 interacting protein); down-regulated in colon and gastric cancers</td>
<td>0.89</td>
<td>1.6</td>
<td>1.11</td>
</tr>
<tr>
<td>NM_004887</td>
<td>CXCL14</td>
<td>chemokine (C-X-C motif) ligand 14; angiogenesis inhibitor, chemotactic factor in dendritic cells</td>
<td>0.81</td>
<td>1.58</td>
<td>2.25</td>
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<tr>
<td>NM_205862</td>
<td>UGT1A6</td>
<td>UDP glucuronosyltransferase 1 family, polypeptide A6, transcript variant 2</td>
<td>0.74</td>
<td>1.6</td>
<td>1.87</td>
</tr>
<tr>
<td>NM_003246</td>
<td>THBS1</td>
<td>thrombospondin 1; possible angiogenic inhibitor</td>
<td>0.65</td>
<td>1.76</td>
<td>1.27</td>
</tr>
<tr>
<td>NM_013253</td>
<td>DKK3</td>
<td>dickkopf homologue 3; possible suppressor for melanoma</td>
<td>0.58</td>
<td>1.7</td>
<td>1.36</td>
</tr>
<tr>
<td>NM_001627</td>
<td>ALCAM</td>
<td>activated leukocyte cell adhesion molecule; possible metastasis suppressor in breast cancer</td>
<td>0.54</td>
<td>1.46</td>
<td>1.73</td>
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<tr>
<td>NM_001005340</td>
<td>GPNMB</td>
<td>glycoprotein (transmembrane) nmbr; possible metastasis suppressor in melanoma</td>
<td>0.30</td>
<td>1.66</td>
<td>1.41</td>
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<tr>
<td>NM_003793</td>
<td>AKR1C3</td>
<td>aldo-keto reductase family 1, member C3; possible suppressor in prostate cancer</td>
<td>0.25</td>
<td>2.12</td>
<td>1.87</td>
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<tr>
<td><strong>Down-regulated genes</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>NM_004219</td>
<td>PTTG1</td>
<td>pituitary tumor-transforming gene 1</td>
<td>−9.34</td>
<td>0.12</td>
<td>0.14</td>
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<td>NM_002808</td>
<td>PSMD2</td>
<td>proteasome 26S subunit 2; upregulated in breast cancer</td>
<td>−2.26</td>
<td>0.76</td>
<td>0.85</td>
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<tr>
<td>NM_001416</td>
<td>EIF4A1</td>
<td>eukaryotic translation initiation factor 4A, isoform 1; metastasis-associated gene in lung cancer</td>
<td>−1.63</td>
<td>0.72</td>
<td>0.81</td>
</tr>
<tr>
<td>NM_004309</td>
<td>ARHGIDA</td>
<td>Rho GDP dissociation inhibitor α/RhoGDIα; related to actin cytoskeletal rearrangements, related to poor prognosis in lung cancer</td>
<td>−1.55</td>
<td>0.82</td>
<td>0.92</td>
</tr>
<tr>
<td>NM_006270</td>
<td>BRAS</td>
<td>related RAS viral oncogene homologue; promotes cell motility and metastasis in cervical cancer</td>
<td>−1.53</td>
<td>0.73</td>
<td>0.7</td>
</tr>
<tr>
<td>NM_001665</td>
<td>RHOG</td>
<td>ras homologue gene family, member G/ARHG; promotes cytoskeletal reorganization and migration, related to lymph node metastasis and poor prognosis in breast cancer</td>
<td>−1.24</td>
<td>0.86</td>
<td>0.74</td>
</tr>
<tr>
<td>NM_004308</td>
<td>ARHGAPI</td>
<td>Rho GTPase activating protein 1/RhoGAP1; modulates Rho-mediated signaling pathways through activation of p21-Rho, cancer relation unknown</td>
<td>−1.21</td>
<td>0.85</td>
<td>0.83</td>
</tr>
</tbody>
</table>

(Continued on the following page)
to the cytoplasm may be related to the malignant phenotype. Therefore, it is reasonable to categorize PTTG1 expression levels by cytoplasmic staining of PTTG1 in ESCC cells. In fact, nuclear PTTG1 staining in 113 ESCC tumors did not correlate with any clinicopathologic features, whereas cytoplasmic PTTG1 staining correlated with several clinical parameters. Mechanisms underlying dysregulation in the subcellular translocation of PTTG1 in ESCC cells remain unclear. However, two different siRNAs directed against PTTG1 effectively knocked down both cytoplasmic and nuclear PTTG1 protein expression in ESCC cells.

The process of lymph node metastasis consists of many steps, including tumor enlargement, lymphangiogenesis, migration into lymphatic capillaries, survival in the lymphatic stream, entry into the subcapsular sinus, invasion of the lymph node cortex, and persistent proliferation (25). The endothelium of lymph capillary has overlapping loose junctions and a discontinuous or absent basement membrane, permitting the passage of large biological macromolecules, pathogens, and migrating cells (26). Once tumor cells reach the adjacent lymphatic capillaries (formation of intratumor lymphatic capillaries may be unnecessary; ref. 27), tumor cells migrate into capillary lumina either through open interendothelial gaps or by inducing the opening of closed gaps (18). As clearly shown in Transwell and Matrigel chamber assays with siRNAs against PTTG1, PTTG1 knockdown reduced cell motility, but interestingly, not penetration through the Matrigel layer. This finding suggests that PTTG1 may promote the entry of tumor cells into lymph capillary lumina by increasing tumor cell motility, which is considered an early stage in the multistep theory of lymph node metastasis. Previously, we have shown that cell motility is closely associated with lymph node metastasis and poor survival (14, 18, 28, 29). For example, reduced expression of motility-related protein 1 and overexpression of chemokine (CC motif) receptor 7 were associated with lymph node metastases in ESCCs (28, 29). Similarly, overexpression of fascin, an actin-bundling protein related to cell motility, was significantly associated with regional lymph node metastasis and poor survival in ESCC patients, and down-regulation of fascin in ESCC cells decreased their motility (18). Moreover, overexpression of osteopontin, an integrin-binding secreted glycoprotein associated with cell motility, was significantly associated with distant lymph node metastasis and poor survival of ESCC patients, and knockdown of osteopontin expression in ESCC cells induced by an inducible short hairpin RNA vector decreased their motility, invasiveness, and popliteal lymph node metastasis from footpads of nude mice (14). As shown in the current study, knockdown of PTTG1 expression in ESCC cells by in vitro and in vivo transfection significantly decreased popliteal lymph node metastases from footpads in nude mice. Thus, PTTG1 may be related to cell motility and lymph node metastasis in ESCC.

To identify potential downstream genes in the PTTG1 pathway, we performed global gene expression profiling using a bead array–based technique and siRNAs directed against PTTG1. As shown in Table 3, siRNAs directed against PTTG1 down-regulated the expression of several Ras and Rho gene family members known to play central roles in cell motility via actin cytoskeleton rearrangement (30). Some of the genes identified by this strategy have exhibited clinical correlation with poor survival or metastasis. Rras, which plays a key role in cell motility by modulating Rho and Rac activities (31), promotes metastasis in cervical cancer (32). Rhog, which regulates cell motility through Rac1 activation (33), is related to lymph node metastasis and poor prognosis in breast cancer patients (34). Argidia, which acts as an inhibitor of Rho family members, is related to poor survival in lung cancer patients (35). Ahrgap1 modulates Rho-mediated signaling pathways through activation of p21-Rho (36), but the relationship between AHRGAP1 and cancer metastasis is unknown. Interestingly, FSCN1 (fascin), which regulates cell motility through its actin-bundling function, was also down-regulated in our study by siRNAs against PTTG1. In contrast, potential tumor suppressors or metastasis suppressors, including Calu (37, 38), Hoxd8 (39), Gclc (40), St13 (41), and Cxcl14 (42), were up-regulated by PTTG1 down-regulation. Thbs1 (thrombospondin 1), a potential angiogenesis inhibitor, was up-regulated by PTTG1 down-regulation. Consistent with this result, Kim et al. (43) reported that Thbs1 expression was suppressed in PTTG1-overexpressing thyroid cells, and that down-regulation of PTTG1 increased Thbs1 expression. Thbs1 also predicts poor survival and represents a marker of tumor aggressiveness in thyroid cancer (44). bFGF and VEGF, which are known PTTG1-related genes, were also included in the gene list derived from our expression profiling. However, their expression levels did not change after PTTG1 knockdown in our experimental setting. Thus, cell motility regulated by PTTG1 could be independent of bFGF or VEGF in ESCC cells. Taken together, these microarray results suggest that PTTG1 may modulate the expression of multiple cell motility–related genes, including Ras-Rho oncogene superfamily members, and provide potentially valuable clues

### Table 3. Genes differentially expressed after anti-PTTG1 siRNA treatment in HSA/c and KYSE140 cells (Cont’d)

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</tr>
</thead>
<tbody>
<tr>
<td>NM_0030088</td>
<td>FSCN1</td>
<td>fascin; actin-bundling protein, promotes cell migration, related to lymph node metastasis in esophageal cancer</td>
<td>−1.13</td>
<td>0.71</td>
<td>0.89</td>
</tr>
<tr>
<td>NM_0022905</td>
<td>ITGA5</td>
<td>integrin, αβ, fibronectin receptor α; related to invasion of bladder cancer</td>
<td>−0.62</td>
<td>0.58</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Abbreviation: SAM, significance analysis of microarrays.

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to understanding the mechanisms underlying cell migration regulated by PTTG1.

In summary, PTTG1 overexpression in ESCC primary tumors was associated with lymph node metastasis and poor survival, and PTTG1 knockdown inhibited ESCC cell motility in vitro and lymph node metastasis in vivo, possibly by regulating Ras-Rho motility-related gene family members. Therefore, it is possible that PTTG1 promotes lymph node metastasis in ESCC by enhancing cell motility. In addition, PTTG1 may represent a useful biomarker of poor survival and lymph node metastasis in ESCC, as well as a promising therapeutic target for the treatment of lymph node metastases in ESCC patients.

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References

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