

an in-silico target of miR-373*, which is downregulated in diffuse-type gastric cancer.

In this study, we identified microRNAs related to the progression of gastric cancer. In breast cancer, tumour invasion and metastasis are initiated by miR-10b,²⁴ which is one of the microRNAs associated with invasion in gastric cancer. miR-21 was selected in the progression signature of both T and stage, and it targets programmed cell death 4 (*PDCD4*) and maspin (*SERPINB5*), resulting in tumour invasion and metastasis.²⁴ Another group showed that miR-21 targets a tumour-suppressor gene, reversion-inducing-cysteine-rich protein with kazal motifs (*RECK*), and that knockdown of miR-21 decreased invasion and migration of gastric cancer cells significantly.³⁰ The microRNAs that were related most significantly to progression of gastric cancer—miR-125b, miR-199a, and miR-100—were also upregulated in pancreatic adenocarcinoma in our previous study.²¹ miR-125b is reportedly related to proliferation of differentiated cells³² and downregulated in breast cancer³⁴ and thyroid anaplastic carcinoma,³² suggesting that this microRNA functions differently in gastric cancer and pancreatic adenocarcinoma. Proapoptotic *BAK1* and *TP53* are proven targets of miR-125b in prostate cancer and neuroblastoma cells, supporting the oncogenic function of miR-125b.^{34,35} Upregulation of miR-199a is associated purportedly with tumour cell growth in cervical carcinoma.³⁶

We identified microRNAs associated with an unfavourable outcome (independent of clinical factors) in specimens from patients treated by curative surgery and adjuvant chemotherapy. Although our findings should be validated in an independent cohort, these microRNAs might help to identify individuals who are candidates for aggressive treatment because of their expression status and who could become candidates for therapeutic targets with antagomirs^{25–27} or by reconstitution with microRNA precursor sequences.²⁸ Three microRNAs selected in the progression analysis were not chosen for the prognostic signature partly because they were associated highly with clinical factors. The difference of the selected microRNAs between overall and disease-free survival is probably caused by the effect of chemotherapy after disease recurrence.

We chose let-7g and let-7b as independent prognostic factors. The Ras family of oncogenes is regulated by the let-7 family in lung cancer,^{37,38} and the high mobility group AT-hook 2 (*HMG2*) oncogene is also targeted by this microRNA family.^{37,38} *HMG2* is regulated negatively by the let-7 family, and high expression of this gene correlates with tumour invasiveness and is an unfavourable prognostic factor in gastric cancer.³⁹ Additionally, in tumour-initiating cells of breast cancer (which have stem cell-like properties), let-7 regulates self-renewal (by silencing *HRAS*) and differentiation (by silencing *HMG2*).²⁴ Administration of let-7 family members inhibits growth of lung cancer in mice.^{37,38} A negative regulator of hedgehog signalling, suppressor of fused

	Hazard ratio (95% CI)	p
Disease-free survival		
Intestinal type (n=45)		
Stage, III-IV vs I-II*	3.2 (1.1–9.1)	0.032
let-7g expression, low vs high*	2.8 (1.0–7.8)	0.043
miR-19a expression, low vs high*	7.5 (2.3–24.6)	0.001
miR-495 expression, high vs low*	4.9 (1.7–14.3)	0.004
Diffuse type (n=56)		
Stage, III-IV vs I-II*	5.5 (1.9–15.7)	0.001
let-7b expression, low vs high*	2.6 (1.1–6.2)	0.031
Overall survival*		
Intestinal type (n=45)		
Stage, III-IV vs I-II*	5.7 (2.0–16.0)	0.001
miR-433 expression, low vs high*	4.4 (1.6–12.2)	0.004
Diffuse type (n=55)		
Stage, III-IV vs I-II*	6.3 (2.1–18.9)	0.001
miR-214 expression, high vs low*	2.7 (1.0–7.3)	0.048
miR-433 expression, low vs high*	2.4 (1.0–5.6)	0.050
*Reference group. For all microRNAs, patients were categorised into high-expression and low-expression groups with the same cutoff values of microRNA expression used in tables 3 and 4. Multivariable analysis was undertaken by stepwise addition and removal of covariates found to be associated with survival in tables 3 and 4. Only final models are shown. *In overall survival of diffuse-type gastric cancer, one patient was censored before first event (patient's death) and these data were removed.		
Table 5: Multivariable Cox regression analysis of disease-free survival and overall survival of patients with intestinal-type and diffuse-type gastric cancer		

(*su[fu]*), is targeted by miR-214 in the development of zebrafish,⁴⁰ and activation of hedgehog signalling is involved in gastric cancer.³² Recently, miR-214 was reported to induce cell survival and cisplatin resistance by targeting *PTEN* in ovarian cancer.⁴¹ miR-433 targets growth factor receptor-bound protein 2 (*GRB2*) in gastric cancer.⁴²

Further studies are needed to establish whether the microRNAs we selected in this study have full potential as either biomarkers or therapeutic targets in gastric cancer. Proving new targets and other biological experiments will clarify the functions and roles of microRNAs in gastric cancer. However, we have shown already that microRNAs can meet criteria for ideal biomarkers and therapeutic targets.²²

Contributors

All authors planned and implemented the investigation. TU, YS, MK, WY, HS, GAC, and CMC had the idea for and designed the experiments. TU, SN, NO, and KY obtained samples and clinical data. TU, HO, MS, HA, and C-gL undertook the experiments. SV, CT, SR, and TU did the statistical analysis. TU, SV, CT, GAC, and CMC wrote the report. All authors critically reviewed the manuscript and approved the final version.

Conflicts of interest

The authors declared no conflicts of interest.

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ORIGINAL ARTICLE

Wnt5a signaling is involved in the aggressiveness of prostate cancer and expression of metalloproteinase

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Wnt5a is a representative ligand that activates the β -catenin-independent pathway in Wnt signaling. Although it has been reported that abnormal activation of the Wnt/ β -catenin-dependent pathway is often observed in human prostate cancer, the involvement of the β -catenin-independent pathway in this cancer is unclear. Abnormal expression of Wnt5a and β -catenin was observed in 27 (28%) and 49 (50%) of 98 prostate cancer cases, respectively, by immunohistochemical analyses. Simultaneous expression of Wnt5a and β -catenin was observed in only five cases, suggesting their exclusive expression. The positive detection of Wnt5a was correlated with high Gleason scores and biochemical relapse of prostate cancer, but that of β -catenin was not. Knockdown and overexpression of Wnt5a in human prostate cancer cell lines reduced and stimulated, respectively, their invasion activities, and the invasion activity required Frizzled2 and Ror2 as Wnt receptors. Wnt5a activated Jun-N-terminal kinase through protein kinase D (PKD) and the inhibition of PKD suppressed Wnt5a-dependent cell migration and invasion. In addition, Wnt5a induced the expression of metalloproteinase-1 through the recruitment of JunD to its promoter region. These results suggest that Wnt5a promotes the aggressiveness of prostate cancer and that its expression is involved in relapse after prostatectomy.

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Keywords: Wnt5a; prostate cancer; Gleason score; invasion; MMP-1

Introduction

Prostate cancer (PCa) is an increasingly prevalent cancer in men, which develops and progresses under the

influence of androgenic steroids (Jemal *et al.*, 2008). PCa screening by assessing serum prostate-specific antigen (PSA) level has led to increased detection of early-stage PCa that can be cured by radical prostatectomy or radiation therapy. Although overall cancer control rates are high for clinically localized diseases, 20–30% of patients will experience recurrence manifested initially as a rising PSA level without clinical or radiographic metastasis (Han *et al.*, 2003). This biochemical relapse is indicative of the presence of prostate tissue and is assumed to represent cancer. Many patients with biochemical relapse have indolent disease that grows slowly and requires no treatment but some will have rapid progression. A critical issue for patients is determination of whether rising PSA represents local or systemic disease, as the former may be cured by salvage radiotherapy and the latter requires hormone therapy. High risk of recurrence is defined according to preoperative PSA level (>20 ng/ml), biopsy Gleason score (≥ 8) and the 1992 American Joint Committee on Cancer clinical T stage ($\geq T2c$) (Partin *et al.*, 1997; D'Amico *et al.*, 2000). These factors are helpful but not perfect due to significant clinical heterogeneity. Identifying molecules that are expressed in clinically localized PCa but associated with PCa invasion and metastasis might significantly improve the prognostic capabilities and management of patients with PCa after a curative approach.

The accumulation of cytoplasmic and nuclear β -catenin has been documented in many malignancies, including breast, gastric, colon, esophageal, hepatic, pancreatic, thyroid, cerebellar and skin carcinoma (Polakis, 2000; Kikuchi, 2003). In PCa, abnormal accumulation of β -catenin has been detected in 20–50% of tumors, and high levels of β -catenin expression are associated with advanced, metastatic and hormone-refractory PCa (Yardy and Brewster, 2005). Although β -catenin was originally identified as a cadherin-binding protein, it is known to be a key molecule in the Wnt signaling pathway. Wnt proteins are a large family of cysteine-rich secreted molecules that exhibit unique expression patterns and distinct functions in development (Logan and Nusse, 2004). The well-established intracellular signaling pathway activated by Wnt proteins is a β -catenin-dependent signaling pathway that is

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highly conserved among species (Logan and Nusse, 2004; Kikuchi *et al.*, 2009). When Wnt acts on its cell-surface receptor, which consists of Frizzled and low-density lipoprotein receptor-related protein 5/6, cytoplasmic β -catenin is stabilized by release from the Axin complex. The accumulated β -catenin is translocated to the nucleus, where it binds to the transcription factor T-cell factor/lymphoid enhancer factor and thereby stimulates the expression of various genes (Hurlstone and Clevers, 2002). At least 19 Wnt members have been shown to be present in mammals to date, and some Wnts, including Wnt1, Wnt3a and Wnt7a, activate the β -catenin pathway. In addition to T-cell factor/lymphoid enhancer factor, β -catenin binds to androgen receptor, and these Wnt ligands also increase androgen receptor-mediated transcription even in the absence of androgen ligands (Verras *et al.*, 2004). Therefore, activation of the β -catenin pathway appears to be involved in the initiation and progression of PCa as shown in other tumors.

Another class of Wnts, including Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6 and Wnt11, activates a β -catenin-independent pathway that primarily modulates cell movement and polarity (Veeman *et al.*, 2003). This pathway is known to activate several protein kinases including Ca^{2+} /calmodulin-dependent protein kinase II, protein kinase C (PKC), c-jun N-terminal kinase (JNK) and Rho-associated kinase. Wnt5a is a representative of the Wnt proteins that activate the β -catenin-independent pathway, which includes multiple pathways, and Wnt5a activates distinct routes (Veeman *et al.*, 2003; Kurayoshi *et al.*, 2007; Kikuchi and Yamamoto, 2008). It has been shown that Wnt5a stimulates migration in some cancer cells and that its expression is correlated with the aggressiveness of melanoma, breast cancer, lung cancer and gastric cancer (Weeraratna *et al.*, 2002; Veeman *et al.*, 2003; Huang *et al.*, 2005; Kurayoshi *et al.*, 2006; Pukrop *et al.*, 2006; Kikuchi and Yamamoto, 2008; Yamamoto *et al.*, 2009), suggesting that Wnt5a has oncogenic properties. Other reports indicate that Wnt5a acts as a tumor suppressor based on the finding that Wnt5a has an ability to inhibit proliferation, migration and invasiveness in thyroid tumor and colorectal cancer cell lines (Dejmek *et al.*, 2005; Kremnevskaja *et al.*, 2005). Although the β -catenin-independent pathway activated by Wnt5a is also involved in tumorigenesis, the relationship between the expression of Wnt5a and PCa is not well understood. This study showed that a high expression level of Wnt5a significantly correlates with biochemical relapse of clinically localized PCa cases treated with radical prostatectomy. It was also shown that Wnt5a promotes invasion activities of PCa cells at least through the activation of JNK and the expression of matrix metalloproteinase-1 (MMP-1).

Results

Immunohistochemical analysis of Wnt5a in PCa tissues
Preceding immunohistochemical studies showed that approximately 30% of 237 gastric cancer cases exhibit high expression levels of Wnt5a (Kurayoshi

et al., 2006). Using the same antibody, we examined the expression of Wnt5a in PCa. In adjacent non-neoplastic prostate tissue including glandular hyperplasia, weak or no staining of Wnt5a was observed in epithelial and stromal cells (Figure 1a). However, PCa tissue showed stronger and more extensive staining than corresponding non-neoplastic mucosa (Figure 1a). In the majority of PCa cases containing Wnt5a-positive tumor cells, more than 50% of the tumor cells showed cytoplasmic staining for Wnt5a. Of 98 PCa cases, 27 (28%) were positive for Wnt5a. In these PCa cases, no tendency of strong staining for Wnt5a at the invasive front was observed. The relationship between Wnt5a staining and clinicopathological characteristics was analyzed. Wnt5a positivity was found more frequently in PCa showing a Gleason score ≥ 8 (12/24, 50%) than in PCa showing a Gleason score ≤ 7 (15/74, 20%, $P=0.0079$, Fisher's exact test) (Supplementary Table S1). Therefore, the expression of Wnt5a may be associated with the aggressiveness of PCa. However, Wnt5a staining did not correlate with age, pT classification or preoperative PSA concentration (Supplementary Table S1).

An immunohistochemical analysis of β -catenin expression in PCa was also performed. Although β -catenin was usually detected at the cell membranes, cytosomal or nuclear accumulation of β -catenin was observed in 49 (50%) of 98 PCa cases (Supplementary Table S2). However, β -catenin staining in cytoplasm and nucleus did not correlate with age, pT classification, Gleason score or preoperative PSA concentration (Supplementary Table S2). These results suggested that the abnormal expression of β -catenin may be involved in the initiation of PCa but not in the aggressiveness of the tumor. The relationship between the expression of Wnt5a and β -catenin in PCa was analyzed further. Wnt5a positivity was found more frequently in cytosomal or nuclear β -catenin-negative cases (22/49, 45%) than in cytosomal or nuclear β -catenin-positive cases (5/49, 10%, $P=0.0002$, Fisher's exact test) (Supplementary Table S1). In the five PCa cases positive for both Wnt5a and cytosomal or nuclear β -catenin, there was a tendency that Wnt5a-positive cancer cells do not show cytosomal or nuclear accumulation of β -catenin (Figure 1b). These findings suggested that Wnt5a and cytosomal and nuclear β -catenin are expressed in an exclusive pattern in PCa.

Relapse of patients with PCa expressing Wnt5a

Next, the relationship between Wnt5a immunostaining and relapse in PCa was examined. Univariate analysis revealed that the expression of Wnt5a ($P=0.0045$, log-rank test) decreases the ratios of relapse-free survival in patients as well as high Gleason score ($P<0.0001$) and high preoperative PSA concentration ($P=0.0167$) (Figure 2a), whereas cytosomal or nuclear accumulation of β -catenin, age and pT classification did not correlate with relapse (Figure 2b). A Cox proportional hazards multivariate model was used to examine the relationship between clinicopathological factors, expression of Wnt5a and β -catenin, and relapse-free survival. Multi-

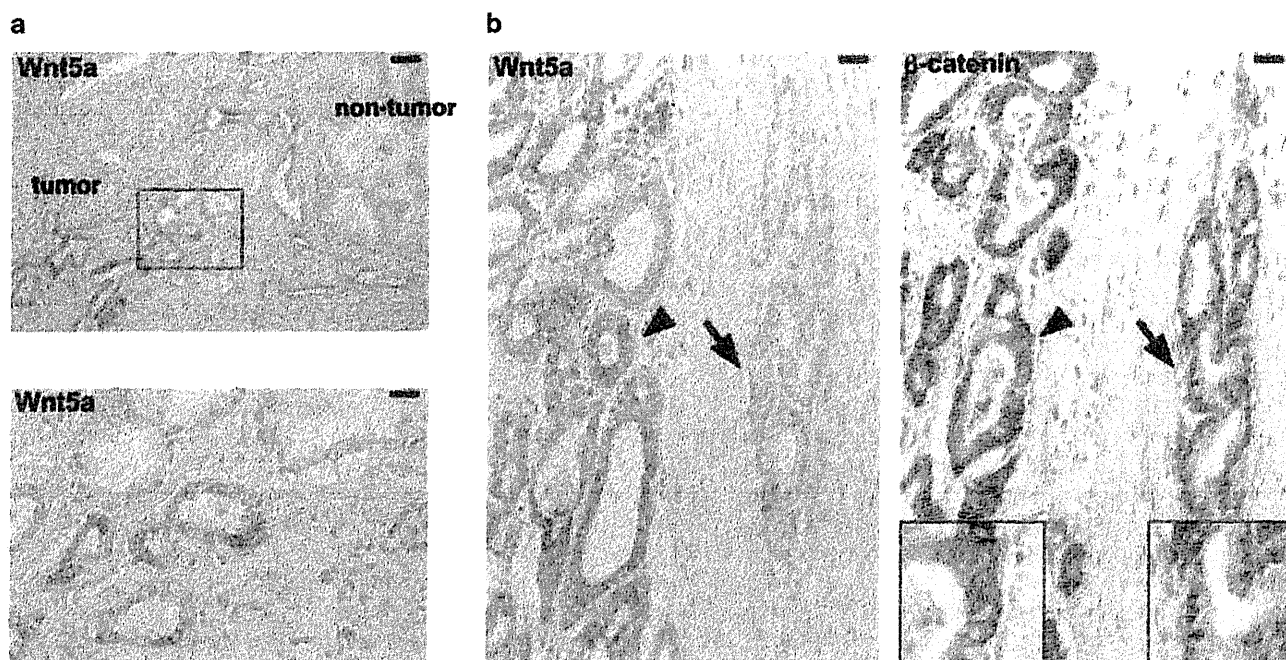


Figure 1 Immunohistochemical analyses of Wnt5a and β -catenin in prostate cancer (PCa). (a) Top panel, expression levels of Wnt5a in nontumor and tumor regions were compared. Bar, 50 μ m. Bottom panel, high-magnification image of the fields indicated by the box in the upper panel. The tumor regions were enlarged. Bar, 12 μ m. (b) A sample of PCa was stained with anti-Wnt5a (left panel) and anti- β -catenin (right panel) antibodies. Bars, 25 μ m. Arrowheads indicate Wnt5a-positive and cytosomal or nuclear β -catenin-negative PCa cells. Arrows indicate Wnt5a-negative and cytosomal or nuclear β -catenin-positive PCa cells. Insets, high-magnification images of the fields indicated by the arrow and arrowhead in the right panel.

variate analysis indicated that Wnt5a staining, Gleason score and preoperative PSA concentration are independent predictors of relapse of PCa, but cytosomal or nuclear β -catenin staining, age and pT classification are not (Table 1). These results suggested that Wnt5a expression contributes directly to the malignant potential of PCa.

Involvement of Wnt5a in migration and invasion of PCa cells

To understand the relationship between the expression of Wnt5a and aggressiveness of PCa, we examined the expression levels of various Wnts in PCa cells (Figure 3a). DU145 and PC3 cells are androgen-independent PCa cells and LNCap cells are androgen-dependent PCa cells. *Wnt5a* mRNA was highly expressed in DU145 and LNCap cells, but PC3 cells showed a low expression level. mRNA expression of *Wnt4* was observed in all cell lines. *Wnt5b* mRNA was detected in DU145 but not in LNCap and PC3 cells. Neither *Wnt3a* nor *Wnt11* mRNA was detected in these cells. Wnt5a siRNA reduced the mRNA level of *Wnt5a* in LNCap cells and suppressed migration activity in transwell assays using a Boyden chamber (Figure 3b, Supplementary Figure S1). Knockdown of Wnt5a in DU145 cells also decreased cell migration (Figure 3c), but knockdown of Wnt5b did not (data not shown). Wnt7a siRNA did not affect cell migration of LNCap and DU145 cells (Figures 3b and c, Supplementary Figure S1). Migration activity in Wnt4-knockdown cells

was decreased to about 70% of control cell (Figure 3c). Wnt4 has been reported to activate both the β -catenin-dependent and β -catenin-independent pathways (Bernard and Harley, 2007), but the role of Wnt4 in cell migration is not well understood. Therefore, we did not study the role of Wnt4 in migration of PCa cells further. It is known that DU145 and PC3 cells, but not LNCap cells, have invasion activities. Whereas control DU145 cells invaded the Matrigel, Wnt5a knockdown cells were less invasive (Figure 3c). Transient overexpression of Wnt5a enhanced the invasion activities of PC3 cells, but that of Wnt5a CA, which is an inactive form of Wnt5a generated by mutating Cys104 to Ala (Kurayoshi *et al.*, 2007), did not (Figure 3d).

Secreted Frizzled-related protein 2 (sFRP2) binds to Wnt proteins and acts as a negative regulator of Wnt signaling (Kawano and Kypta, 2003). DU145 cells were allowed to migrate in scratch-wound cultures, resulting in wound closure after 24 h, and the migration of DU145 cells in scratch-wound cultures was inhibited by the addition of sFRP2 conditioned medium (CM) (Figure 3e). Furthermore, an anti-Wnt5a antibody suppressed the migration of DU145 cells in scratch-wound cultures (Figure 3f). Taken together, these results indicated that Wnt5a stimulates cell migration and invasion in PCa cells.

Mechanism of Wnt5a-induced invasion by PCa cells

The mechanism by which Wnt5a induces invasion of DU145 and PC3 cells was examined as an *in vitro* model

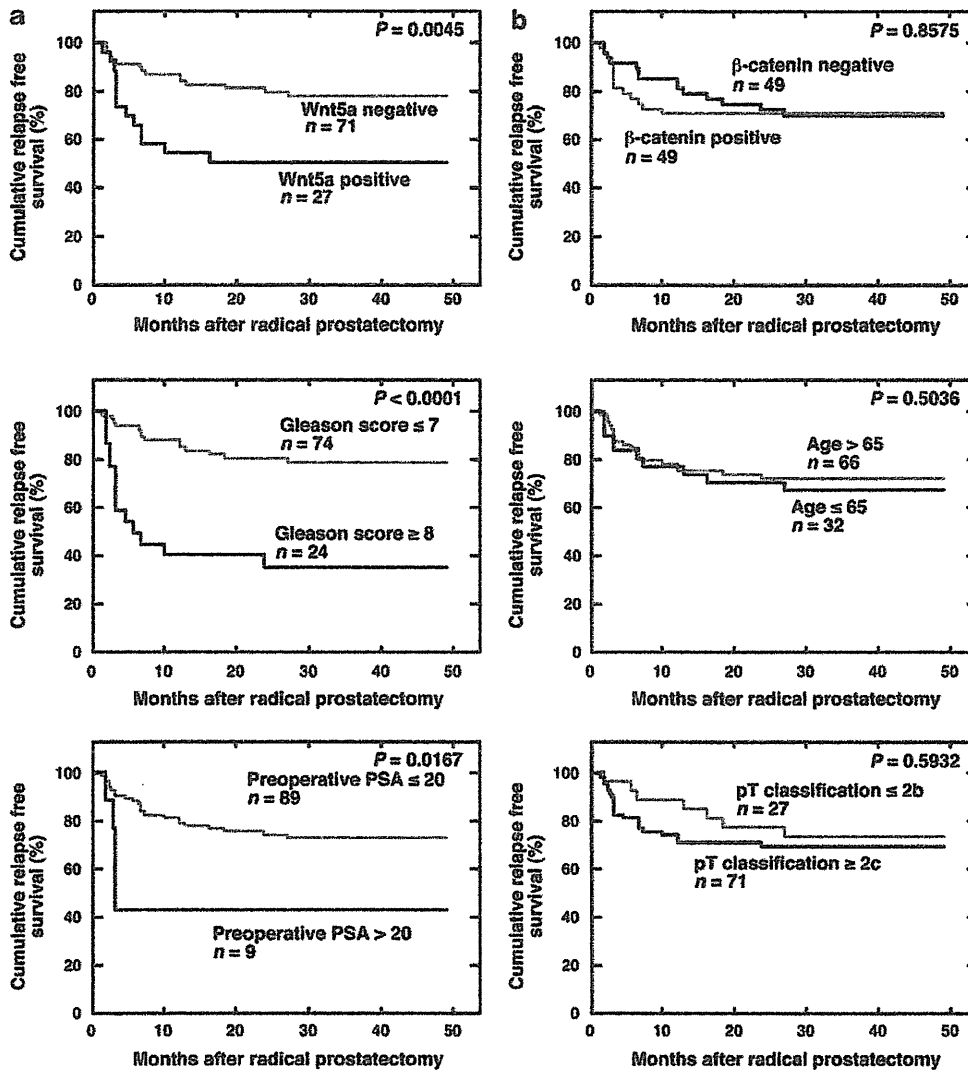


Figure 2 Relapse-free survival of patients with prostate cancer (PCa) expressing Wnt5a or β -catenin. (a) Kaplan–Meier curves of patients with PCa with Wnt5a-negative or Wnt5a-positive PCa (upper panel), with low Gleason score (≤ 7) or high Gleason score (≥ 8) PCa (middle panel), and with low preoperative prostate-specific antigen (PSA) concentration (≤ 20 ng/ml) or high preoperative PSA concentration (> 20 ng/ml) (lower panel). (b) Kaplan–Meier curves of patients with PCa with or without β -catenin expression in the cytoplasm and nucleus (upper panel), with younger (≤ 65) or older (> 65) age (middle panel) and with low pT classification ($\leq 2b$) or high pT classification ($\geq 2c$) PCa (lower panel).

of invasive PCa cells. Frizzled (Fz) family members are known to function as Wnt receptors (Wang *et al.*, 2006). Fz2, Fz6 and Fz7 were expressed highly in DU145 cells compared with other Fzs (Supplementary Figure S2a). Wnt5a bound to Fz2, Fz5 and Fz8 but not to Fz6 and Fz7 (Sato *et al.*, 2010) (data not shown), and Ror2, a single transmembrane protein, is known to function as a Wnt5a receptor (Oishi *et al.*, 2003). To examine which receptor(s) are involved in cell migration of DU145 cells, we depleted Wnt receptors by siRNA (Supplementary Figure S2b). Knockdown of Fz2 and Ror2 but not Fz6 reduced cell invasion by DU145 cells significantly (Figure 4a). Knockdown of Fz7 showed a tendency to decrease cell invasion, but the difference was not statistically significant (Figure 4a). Wnt5a induced the phosphorylation of protein kinase D (PKD)/PKC μ in DU145 cells (Figure 4b). PKD is a protein kinase, which

not only is a direct target of diacylglycerol but also lies downstream of novel PKCs (Rozenfurt *et al.*, 2005). Therefore, these results suggested that Wnt5a activates novel PKCs. Staurosporine, a PKC inhibitor, indeed suppressed Wnt5a-dependent migration and invasion activities of DU145 cells (Figure 4b). Furthermore, Gö6976, an inhibitor that is relatively specific for PKD, suppressed Wnt5a-dependent migration and invasion activities of DU145 cells (Figure 4b). These results suggested that PKD activation by Wnt5a probably through the activation of novel PKCs is involved in Wnt5a-dependent migration and invasion. In addition, Wnt5a activated small G protein Rac, which has a role in cell migration (Figure 4c).

It has been reported that many genes associated with aggressive behavior, including Wnt5a and MMP-9, were increased in androgen-independent metastatic tumors

Table 1 Multivariate analysis of factors influencing relapse-free survival

	Hazard ratio (95% CI)	χ^2	P-value
<i>Wnt5a staining</i>			
Negative	1 (Reference)	3.907	0.0312
Positive	2.451 (1.007–5.960)		
<i>Cytosomal or nuclear β-catenin staining</i>			
Negative	1 (Reference)	1.681	0.1947
Positive	1.782 (0.744–4.265)		
<i>Age</i>			
≤ 65	1 (Reference)	0.101	0.7507
> 65	1.138 (0.513–2.525)		
<i>pT classification</i>			
$\leq 2b$	1 (Reference)	0.161	0.6882
$\geq 2c$	1.215 (0.469–3.148)		
<i>Gleason score</i>			
≤ 7	1 (Reference)	10.976	0.0009
≥ 8	3.912 (1.745–8.769)		
<i>Preoperative PSA concentration</i>			
≤ 20	1 (Reference)	4.574	0.0325
> 20	3.176 (1.101–9.161)		

Abbreviations: CI, confidence interval; PSA, prostate-specific antigen.

and that MMP-1 is involved in invasion by DU145 cells (Stanbrough *et al.*, 2006; Zeng *et al.*, 2006). The stimulation of DU145 cells with Wnt5a increased the expression of MMP-1 (collagenase) mRNA but not those of MMP-2 (gelatinase A), MMP-3 (stromelysin-1) or MMP-9 (gelatinase B) mRNA, and knockdown of Wnt5a decreased the levels of MMP-1 mRNA (Figure 5a). Consistent with these results, Wnt5a increased the protein levels of MMP-1 (Figure 5a). As shown in transient expression of Wnt5a in PC3 cells, the invasion activities of PC3 cells were also enhanced by stable expression of Wnt5a, and knockdown of MMP-1 suppressed the invasion activities (Figure 5a). To evaluate the role of Wnt5a on MMP-1 promoter activity, we transfected the MMP-1 5'-flanking region containing two activator protein-1 (AP-1) sites (-517/+60) with luciferase gene into PC3 cells (Figure 5b). Wnt5a increased the promoter activity (Figure 5b). It was reported that the proximal AP-1 site at -72 is necessary for the phorbol ester-induced expression of MMP-1 (Hall *et al.*, 2003). The basal reporter gene activity was decreased by introducing mutations in this area (*AP-1 mut*), and Wnt5a did not stimulate it (Figure 5b). Consistent with these results, Wnt5a indeed induced the phosphorylation of JNK at Thr183 and Tyr185, which indicates the activation of JNK, in DU145 cells (Figure 5c). In addition, Gö6976 suppressed Wnt5a-dependent JNK activation (Figure 5c), suggesting Wnt5a activates JNK through PKD. c-Jun or JunD has been shown to bind to the AP-1 site in the promoter region of MMP-1 in MKN45 and U937 cells (Doyle *et al.*, 1997; Wu *et al.*, 2006). In a chromatin immunoprecipitation assay, MMP-1 promoter occupancy of JunD was decreased in DU145/Wnt5a knockdown cells compared with DU145/control cells (Figure 5d). Furthermore, Gö6976 interfered the binding of JunD to MMP-1 promoter, but knockdown of Rac did not affect the

Wnt5a-induced binding of JunD and MMP-1 promoter (Figure 5d). Taken together, these results suggested that Fz2 and Ror2 function as Wnt5a receptors in this signaling of PCa cells and that PKD and JNK mediate Wnt5a-dependent expression of MMP-1 through the recruitment of JunD to the AP-1 site of the MMP-1 promoter.

Discussion

Clinical relevance of Wnt5a expression in PCa

PCa is the most commonly diagnosed malignancy, and its incidence is rising in many countries (Hsing *et al.*, 2000; Jemal *et al.*, 2008). The present results showed that the expression of Wnt5a is correlated with a prostatectomy Gleason score ≥ 8 . Gleason score is the most frequently used grading system for PCa and is a powerful prognostic indicator (Gleason and Mellinger, 1974). It has also been reported that prostatectomy Gleason score is a predictor of distant metastasis (Pound *et al.*, 1999). When the Gleason score was ≥ 8 , the probability of distant metastasis was $> 65\%$ at 5 years. In the present cases, patients with a Gleason score ≥ 8 indeed showed a significantly higher risk of biochemical relapse. Furthermore, multivariate analyses showed that the expression of Wnt5a is an independent predictor of biochemical relapse, along with prostatectomy Gleason score and preoperative PSA concentration, indicating that Wnt5a might be a good indicator of the recurrence of PCa. Biochemical relapse indicates the presence of PCa, which may have already migrated to distant sites when the prostatectomy was performed. PCa cells positive for Wnt5a expression could have an ability to invade. Knockdown and overexpression of Wnt5a in PCa cells indeed inhibited and activated, respectively, their migration and invasion activities. Taken together with the observations that sFRP2 and anti-Wnt5a antibody inhibited migration of PCa cells, it is conceivable that Wnt5a is a candidate molecular target of therapy for PCa.

It has been reported that high levels of β -catenin are associated with aggressiveness in PCa (Yardy and Brewster, 2005). Among the current 98 cases, PCa abnormally expressing both Wnt5a and β -catenin was observed in only 5 cases. This is similar to the situation in cases of gastric cancer (Kurayoshi *et al.*, 2006). At present the reason why the expression of Wnt5a and β -catenin is mutually exclusive is not known.

Mechanism by which Wnt5a promotes aggressiveness of PCa

How is Wnt5a involved in the aggressiveness of PCa? Wnt5a increased MMP-1 mRNA and protein levels in PCa cells, but it did not induce the expression of MMP-2, MMP-3 and MMP-9 mRNAs. MMPs are zinc-containing endopeptidases that degrade extracellular matrix components and are associated with cancer cell invasion and metastasis (Egeblad and Werb, 2002). It was suggested that upregulation of MMP-1 is an

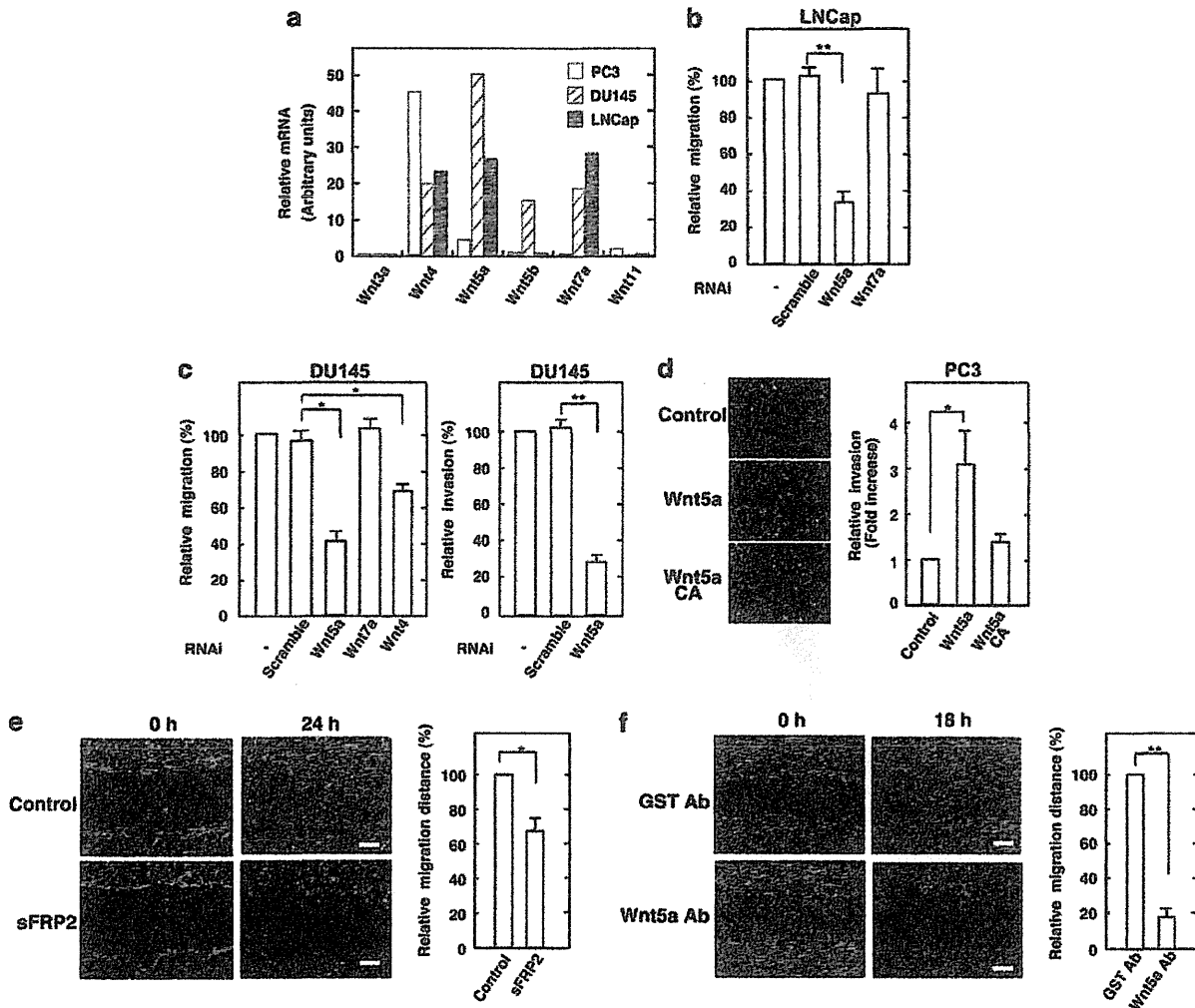


Figure 3 Wnt5a is involved in migration and invasion of prostate cancer (PCa) cells. (a) The mRNA levels of various Wnts in PC3, DU145 and LNCap cells were quantified by quantitative reverse transcription (RT)-PCR. (b) LNCap cells transfected with *scrambled* (control), *Wnt5a* or *Wnt7a* siRNA were placed in transwell chambers for a migration assay toward fibronectin. In three independent fields, 130 ± 7 untransfected cells transmigrated. Relative migration activities were expressed as percentages for the migration of untransfected cells. The results shown are means \pm s.e. from three independent experiments. $**P < 0.01$. (c) DU145 cells transfected with *scrambled*, *Wnt5a*, *Wnt7a* or *Wnt4* siRNA were placed in noncoated (left panel) or Matrigel-coated (right panel) transwell chambers for migration and invasion assays. In three independent fields, 189 ± 11 untransfected cells transmigrated and 34 ± 6 untransfected cells invaded. Relative migration and invasion activities were expressed as percentages for the migration and invasion of untransfected cells. $*P < 0.05$; $**P < 0.01$. (d) Left panel, PC3 cells transfected with pPGK empty vector (control), pPGK/wild-type Wnt5a (*Wnt5a*), *Wnt5a* or pPGK/*Wnt5a*^{C104A} (*Wnt5a* CA) were placed in Matrigel-coated transwell chambers for an invasion assay. Right panel, in eight independent fields, 22 ± 12 of PC3 cells transfected with empty vector invaded. Relative invasion activities were expressed as fold increases compared with the invasion of transfectants with empty vector. $*P < 0.05$. (e) Control or secreted Frizzled-related protein 2 (sFRP2) conditioned medium (CM) was added to DU145 cells and then the cells were wounded. The culture was further continued for 24 h. Bars, 200 μ m. Right graph, migration distances were measured and expressed as percentages of the migration in the presence of control CM. $*P < 0.05$. (f) DU145 cells incubated with anti-glutathione *S*-transferase (GST) or anti-Wnt5a antibody (10 μ g/ml) were wounded. The culture was continued for 18 h. Bars, 200 μ m. Right graph, migration distances were measured and expressed as percentages of the migration in the presence of anti-GST antibody. $**P < 0.01$.

important factor in the aggressiveness of PCa and bone marrow metastasis (Hart *et al.*, 2002). This study showed that knockdown of MMP-1 indeed suppressed Wnt5a-dependent invasion of PC3 cells *in vitro*.

Although how MMP-1 is overexpressed in PCa is not clear, one report showed that a pathway involving FAK, PI3K and PKC δ activated by engagement of integrin α 5 β 1 with fibronectin regulates the expression of MMP-1 in DU145 cells (Zeng *et al.*, 2006). It was also shown

previously that Wnt5a induces the expression of MMP-1 in endothelial cells although the mechanism was not known (Masckauchan *et al.*, 2006). This study found that Wnt5a induces the phosphorylation of PKD/PKC μ , which is a direct target of novel PKCs (PKC δ , PKC ϵ , PKC η and PKC θ), and activates JNK. Furthermore, it was shown that Wnt5a signaling recruits JunD to the AP-1 site of the *MMP-1* promoter region. These results were consistent with the previous observations

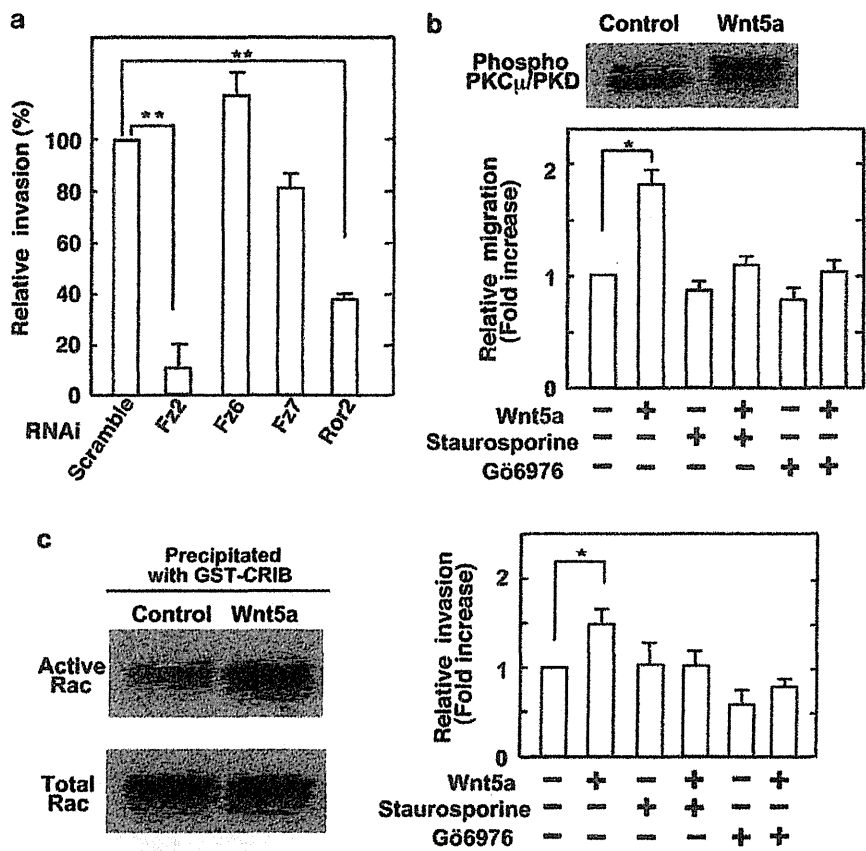


Figure 4 Wnt5a stimulates cell migration and invasion by activating protein kinase C (PKC). (a) DU145 cells transfected with *scrambled*, *Fz2*, *Fz6*, *Fz7* or *Ror2* siRNA were placed in Matrigel-coated transwell chambers for an invasion assay. In three independent fields, 37 ± 7 transfectants with *scrambled* siRNA invaded. Relative invasion activities were expressed as fold increases compared with the transfectants with *scrambled* siRNA. The results shown are means \pm s.e. from three independent experiments. $**P < 0.01$. (b) Top panel, DU145 cells were stimulated with 300 ng/ml Wnt5a for 30 min, then the cell lysates were probed with anti-phospho protein kinase D (PKD)/PKC μ antibody. Middle panel, after DU145 cells were treated with 1.25 nM staurosporine or 100 nM Gö6976 for 2 h, the cells were subjected to the transwell migration assay in the presence or absence of 200 ng/ml Wnt5a. Relative migration activities were expressed as fold increases compared with that of cells in the absence of Wnt5a and without staurosporine. Bottom panel, after DU145 cells were treated with 2.5 nM staurosporine or 100 nM Gö6976 for 2 h, the cells were subjected to the Matrigel invasion assay in the presence or absence of 600 ng/ml Wnt5a. $*P < 0.05$. (c) DU145 cells were treated with 50 ng/ml Wnt5a for 1 h, and then cells were lysed and probed with anti-Rac1 antibody. The same lysates were incubated with glutathione *S*-transferase (GST)-Cdc42/Rac-interacting binding domain (CRIB) immobilized on glutathione-sepharose to examine the activation of Rac. The total lysates and precipitates were probed with anti-Rac1 antibody. The results shown are representative of three independent experiments.

that the AP-1 site in the promoter region of the *MMP-1* gene is critical for its transcriptional regulation (Angel *et al.*, 1987) and that c-Jun and JunD bind to the AP-1 site (Doyle *et al.*, 1997; Wu *et al.*, 2006). In addition, knockdown of *Fz2* and *Ror2* reduced Wnt5a-dependent invasion and increment in *MMP-1* mRNA. From these results, it is suggested that the binding of Wnt5a to *Fz2* and/or *Ror2* stimulates the expression of *MMP-1* by the recruitment of JunD to the AP-1 binding site of the promoter region of the *MMP-1* gene through the activation of PKC and JNK.

As shown in other cells (Kurayoshi *et al.*, 2006, 2007), Wnt5a activated Rac, which stimulates cell migration, in DU145 cells. However, the Wnt5a-Rac pathway was not involved in the expression of *MMP-1*. Therefore, it is also possible that Wnt5a activates Rac to stimulate cell migration independently of transcription and that this

pathway cooperates with the Wnt5a/PKC pathway to stimulate cell invasion.

Although evidence has been accumulated that Wnt5a is expressed in various cancers (Weeraratna *et al.*, 2002; Veeman *et al.*, 2003; Huang *et al.*, 2005; Kurayoshi *et al.*, 2006; Pukrop *et al.*, 2006; Kikuchi and Yamamoto, 2008; Yamamoto *et al.*, 2009), how Wnt5a is upregulated in cancer cells has not been determined. It has been shown that Wnt5a is upregulated at the transcriptional level in PCa by hypomethylation in the 5'-untranslated region and that three CpG sites were consistently methylated in normal tissues but not in primary PCa (Wang *et al.*, 2007). It was also reported that membrane type 1-MMP is upregulated in PCa species and that membrane type 1-MMP-induced phenotypic changes are dependent on the expression of Wnt5a (Cao *et al.*, 2008). It is intriguing to speculate

that Wnt5a induced by membrane type 1-MMP upregulates MMP-1 and these three molecules work cooperatively to stimulate cell migration and invasion in PCa cells. Various alterations, including gene amplification, genetic mutations, transcriptional activation and epigenetic alterations, could upregulate Wnt5a expression in PCa. Further studies will be necessary to understand the functions of Wnt5a and the pathological significance of the abnormal expression of Wnt5a in cancer cells.

Materials and methods

Materials and chemicals

The *MMP-1* promoter-*luciferase* constructs and pGEX- α PAK-CRIB were provided by Dr I Clark (University of East Anglia, Norwich, UK) (Hall *et al.*, 2003) and Dr K Kaibuchi (Nagoya University, Nagoya, Japan), respectively. DU145, LNCap and PC3 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. PC3 cells stably expressing mouse Wnt5a were generated by selection with 200 μ g/ml G418. Wnt5a was purified to homogeneity, and an anti-Wnt5a antibody was generated as described previously (Kurayoshi *et al.*, 2006, 2007). sFRP2 CM was prepared from culture medium of HEK293T cells stably expressing sFRP2 as described previously (Kurayoshi *et al.*, 2006). Control CM and Wnt5a CM were prepared as described previously (Kurayoshi *et al.*, 2007).

Immunohistochemistry

Formalin-fixed and paraffin-embedded samples were sectioned, deparaffinized and stained with hematoxylin and eosin to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistochemically. For immunostaining of Wnt5a, a Dako CSA Kit (Dako, Carpinteria, CA, USA) was used according to the manufacturer's recommendation. Sections were pretreated in a microwave oven in citrate buffer for 30 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H₂O₂-methanol for 10 min, the sections were incubated with normal goat serum (Dako) for 20 min to block nonspecific antibody binding sites. The anti-Wnt5a antibody was incubated with tissue samples for 15 min at room temperature and detected by incubating for 15 min with biotinylated goat anti-rabbit immunoglobulins, and the signal was amplified and visualized using the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. The specificity of the anti-Wnt5a antibody has been characterized previously (Kurayoshi *et al.*, 2006, 2007).

For immunostaining of β -catenin, a Dako LSAB Kit (Dako) was used in accordance with the manufacturer's recommendations. After blocking nonspecific antibody binding sites, the samples were incubated with mouse monoclonal anti- β -catenin (1:20; BD Bioscience, San Jose, CA, USA), and followed by incubation with biotinylated anti-mouse IgG and peroxidase-labeled streptavidin for 10 min each. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. The staining of Wnt5a and cytosomal or nuclear β -catenin was classified according to the percentage of stained cancer cells in the tumor region. When more than 50% of cancer cells were stained, the immunostaining was considered positive.

Tissue samples

Ninety-eight primary tumors were collected from patients diagnosed with PCa who underwent surgery during the period 2000 through 2002 at the Department of Urology, Hiroshima University Hospital (Hiroshima, Japan). Identifying information for all samples was removed before analysis for strict privacy protection. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government, and the study was approved by the Ethical Committee for Human Genome Research of Hiroshima University (Hiroshima, Japan).

All patients were treated by radical prostatectomy and bilateral lymphadenectomy for clinically localized PCa and were confirmed to be node negative by pathological examination. None of the patients were treated preoperatively with hormonal or radiation therapy, and none had secondary cancer. All 98 specimens were archival, formalin-fixed and paraffin-embedded tissues. Tumor staging was performed according to the TNM classification system (Sobin and Wittekind, 2002). After prostatectomy, the serum PSA level was measured by E-test Tosoh II Assay (Tosoh, Tokyo, Japan). Patients were followed up by PSA measurement monthly during the first 6 months after prostatectomy and then every 3 months thereafter. Biochemical relapse was defined as a PSA level of 0.2 ng/ml or greater.

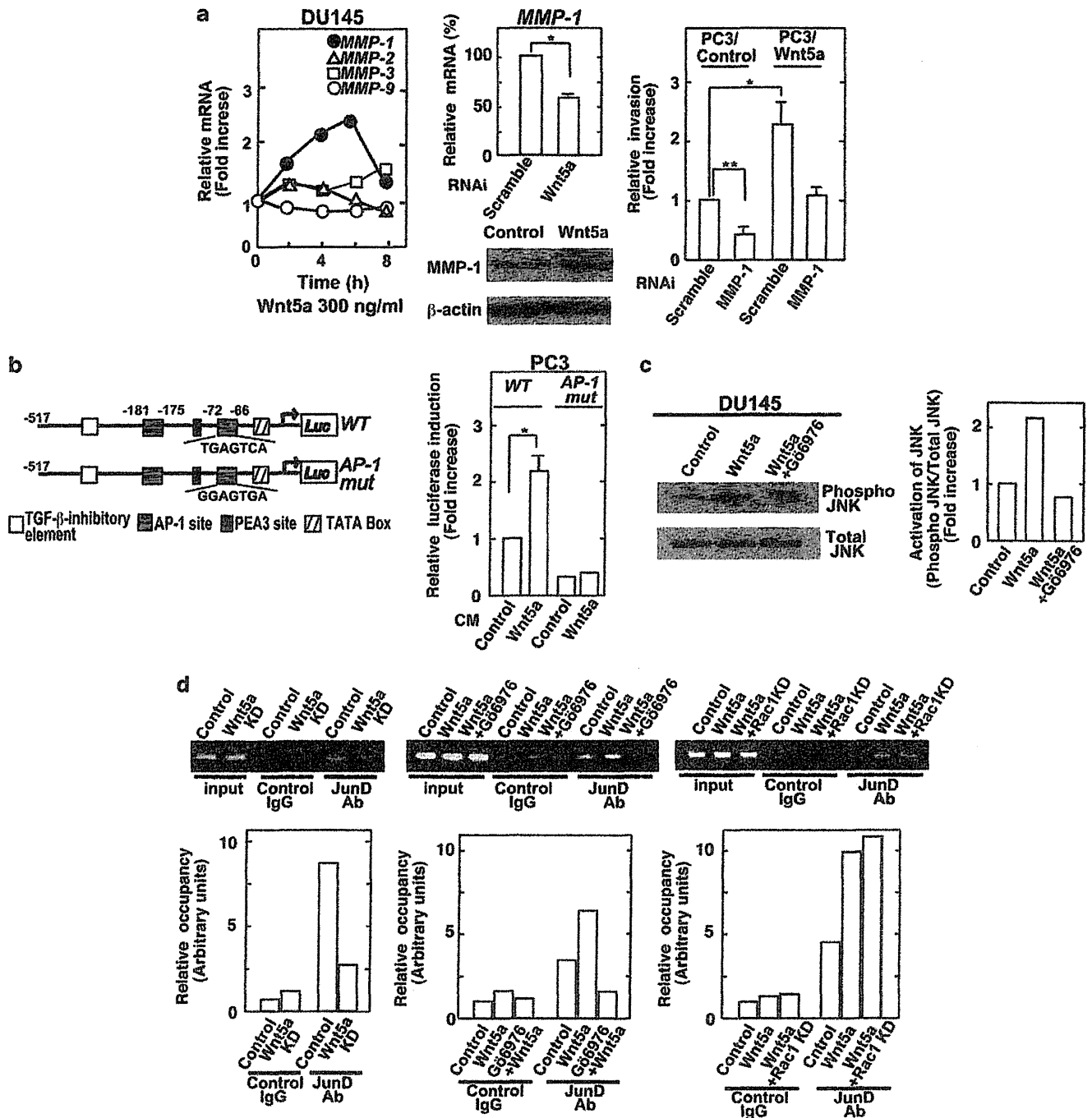
Statistical methods

Correlations between clinicopathological parameters and Wnt5a or β -catenin positivity were analyzed by Fisher's exact test. Kaplan-Meier curves were constructed, and differences between relapse-free survival curves were tested for statistical significance by log-rank test (Mantel, 1966). Cox proportional hazards multivariate model was used to examine the association of clinical and pathological factors and the expression of Wnt5a or β -catenin with relapse-free survival. Statistical analyses for Figures 3–5 were carried out using Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Cell migration and invasion assays

To measure the cell migration activity, we performed transwell assays using a modified Boyden chamber (tissue culture treated, 6.5-mm-diameter, 10- μ m-thickness, 8- μ m-pores; Transwell, Costar, Cambridge, MA, USA) as described previously (Kobayashi *et al.*, 2006). The lower surface of the filters was coated with 10 μ g/ml fibronectin for LNCap cells and 10 μ g/ml type I collagen for DU145 cells. DU145 and LNCap cells (2.5×10^4 cells in 100 μ l) suspended in serum-free RPMI-1640 medium containing 0.1% bovine serum albumin were applied to the upper chamber. The same medium was added to the lower chamber. After the cells were incubated at 37 °C for 4–8 h, the number of cells that migrated to the lower side of the upper chamber was counted. Relative cell migration was expressed as a percentage of migrated cells with siRNA treatment compared to those without treatment. The invasive potentials of DU145 and PC3 cells were analyzed using a Matrigel-coated modified Boyden chamber (Becton, Dickinson and Company, Bedford, MA, USA). RPMI-1640 medium containing 10% fetal bovine serum was added to the lower chamber. The incubations of DU145 and PC3 cells were continued for 24 and 4 h, respectively.

To carry out the wound-healing assay, we plated the cells onto fibronectin-coated coverslips. The monolayer of DU145 cells was then scratched manually with a plastic pipette tip, and after being washed with PBS, the wounded monolayers of



cells were allowed to heal for 18–24 h in RPMI-1640 medium containing 10% fetal bovine serum. The length of the wounds was measured and expressed as a percentage of the initial length at zero time (Kobayashi *et al.*, 2006). When necessary, the anti-Wnt5a antibody (10 μg/ml) or CM containing sFRP2 was added to the medium.

Chromatin immunoprecipitation assay

Cells (2 × 10⁶) were cross-linked with 1% formaldehyde for 10 min at room temperature. The cell pellets were lysed with SDS lysis buffer and sonicated to shear DNA to a size range

between 200 and 1000 bp. Sheared chromatin samples were diluted in chromatin immunoprecipitation buffer and incubated for 12 h at 4 °C with 6 μg of anti-JunD antibody (sc-74; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or negative control IgG (Diagenode, Liège, Belgium). Immunocomplexes were collected, and the DNA fragments were purified using DNA purifying slurry (Diagenode). After incubation at 55 °C for 30 min to reverse protein/DNA cross-links, the purified DNA was used as a template for PCR. Forward and reverse primers were as follows: fragment containing AP-1 sites of *MMP-1* promoter, 5'-TGTCTCCTTCGCACACATCT-3' and 5'-TGCATACTGGCCTTTGTCTT-3'.

Figure 5 Wnt5a induces the expression of matrix metalloproteinase-1 (MMP-1). (a) Left panel, DU145 cells were treated with 300 ng/ml Wnt5a for the indicated periods of time, and then *MMP-1*, *MMP-2*, *MMP-3* and *MMP-9* mRNA levels were quantified. Middle upper panel, *MMP-1* mRNA levels in DU145 cells transfected with *scrambled* or *Wnt5a* siRNA were quantified. The results shown are means \pm s.e. from three independent experiments. * $P < 0.05$. Middle lower panel, DU145 cells were stimulated with 300 ng/ml Wnt5a for 10 h, then the cell lysates were probed with anti-MMP-1 antibody. β -Actin is a loading control. Right panel, PC3/control and PC3/Wnt5a cells were transfected transiently with *scrambled* or *MMP-1* siRNA, and these cells were subjected to the Matrigel invasion assay. Relative invasion activities were expressed as fold increases compared with the invaded cell numbers in PC3/control cells transfected with *scrambled* siRNA. The results shown are means \pm s.e. from three independent experiments. * $P < 0.05$; ** $P < 0.01$. (b) Left panel, the *MMP-1-luciferase* constructs used in this study. Right panel, after PC3 cells were transfected with pGL3/*MMP-1* -517/+60-*Luc* with or without mutations in the proximal activator protein-1 (AP-1) site (*AP-1 mut* or *WT*, respectively), the cells were stimulated with control or Wnt5a conditioned medium (CM) for 10 h. Luciferase activities were expressed as fold increases compared with that of *WT* transfectants treated with control CM. The results shown are means \pm s.e. from three independent experiments. * $P < 0.05$. WT, wild type. (c) After the treatment of G66976 for 2 h, DU145 cells were stimulated with 450 ng/ml Wnt5a for 1 h. The cell lysates were probed with anti-phospho-c-jun N-terminal kinase (JNK) antibody. Right panel, the activity of JNK was calculated by dividing the band intensity of phosphorylated JNK by that of total JNK and expressed as fold increases compared with control DU145 cells. The results shown are representative of three independent experiments. (d) Left panel, chromatin from DU145/control and DU145/Wnt5aKD cells were immunoprecipitated with anti-JunD antibody or control IgG. KD, knockdown. Middle panel, after DU145 cells were stimulated with 450 ng/ml Wnt5a for 1 h in the presence or absence of 100 nm G66976, chromatin was collected and was immunoprecipitated with anti-JunD antibody or control IgG. Right panel, after DU145 cells were transfected with *scrambled* or *Rac1* siRNA, the cells were stimulated with 450 ng/ml Wnt5a for 1 h. Chromatin from each cell was immunoprecipitated with anti-JunD antibody or control IgG. The immunoprecipitated samples were analyzed by real-time PCR for the *MMP-1* promoter region containing two AP-1 sites. The relative amounts of DNA fragments containing AP-1 sites immunoprecipitated with anti-JunD antibody were expressed as arbitrary units compared with that with control IgG in control DU145 cells. The results shown are representative of three independent experiments.

Reporter gene assay

PC3 cells were transfected with pGL3/*MMP-1* -517/+60-*Luc* and pME18S/*LacZ*. At 24 h after transfection, the cells were cultured in serum-free medium for 24 h and stimulated with Wnt5a CM for further 10 h, and then the luciferase activities were measured with PicaGene reagent (Toyo Ink, Tokyo, Japan). β -Galactosidase activities were determined to normalize the transfection efficiency.

Others

Rac activity was assayed using glutathione *S*-transferase fusion Cdc42/Rac-interacting binding domain as described previously (Kurayoshi *et al.*, 2006).

Conflict of interest

The authors declare no conflict of interest.

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Serial analysis of gene expression of esophageal squamous cell carcinoma: *ADAMTS16* is upregulated in esophageal squamous cell carcinoma

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Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies worldwide. To identify potential diagnostic markers for ESCC and therapeutic targets for ESCC, we used Serial Analysis of Gene Expression (SAGE) on one ESCC sample. We obtained a total of 14 430 tags, including 5765 that were unique. By comparing SAGE tags from the ESCC sample with those from normal human squamous esophagus, we found several genes that were differentially expressed between ESCC and normal squamous esophagus. Among these, we focused on the ADAM metalloproteinase with thrombospondin type 1 motif, 16 (*ADAMTS16*) gene because quantitative RT-PCR analysis showed a high level of *ADAMTS16* expression in eight out of 20 ESCC samples (40%), but not in 15 kinds of normal tissues. Western blot analysis also showed upregulation of *ADAMTS16* protein in ESCC tissues. Furthermore, *ADAMTS16* protein was detected in culture media from the TE5 esophageal cancer cell line. Knockdown of *ADAMTS16* in TE5 cells inhibited both cell growth and invasion ability. Our present SAGE data provide a list of genes potentially associated with ESCC. *ADAMTS16* could be a novel diagnostic and therapeutic target for ESCC. (*Cancer Sci* 2010; 101: 1038–1044)

Human esophageal cancer occurs worldwide with a variable geographic distribution and ranks eighth in order of occurrence and sixth as a leading cause of cancer mortality, affecting men more than women.⁽¹⁾ There are two main forms, each with distinct etiologic and pathologic characteristics, esophageal squamous cell carcinoma (ESCC) and adenocarcinoma. ESCC is the most frequent subtype of esophageal cancer, although the incidence of adenocarcinoma in the USA and UK is increasing faster than other esophageal malignancies. Most ESCC is diagnosed at an advanced stage, and even superficial ESCC that appears to extend no further than the submucosa metastasizes to the lymph nodes in 50% of cases.⁽²⁾ In spite of the use of modern surgical techniques combined with various treatment modalities, such as chemoradiotherapy (CRT), the overall 5-year survival rate of ESCC still remains at 40–60%.⁽³⁾ Therefore, identification of new diagnostic markers for ESCC and new therapeutic targets for ESCC is important.

Better knowledge of changes in gene expression that occur during carcinogenesis might lead to improvements in diagnosis, treatment, and prevention of ESCC. Genes encoding transmembrane/secretory proteins expressed specifically in cancers may be ideal diagnostic biomarkers.⁽⁴⁾ Moreover, if the gene product functions in the neoplastic process, the gene is not just a biomarker but might also be a therapeutic target.⁽⁵⁾ To identify potential markers for early detection of ESCC and therapeutic targets for ESCC, comprehensive gene expression analysis could be useful. Studies on differential global gene expression profiling in ESCCs using cDNA and oligonucleotide arrays have been carried

out in various populations.^(6,7) Although many studies have been done on gene expression profiling of specific tumor types, and differentially expressed genes in these tumors have been reported, few of these studies have resulted in clinical applications. However, among the comprehensive methods used to analyze transcript expression levels, Serial Analysis of Gene Expression (SAGE) is a common approach.⁽⁸⁾ We previously carried out SAGE on four primary gastric cancer tissues⁽⁹⁾ and identified several gastric cancer-specific genes.⁽¹⁰⁾ Of these genes, regenerating islet-derived family, member 4 (*REG4*, which encodes Reg IV) and olfactomedin 4 (*OLFM4*, also known as GW112 or hGC-1) are highly sensitive serum markers for gastric cancer.^(11,12) However, SAGE analysis on ESCC tissue has been done in only one case.⁽¹³⁾

In the present study, we generated the SAGE library from one ESCC sample. By comparing SAGE tags from ESCC samples with those from normal human squamous esophagus (Gene Expression Omnibus accession number, GSM52501),⁽¹⁴⁾ we found several genes and tags that were differentially expressed between ESCC and normal squamous esophagus. Among these, we focused on the ADAM metalloproteinase with thrombospondin type 1 motif, 16 (*ADAMTS16*) gene because it is frequently overexpressed in ESCC, and *ADAMTS16* expression is narrowly restricted among various normal tissues. In addition, the amino acid sequence of the *ADAMTS16* protein suggests that it might be secreted. *ADAMTS* has been described as part of a family of zinc-dependent proteases (metzincin family) that play important roles in a variety of normal and pathological conditions, including arthritis and cancer.^(15,16) Although expression of *ADAMTS16* in some organs has been reported, the relationship with cancers, including ESCC, has not been studied.

Materials and Methods

Tissue samples. For SAGE analysis, one primary ESCC (75-year-old male, T2N0M0) sample was used (Fig. 1). We confirmed microscopically that the tumor specimens consisted mainly (>80%) of carcinoma tissue. For quantitative RT-PCR analysis, 20 ESCC tissue samples and corresponding non-neoplastic mucosa samples were used. For Western blot analysis, four ESCC tissue samples and corresponding non-neoplastic mucosa samples were used. The samples were obtained from surgeries at Hiroshima University Hospital and affiliated hospitals. Samples were frozen immediately in liquid nitrogen and stored at -80°C until use. Fifteen kinds of normal tissue samples, including heart, lung, esophagus, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral

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Fig. 1. Histological features of the esophageal squamous cell carcinoma sample analyzed by Serial Analysis of Gene Expression. The formalin-fixed, paraffin-embedded section was stained with H&E.

leukocytes, spleen, skeletal muscle, brain, and spinal cord, were purchased from Clontech (Palo Alto, CA, USA). Histological classification was based on the World Health Organization system. Tumor staging was done according to the TNM stage grouping system.⁽¹⁷⁾ For strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Serial analysis of gene expression. SAGE was carried out according to SAGE protocol version 1.0e (June 23, 2000). Tags were extracted from the raw sequence data with SAGE2000 analysis software version 4.12, kindly provided by Dr. Kenneth W. Kinzler (Ludwig Center for Cancer Genetics and Therapeutics and Howard Hughes Medical Institute, Johns Hopkins Kimmel Cancer Center, Baltimore, MD, USA).

Quantitative RT-PCR. Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 µg total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). PCR was carried out with a SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA, USA). *ADAMTS16* primer sequences were 5'-TCT CAT AGG AGT CGC CTC TGC-3' and 5'-CGA GTG GAG CCC TCA CAG AA-3'. Squamous cell carcinoma antigen A1 (*SCCA1*) primer sequences were 5'-GAA TGG TGG ATA TCT TCA ATG GG-3' and 5'-GAT AGC ACG AGA CCG CGG-3'. Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA was done with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously.⁽¹⁸⁾ Actin-beta-specific PCR products were amplified from the same RNA samples and served as internal controls.

Cell line and RNAi. Human esophageal cancer-derived cell lines, TE1, TE3, TE5, TE7, and TE13, were kindly provided by Dr. Tetsuro Nishihara (Tohoku University School of Medicine, Miyagi, Japan).⁽¹⁹⁾ All cell lines were maintained in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% FBS (Whittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. To knockdown the endogenous *ADAMTS16*, RNAi was carried out. siRNA oligonucleotides for *ADAMTS16* and a negative control were purchased from Invitrogen (Carlsbad, CA, USA). Three independent oligonucleotides were used for *ADAMTS16* siRNA. The *ADAMTS16* siRNA1 sequence was 5'-CCA GUA UUA UCA CAU GGU CAC CAU U-3'. The *ADAMTS16* siRNA2

sequence was 5'-ACA GAG ACC UGA AGU UUC AAG UAA A-3'. The *ADAMTS16* siRNA3 sequence was 5'-GAG UAU AAG UCU UGC UUA CGG CAU A-3'. Transfection was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, 60 pmol siRNA and 10 µL Lipofectamine RNAiMAX were mixed in 1 mL RPMI medium (10 nmol/L final siRNA concentration). After 20 min of incubation, the mixture was added to the cells and these were plated on dishes for each assay. Forty-eight hours after transfection, cells were analyzed for all experiments.

Western blot analysis. For Western blot analysis, tissue samples or cells were lysed as described previously.⁽²⁰⁾ The culture media were concentrated with the Protein Concentrate Kit (Takara Bio, Shiga, Japan). The lysates (40 µg) were solubilized in Laemmli sample buffer by boiling, then subjected to 8% SDS-PAGE followed by electrotransfer onto a nitrocellulose filter. The filter was incubated with the primary antibody against *ADAMTS16* (rabbit polyclonal, dilution 1:500; Abcam, Cambridge, UK). Peroxidase-conjugated antirabbit IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences). β-actin antibody (Sigma Chemical, St. Louis, MO, USA) was also used as a loading control.

Cell growth and *in vitro* invasion assays. The cells were seeded at a density of 2000 cells per well in 96-well plates. Cell growth was monitored after 1 and 2 days by MTT assay.⁽²¹⁾ Modified Boyden chamber assays were carried out to examine invasiveness. Cells were plated at 10 000 cells per well in RPMI-1640 medium plus 1% serum in the upper chamber of a Transwell insert (8 µm pore diameter; Chemicon, Temecula, CA, USA) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber. After 1 and 2 days, cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained with CyQuant GR dye (Chemicon, Temecula, CA, USA) to assess the number of cells.

Statistical methods. Correlations between clinicopathologic parameters and *ADAMTS16* mRNA expression were analyzed by Fisher's exact test. A *P* value of <0.05 was considered statistically significant.

Results

Generation of SAGE data and comparison of expression patterns in ESCC and normal squamous esophagus. A total of 14 430 tags was generated, including 5765 that were unique. Then we compared SAGE tags from the ESCC sample with those from normal squamous esophagus (Gene Expression Omnibus accession number, GSM52501), which contained a total of 50 508 tags including 14 835 unique tags. The 20 most upregulated tags and the 20 most downregulated tags are shown in Tables 1 and 2. The upregulated tags included *ADAMTS16*, immunoglobulin heavy constant gamma 1 (*IGHG1*), 2-oxoglutarate and iron-dependent oxygenase domain containing 1 (*OGFOD1*), nuclear transport factor 2 (*NUTF2*), and RING1 and YY1 binding protein (*RYBP*), whose expressions have not been investigated in ESCC. The downregulated tags included S100 calcium binding protein A9 (*S100A9*), keratin 4 (*KRT4*), cystatin B (*CSTB*), exportin 7 (*XPO7*), keratin 6C (*KRT6C*), and epithelial membrane protein 1 (*EMPI*). Downregulation of some of these genes has been reported previously.⁽¹³⁾ To identify novel biomarkers for ESCC diagnosis and novel targets for ESCC treatment, we focused on genes that were upregulated in the ESCC sample. Of the upregulated genes, we decided to analyze *ADAMTS16* expression because the amino acid sequence of the *ADAMTS16* protein suggests that it might be secreted.

mRNA expression of *ADAMTS16*. Because genes expressed at high levels in tumors and at greatly reduced levels in normal tissues are ideal diagnostic markers and therapeutic targets,⁽⁴⁾

Table 1. Twenty most upregulated tags in esophageal squamous cell carcinoma (ESCC) compared to normal squamous esophagus (normal)

Tag sequence	Tags per million		Symbol	Description
	ESCC	Normal		
TCCCCTACAT	2564† (37)‡	0 (0)	<i>ADAMTS16</i>	ADAM metalloproteinase with thrombospondin type 1 motif, 16
GAAATAAAGC	2495 (36)	0 (0)	<i>IGHG1</i>	Immunoglobulin heavy constant gamma 1 (G1m marker)
TTCGGTTGGT	2148 (31)	0 (0)	<i>OGFOD1</i>	2-Oxoglutarate and iron-dependent oxygenase domain containing 1
AGGCATTGAA	5336 (77)	20 (1)	<i>NUTF2</i>	Nuclear transport factor 2
CAGTTACAAA	5544 (80)	40 (2)	<i>RYBP</i>	RING1 and YY1 binding protein
TGGAAATGAC	1317 (19)	0 (0)	<i>COL1A1</i>	Collagen, type I, alpha 1
GGCGTTTAGA	2079 (30)	20 (1)	No match	No match
ACCAAAAACC	1663 (24)	20 (1)	<i>COL1A1</i>	Collagen, type I, alpha 1
GGCAGCACAA	1455 (21)	20 (1)	<i>NBEAL2</i>	Neurobeachin-like 2
TTTATTAGAA	1455 (21)	20 (1)	<i>CCDC75</i>	Coiled-coil domain containing 75
AGCCAAAAAA	2980 (43)	40 (2)	<i>MAP3K12</i>	Mitogen-activated protein kinase kinase kinase 12
GCTTTCATTG	2495 (36)	40 (2)	<i>NUCKS</i>	Nuclear casein kinase and cyclin-dependent kinase substrate 1
			<i>GPX2</i>	Glutathione peroxidase 2 (gastrointestinal)
ATGTGAAGAG	901 (13)	0 (0)	<i>SPARC</i>	Secreted protein, acidic, cysteine-rich (osteonectin)
CTCCCCAAA	693 (10)	0 (0)	<i>KLK10</i>	Kallikrein-related peptidase 10
			<i>IGHA2</i>	Immunoglobulin heavy constant alpha 2 (A2m marker)
GCTTAAAAAA	693 (10)	0 (0)	<i>CORO1C</i>	Coronin, actin binding protein, 1C
ATTTGAGAGT	624 (9)	0 (0)	<i>MYH9</i>	Myosin, heavy chain 9, non-muscle
CTTTATTCCA	624 (9)	0 (0)	<i>WWC2</i>	WW and C2 domain containing 2
TCAAGCCATC	624 (9)	0 (0)	<i>BLMH</i>	Bleomycin hydrolase
			<i>PCYT2</i>	Phosphate cytidyltransferase 2, ethanolamine
TTTTCCAATT	624 (9)	0 (0)	<i>UTP3</i>	UTP3, small subunit (SSU) processome component, homolog (<i>S. cerevisiae</i>)
TTGCTCACAA	1178 (17)	20 (1)	<i>ABHD12B</i>	Abhydrolase domain containing 12B

†Absolute tag counts are normalized to 1 000 000 total tags/sample. ‡Number in parentheses indicates the absolute tag counts.

Table 2. Twenty most downregulated tags in esophageal squamous cell carcinoma (ESCC) compared to normal squamous esophagus (normal)

Tag sequence	Tags per million		Symbol	Description
	ESCC	Normal		
GTGGCCACGG	0 (0)	25 283† (1277)‡	<i>S100A9</i>	S100 calcium binding protein A9 (calgranulin B)
GGCAGAGAAG	0 (0)	8454 (427)	<i>KRT4</i>	Keratin 4
ATGAGCTGAC	0 (0)	3762 (190)	<i>CSTB</i>	Cystatin B (stefin B)
			<i>XPO7</i>	Exportin 7
GAAGCACAAG	0 (0)	2475 (125)	<i>KRT6C</i>	Keratin 6C
TAATTTGCAT	0 (0)	2455 (124)	<i>EMP1</i>	Epithelial membrane protein 1
			<i>GNA13</i>	Guanine nucleotide binding protein (G protein), alpha 13
AAAGCGGGGC	0 (0)	2356 (119)	<i>KRT13</i>	Keratin 13
TGTGTTGAGA	0 (0)	2257 (114)	<i>EEF1A1</i>	Eukaryotic translation elongation factor 1 alpha 1
CACAAACGGT	0 (0)	2079 (105)	<i>TSPAN9</i>	Tetraspanin 9
			<i>RPS27</i>	Ribosomal protein S27
TGGTGTGAG	0 (0)	1841 (93)	<i>RPS18</i>	Ribosomal protein S18
GCCAATCCAG	0 (0)	1802 (91)	<i>CRNN</i>	Cornulin
GGCAAGCCCC	0 (0)	1782 (90)	<i>RPL10A</i>	Ribosomal protein L10a
			<i>PTPRG</i>	Protein tyrosine phosphatase, receptor type, G
AAGGAGATGG	0 (0)	1722 (87)	<i>RPL31</i>	Ribosomal protein L31
			<i>ZNF434</i>	Zinc finger protein 434
CTGTCACCCT	0 (0)	1564 (79)	<i>SPRR1A</i>	Small proline-rich protein 1A
			<i>BTC</i>	Betacellulin
TAAGGAGCTG	0 (0)	1485 (75)	<i>RPS26</i>	Ribosomal protein S26
			<i>ANK2</i>	Ankyrin 2, neuronal
ACCTGGAGGG	0 (0)	1386 (70)	<i>SBSN</i>	Suprabasin
			<i>PCBP1</i>	Poly(rC) binding protein 1
ACGTGTGTAA	0 (0)	1386 (70)	No match	No match
CAAATCCAAA	0 (0)	1366 (69)	No match	No match
GCCGAGGAAG	0 (0)	1346 (68)	<i>RPS12</i>	Ribosomal protein S12
			<i>NCKAP5L</i>	NCK-associated protein 5-like
TGTGCTAAAT	0 (0)	1346 (68)	<i>USP36</i>	Ubiquitin specific peptidase 36
			<i>RPL34</i>	Ribosomal protein L34
GGGTCTGAGG	0 (0)	1307 (66)	<i>SLURP1</i>	Secreted LY6/PLAUR domain containing 1
			<i>PTPRG</i>	Protein tyrosine phosphatase, receptor type, G

†The absolute tag counts are normalized to 1 000 000 total tags/sample. ‡Number in parentheses indicates the absolute tag counts.

quantitative RT-PCR of *ADAMTS16* was carried out in 20 ESCC samples and in 15 kinds of normal tissue (liver, kidney, heart, colon, brain, bone marrow, skeletal muscle, lung, small intestine, spleen, spinal cord, stomach, pancreas, leukocyte, and esophagus) (Fig. 2a). Among the various normal tissues, obvious *ADAMTS16* expression was found in normal brain, spinal cord, pancreas, and kidney, as reported elsewhere.⁽²²⁾ Expression of *ADAMTS16* in these normal tissues was highest in spinal cord; however, in ESCC, high levels of *ADAMTS16* mRNA expression (more than twice the mRNA expression of spinal cord) were found in eight out of 20 cases (40%). *ADAMTS16* expression in two ESCC cases (Cases 16 and 17) was 10-fold higher than in spinal cord. High levels of *ADAMTS16* mRNA expression were not correlated with any clinicopathologic characters (data not shown). Among five cases at stage I ESCC, a high level of *ADAMTS16* mRNA was detected in one case (20%). These results indicate that *ADAMTS16* expression is highly specific for cancer, at least in ESCC.

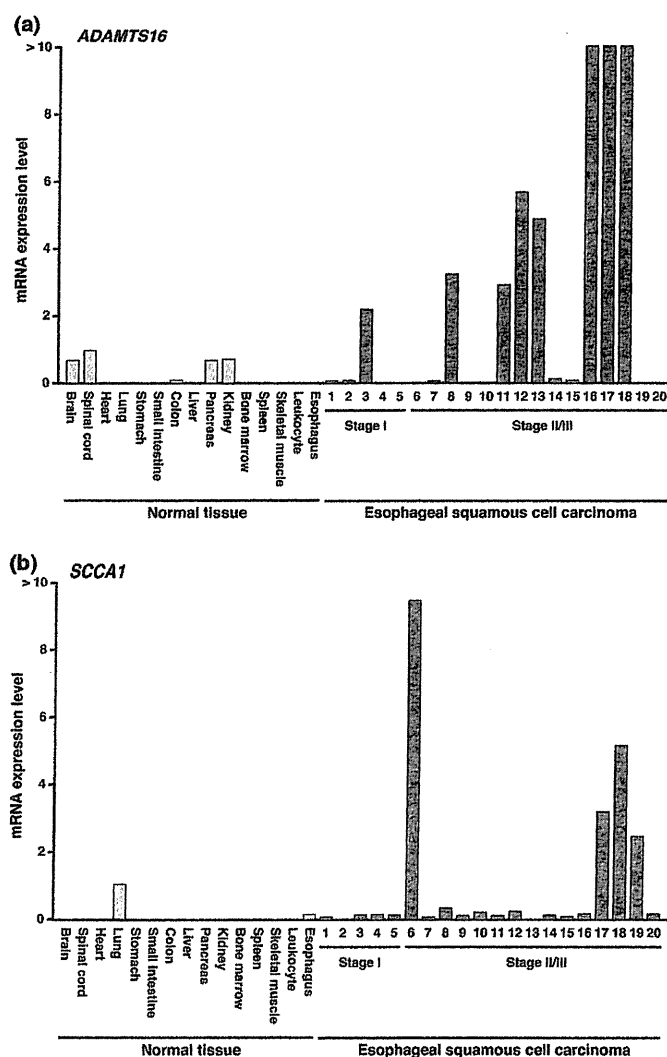


Fig. 2. Quantitative RT-PCR analysis of *ADAMTS16* and *SCCA1* in 15 kinds of normal tissues and 20 esophageal squamous cell carcinoma tissues. (a) mRNA expression level of *ADAMTS16*. The units are arbitrary, and we calculated *ADAMTS16* mRNA expression by standardization of the expression in normal spinal cord to 1.0. (b) mRNA expression level of *SCCA1*. The units are arbitrary, and we calculated *SCCA1* mRNA expression by standardization of the expression in normal lung to 1.0.

Serum squamous cell carcinoma antigen (SCC antigen) detected in the normal squamous epithelium and in ESCC has been considered a useful tumor marker for ESCC.⁽²³⁾ SCC antigen predicts recurrence or progression of the disease and has been used extensively for this purpose. However, clinical use of this marker has been restricted because of lack of sensitivity.⁽²⁴⁾ Therefore, there is an urgent need for new biomarkers for ESCC. To evaluate the usefulness of determining *ADAMTS16* expression as a tumor marker, we measured expression levels of SCC antigen and compared them with *ADAMTS16* levels. Because a measurement system for serum levels of *ADAMTS16* is not available, we investigated the mRNA expression levels of *SCCA1*, which encodes SCC antigen, by quantitative RT-PCR (Fig. 2b). In 15 kinds of normal tissue, expression of *SCCA1* was highest in lung; however, in ESCC, high levels of *SCCA1* mRNA expression (more than twice the mRNA expression levels of lung) were found in four of 20 cases (20%). Among five cases at stage I ESCC, high levels of *SCCA1* mRNA were not detected. These results indicate that *ADAMTS16* might serve as a more sensitive biomarker than SCC antigen. We calculated the ratio of *ADAMTS16* mRNA expression levels between ESCC tissue (T) and corresponding non-neoplastic mucosa (N). T/N ratios >2-fold higher were considered to represent overexpression. *ADAMTS16* overexpression was observed in 13 of 20 ESCC cases (65%). Among five cases at stage I ESCC, *ADAMTS16* overexpression was found in one case (20%). We then investigated the relation of *ADAMTS16* expression to clinicopathologic characters (Table 3). We found that *ADAMTS16* overexpression correlated to the advanced T classification and tumor stage.

ADAMTS16 protein expression. Analysis of the amino acid sequence of the *ADAMTS16* protein suggests that it might be secreted. To investigate whether *ADAMTS16* is a secreted protein, we used Western blot analysis in five esophageal cancer cell lines. Moderate to high *ADAMTS16* expression was noted in TE1, TE3, and TE5 cells as a band of approximately 136 kDa, and the other two remaining cell lines (TE7 and TE13) had low or absent *ADAMTS16* expression (Fig. 3a). Next, we examined the transition of *ADAMTS16* expression by Western blot analysis of cell extracts of TE5 transfected with *ADAMTS16* specific siRNAs. Three types of siRNAs (siRNA1–3) were transfected into TE5. The expression of *ADAMTS16* in TE5 was substantially suppressed by treatment with siRNA2

Table 3. Relationship between *ADAMTS16* expression and clinicopathologic characteristics in esophageal squamous cell carcinoma

	<i>ADAMTS16</i> expression		P value*
	Overexpression	No overexpression	
Age (years)			
≤65	8 (80%)	2 (20%)	0.3498
>65	5 (50%)	5 (50%)	
Sex			
Male	11 (69%)	5 (31%)	0.5868
Female	2 (50%)	2 (50%)	
T classification			
T1	2 (29%)	5 (71%)	0.0215
T2/3	11 (85%)	2 (15%)	
N classification			
N0	3 (43%)	4 (57%)	0.1736
N1	10 (77%)	3 (23%)	
Stage			
Stage I	1 (20%)	4 (80%)	0.0307
Stage II/III	12 (80%)	3 (20%)	

*Fisher's exact test. N, node; T, tumor.

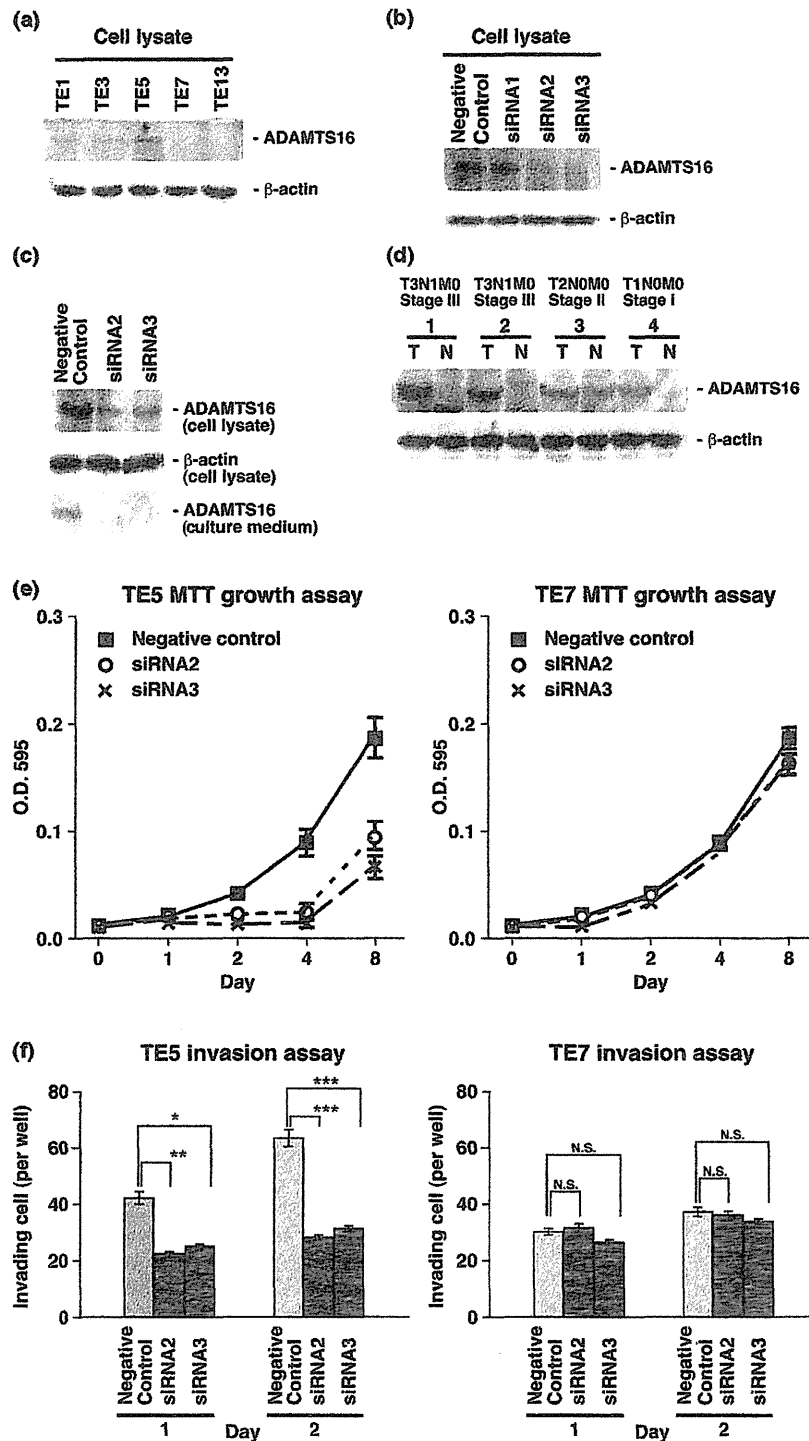


Fig. 3. ADAMTS16 protein expression and functional analysis. (a) Western blot analysis of ADAMTS16 in five esophageal squamous cell carcinoma (ESCC) cell lines. (b) Western blot analysis of ADAMTS16 in cell lysates from TE5 cells transfected with the negative control siRNA and ADAMTS16 siRNA (siRNA1–3). (c) Western blot analysis of ADAMTS16 in cell lysates and culture media from TE5 cells transfected with the negative control siRNA and ADAMTS16 siRNA (siRNA2 and 3). (d) Western blot analysis of ADAMTS16 in four ESCC samples (T) and corresponding non-neoplastic mucosa samples (N). (e) Effect of ADAMTS16 knockdown on cell growth of TE5 and TE7 cells. Cell growth was assessed by an MTT assay at 1, 2, 4, and 8 days after seeding on 96-well plates. Bars and error bars, mean and SE of three different experiments. O.D., optical density. (f) Effect of ADAMTS16 knockdown on cell invasion of TE5 and TE7 cells. TE5 and TE7 cells transfected with negative control siRNA and ADAMTS16 siRNA (siRNA2 and 3) were incubated in Boyden chambers. After 1 and 2 days, invading cells were counted. Bars and error bars, mean and SE of three different experiments. N.S., not significant. * $P = 0.0006$; ** $P = 0.0003$; *** $P < 0.0001$.

and siRNA3, but not with siRNA1 (Fig. 3b). Therefore, to knock down the endogenous ADAMTS16, we used siRNA2 and siRNA3 in the following experiments. A Western blot was car-

ried out of siRNA (siRNA2 and siRNA3)-transfected TE5 cell extracts and culture media (Fig. 3c). In negative control siRNA-transfected TE5 cells, ADAMTS16 protein was detected in

culture media as well as cell extracts; however, in ADAMTS16 siRNA-transfected TE5 cells, ADAMTS16 protein was low or absent in culture media as well as cell extracts. These results clearly indicate that ADAMTS16 is a secreted protein.

Next, expression of ADAMTS16 protein was analyzed by a Western blot of four ESCC tissue samples and corresponding non-neoplastic mucosa samples (Fig. 3d). Among the four ESCC samples, ADAMTS16 protein expression was detected in all; however, of the four corresponding non-neoplastic mucosa samples, ADAMTS16 protein expression was found in only one sample. These results indicate that ADAMTS16 protein is overexpressed in ESCC tissue, and can serve as a serum tumor marker for ESCC.

Effect of ADAMTS16 inhibition on cell growth and invasive activity of esophageal cancer cells. High levels of ADAMTS16 mRNA expression were correlated with T classification of ESCC tissues; however, the biological significance of ADAMTS16 in ESCC has not been studied. To investigate the possible antiproliferative effects of ADAMTS16 knockdown, we carried out an MTT assay 8 days after siRNA transfection (Fig. 3e). TE5 cells were selected for high ADAMTS16 expression. ADAMTS16 siRNA2-transfected and siRNA3-transfected TE5 cells showed significantly reduced viability relative to negative control siRNA-transfected TE5 cells. We carried out the same assay using one additional esophageal cancer cell line that did not express ADAMTS16 (TE7). Reduced cell viability was not observed in siRNA2- or siRNA3-transfected TE7 cells compared with negative control siRNA-transfected TE7 cells.

Next, to determine the possible role of ADAMTS16 in the invasiveness of esophageal cancer cells, we used a Transwell invasion assay (Fig. 3f). On day 1, although there was no difference in cell viability between ADAMTS16 knockdown TE5 cells and negative control siRNA-transfected TE5 cells, the invasiveness of ADAMTS16 knockdown TE5 cells was 40% less than that of the negative control siRNA-transfected TE5 cells. On day 2, the invasiveness of ADAMTS16 knockdown TE5 cells was 50% less than that of the negative control siRNA-transfected TE5 cells; however, as ADAMTS16 knockdown cells showed significantly reduced cell viability, the cell number difference observed in the invasion assay might be caused by the reduced cell viability. In contrast, invasion ability was not significantly different between ADAMTS16 knockdown TE7 cells and negative control siRNA-transfected TE7 cells. These results indicate that ADAMTS16 stimulates cell growth and invasion in esophageal cancer cells.

Discussion

In spite of improvement to modern surgical techniques and adjuvant CRT, ESCC is known to reveal the worst prognosis among malignant tumors. Therefore, it is now urgently required to develop novel diagnostic biomarkers and therapeutic targets for a better choice of adjuvant treatment modalities for individual patients. In the present study, we carried out a genome-wide expression profile analysis of one ESCC tissue sample by SAGE, and identified upregulated and downregulated genes in ESCC. Among these, we further investigated ADAMTS16. Quantitative RT-PCR revealed that ADAMTS16 mRNA expression was frequently upregulated in ESCC, and was narrowly restricted in normal tissues. Western blot analysis also showed upregulation of ADAMTS16 protein in ESCC. Furthermore, ADAMTS16 protein was detected in culture media from TE5 cells. Taken together, these results suggest that ADAMTS16 has potential as a serum tumor marker for ESCC. Because the frequency of high levels of ADAMTS16 mRNA expression (40%) was greater than the frequency of high levels of SCCA1 mRNA expression (20%), serum concentrations of ADAMTS16 might

serve as a sensitive biomarker for ESCC. In contrast, because ADAMTS16 mRNA overexpression was correlated with advanced T classification and tumor stage, serum concentrations of ADAMTS16 might not be suitable for early detection of ESCC. Serum concentrations of ADAMTS16 should be measured in patients with ESCC.

In the present study, ADAMTS16 mRNA overexpression correlated to the advanced T classification and tumor stage. Knockdown of ADAMTS16 by RNAi inhibited the cell growth and invasion ability of TE5 cells. Because expression of ADAMTS16 was highly specific to ESCC, it could be a good therapeutic target with less adverse effects for ESCC. Although the function of ADAMTS16 is poorly understood, members of the metzincin family are known to process a number of growth factors, cytokines and signaling molecules in addition to matrix substrates.⁽²⁵⁾ However, it has been reported that the forced expression of ADAMTS16 has no effect on expression levels of most of the ADAMTS, TIMP, and MMP genes. In the present study, we also used ELISA to measure levels of epidermal growth factor (EGF) and transforming growth factor (TGF)- α in culture media from TE5 cells transfected with ADAMTS16 siRNA and negative control siRNA; however, levels of EGF and TGF- α were not significantly different (data not shown). Therefore, growth factors or cytokines, such as EGF or TGF- α , are not likely to be involved in mechanisms of cell growth inhibition and invasion ability following knockdown of ADAMTS16.

Although ADAMTS16 protein upregulation was observed in ESCC tissues by Western blot analysis, expression and distribution of ADAMTS16 protein in ESCC tissues remains unclear. Therefore, immunohistochemical analysis should be undertaken. Unfortunately, the antibody against ADAMTS16 used in the present study is not suitable for immunostaining because the antibody against ADAMTS16 detected multiple bands on Western blots. Production of a specific antibody against ADAMTS16 is required. Furthermore, ADAMTS16 expression at mRNA and protein levels should be examined in several more tissues from stage I ESCC in the near future.

In addition to ADAMTS16, other upregulated and downregulated genes in ESCC were found. The upregulated group of genes identified by SAGE contains genes whose expression has not been investigated in ESCC. Upregulation of two genes related to the immunoglobulin heavy chain (*IGHG1* and *IGHA2*) was found in the present study. Previously, genes involved in the immune response have been shown as characteristically upregulated in long-term ESCC survivors who were treated with CRT.⁽²⁶⁾ Therefore, the ESCC case analyzed by SAGE in the present study might be sensitive to CRT. *OGFOD1* is a 2-oxoglutarate and Fe(II)-dependent oxygenase, a class of enzymes that catalyze a variety of reactions typically involving the oxidation of an organic substrate using a dioxygen molecule.⁽²⁷⁾ To our knowledge, association between cancer and *OGFOD1* has not been investigated. *NUTF2* encodes nuclear transport factor 2 (NTF2), which is a small GDP Ran binding protein. The main function of NTF2 is to facilitate transport of certain proteins into the nucleus through interaction with nucleoporin FxFG.⁽²⁸⁾ It is also involved in regulating multiple processes, including cell cycle and apoptosis.⁽²⁹⁾ However, no studies have analyzed NTF2 expression in human cancer, including ESCC. *RYBP* is a member of the polycomb group, and it has been reported that RYBP interacts with MDM2 and decreases MDM2-mediated p53 ubiquitination, leading to stabilization of p53 and an increase in p53 activity.⁽³⁰⁾ RYBP induces cell cycle arrest and is involved in the p53 response to DNA damage. Expression of RYBP is decreased in hepatocellular carcinoma and lung cancer tissues.⁽³⁰⁾ Therefore, upregulation of RYBP should be confirmed in a large number of ESCC cases. In contrast, downregulated genes identified by SAGE in the present study were

similar to genes previously reported as downregulated in ESCC.⁽¹³⁾

In conclusion, our present SAGE data provide a list of genes potentially associated with ESCC. Because our list is based on one ESCC case, expression analysis in a large number of cases is required. A high level of *ADAMTS16* expression was detected in ESCC, and expression of *ADAMTS16* was narrowly restricted. Production of a specific antibody against *ADAMTS16* protein and establishment of a measurement system for serum samples are needed to clarify whether *ADAMTS16* serves as a serum marker for early detection and a good therapeutic target for ESCC.

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