TMPRSS4 Expression as a Marker of Recurrence in Patients with Lung Cancer

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Abstract. *Background: Postoperative recurrence is a significant problem associated with a poor prognosis. However, there is currently no consensus regarding biomarkers of recurrence. Materials and Methods: We performed a microarray expression analysis using a combination of tumor tissues (n=2) and cell lines. We prioritized and validated candidate protein expression levels in the primary tumors. Results: We prioritized 18 genes found to be up-regulated by more than four-fold in both A925LPE3 cell lines compared to the A925L cell line (lung adenocarcinoma) and in the cases of recurrence versus no recurrence, in order to find genes highly causative of metastasis. Among them, we selected transmembrane protease, serine 4 (TMPRSS4) and identified positive expression of TMPRSS4 in 93 (57.8%) patients. A significant negative association was observed only between the TMPRSS4 expression level and the N status. The univariate logistic regression models indicated that TMPRSS4 expression was an independent predictor of recurrence, as was the T and N status. Conclusion: TMPRSS4 expression is associated with postoperative recurrence. In addition, the current survival curves demonstrated that TMPRSS4 expression is associated with statistically significant differences in survival among patients with lung adenocarcinoma. TMPRSS4 staining can be used to predict the prognosis of such patients after surgery.*

Lung cancer is the leading cause of cancer-related death in the majority of countries worldwide (1), and the treatment results are by no means satisfactory. For example, postoperative recurrence is a significant problem associated with a poor prognosis, and patients with disease at the same stage exhibit wide variation in their prognosis after curative resection. Therefore, the current TNM staging system may have reached the limits of its usefulness (2). In addition to the TNM classification, biomarkers based on molecular techniques have been proven to be independent factors predicting the risk of recurrence as clinical parameters (2). Although a great deal of effort has been made to identify prognostic markers following surgery, there is currently no consensus regarding this issue in clinical practice. We also examined the frequency and clinical significance of transmembrane protease, serine 4 (TMPRSS4) expression in a retrospective series of 161 patients resected for adenocarcinoma of the lung.

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Materials and Methods

The Institutional Review Board of our University approved this study (H25-185).

Case selection for the microarray. Tumor samples were obtained from 668 patients with primary lung cancer who had undergone surgical resection between 2005 and 2011 at our Department. Two hundred and thirty-five of these patients had double cancer, including multiple lung cancer, 38 of whom underwent incomplete resection. Thirteen and seven cases were positive and suspicious on washing cytology, respectively. Furthermore, the number of cases with a carcinoembryonic antigen (CEA) level of more than 2.5 ng/ml and those treated with limited resection was 112 and 21, respectively. As a result, 426 patients were excluded from further analyses. Therefore, 242 tumor specimens were qualified as candidates for the evaluation. Among them, we selected two available cases with frozen specimens followed-up for a three-year period after surgery by the same operator over the same period to avoid selection bias. The tumors were derived from one case without recurrence for a long-term survivor with advanced disease after resection (Figure 1) and a case of recurrence despite pathological stage IA disease (Figure 2).

Cell line selection for the microarray. A human lung adenocarcinoma cell line, A925L, established from a surgical specimen obtained from a male Japanese patient (T2N2M0, stage IIIA), was maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum

(FBS), penicillin (100 U/ml) and streptomycin (50 g/ml), in a humidified CO_2 incubator at 37˚C (3). A925LPE3 cells have a high potential to produce thoracic tumors and pleural effusion in mice. A925L and A925LPE3 harbor an *EML4–ALK* gene fusion (variant 5a) and are sensitive to ALK-TKIs. We used A925L and A925LPE3 cell lines as a model for cells with parental and highly metastatic potential, respectively. We previously established *in vivo* imaging models for pleural carcinomatosis, bone metastasis and brain metastasis (3).

Microarray expression analysis. Total RNA was extracted from each serum sample from the cases and cell lines as stated before using "3D-Gene" RNA extraction reagent obtained from a liquid sample kit (Toray Industries, Inc., Tokyo, Japan) using a combination of formalin-fixed, paraffin-embedded (FFPE) tissues and cell lines. Total RNA was also obtained from sections in accordance with the manufacturer's instructions. Hybridization was performed using the supplier's protocols (www.3d-gene.com). The amplified RNA was hybridized for 16 hours to the highly sensitive DNA microarray, 3D-Gene Human Oligo chip 25 k, ver. 2.1 (Toray Industries, Tokyo, Japan), which permits the detection of 24,460 mRNAs. The DNA microarray product was washed according to the manufacturer's instructions, followed by image scanning using a 3D-Gene® Scanner 3000 (Toray Industries) and data processing using 3D-Gene® Extraction 2.0.0.4 (Toray Industries). At a laser power setting of 60%, the photomultiplier sensitivity was adjusted so that the fluorescence signal intensity from the two fluorophores, Cy3 and Cy5, at the gain control spots on the microarray was comparable. The ratio of Cy3 and Cy5 signal intensity (Cy3/Cy5, R) was log translated and used as a calibration factor [Log2(R)] to standardize the signal intensity (4). The mean intensity and standard deviation (SD) of the background signal were calculated using the signal intensity of the blank spots residing within the 95% confidence interval (95% CI). Genes with a background signal greater than 2×SD were considered to exhibit positive expression. The mean background level was subtracted from the signals of the detected genes (5, 6). In this study, the mRNA expression profiles of the following one paired-clinical condition were statistically compared: i) the case without recurrence *versus* the recurrent case, and ii) the cell line with parental *versus* that with highly metastatic potential.

Patients, clinical features and follow-up. Tumor samples were obtained from 410 patients with primary lung cancer who had undergone surgical resection between 2003 and 2007 at our Department. The number of patients with lung adenocarcinoma was 281. Five of these patients had stage IV disease and 28 underwent incomplete resection. The tumor samples from 87 patients were too small to be evaluated with immunohistochemical (IHC) staining to determine the *TMPRSS4* status. As a result, these 120 patients were excluded from the further analysis. Therefore, 161 tumor specimens were evaluated. All of the patients were Japanese, consisting of 90 males and 71 females in this series, with a median age of 71 years (range=23-88 years). There were 67 never smokers, 45 former smokers and 49 current smokers. The former smokers were defined as those who had quit smoking at least three years before the time of surgery. The tumor stage was classified according to the TNM Classification for Lung Cancer (7). Based on the pathological stage, 98 patients had tumors of stage IA, 30 patients had tumors of stage IB, seven patients had tumors of stage IIA, 10 patients had tumors of stage IIB, 13 patients had tumors of stage IIIA and three patients had tumors of stage IIIB. Twenty-one (13.0%) patients received adjuvant chemotherapy, as follows: carboplatin plus paclitaxel (n=13), carboplatin plus gemcitabine (n=6) and tegafur–uracil (n=2) (8).

The patients were generally followed-up every month within the first postoperative year and at approximately 2- to 4-month intervals thereafter. The evaluations included a physical examination, chest roentgenography, analysis of blood chemical parameters and measurements of the tumor marker levels. Chest and abdominal computed tomography, brain magnetic resonance imaging and bone scintiscan assessments were performed every 6 months for 3 years after surgery. Additional examinations were performed if any signs or symptoms of recurrence were detected. Follow-up was conducted for all patients. The median follow-up period was 61.0 months.

IHC staining of FFPE tumor samples. IHC staining was carried out using serial sections obtained from the same FFPE blocks according to previously described methods (9). Briefly, all tissue specimens were fixed in formalin and processed similarly, according to standard histological practices. A 3-μm-thick FFPE tissue section was prepared from each specimen. All specimens were stained with hematoxylin–eosin for a histological diagnosis. The sections were briefly immersed in citrate buffer [0.01 mol/l citric acid (pH 6.0)] and then incubated twice for 10 min at 121˚C in a high-pressure sterilization oven for antigen retrieval. The sections were then incubated with TMPRSS4 antibody (Proteintech: 11283-1-AP; Proteintech Group, Inc., Chicago, IL, USA) diluted at 1:50 in phosphate-buffered saline overnight at 4˚C (10).Thereafter, IHC staining was performed using the labeled polymer method (Histofine Simple Stain MAX-PO kit; Nichirei, Tokyo, Japan) according to the manufacturer's instructions (9). The positive control for TMPRSS4 was the colon cancer specimens. The negative control used rabbit IgG (Dako, Glostrup, Denmark) instead of the primary antibody.

Evaluation of the stained specimens. Following IHC detection of the protein expression in each specimen, the percentage of immunoreactive tumor cells in five ×400 fields selected randomly on one slide was recorded, and the final count of positively stained tumor cells was determined as the average number of positively immunostained cells. Initially, four groups were assigned for the assessment of proportional scores for positive staining according to the frequency of positively stained tumor cells (0, none; 1, <25%; 2, <25-50%; 3, >50%). In order to determine the presence of any correlations with the clinicopathological characteristics, the protein expression scores were divided into positive and negative groups. The TMPRSS4 expression status in the cytoplasm of the tumors was categorized as negative when the score was 0-2 and positive when the score was 3. The slides were examined independently by two of the investigators (Y.C. and Y.K.), who were blinded to the patients' clinicopathological data. The concordance rate was 87.1%. When discrepancies were found between the two investigators, a consensus was reached via simultaneous examination by both investigators using a double-headed microscope.

Statistical analysis. Statistical significance was evaluated using the Chi-square test or Fisher's exact test. The Kaplan–Meier method was used to estimate the probability of survival, and survival differences were analyzed according to the log-rank test. Univariate logistic regression models were used to evaluate independent associations.

The odds ratio (OR) and 95% CI were calculated for each variable. Differences were considered to be statistically significant for $p<0.05$. The data were analyzed using the Stat View software package (Abacus Concepts, Inc., Berkeley, CA, USA).

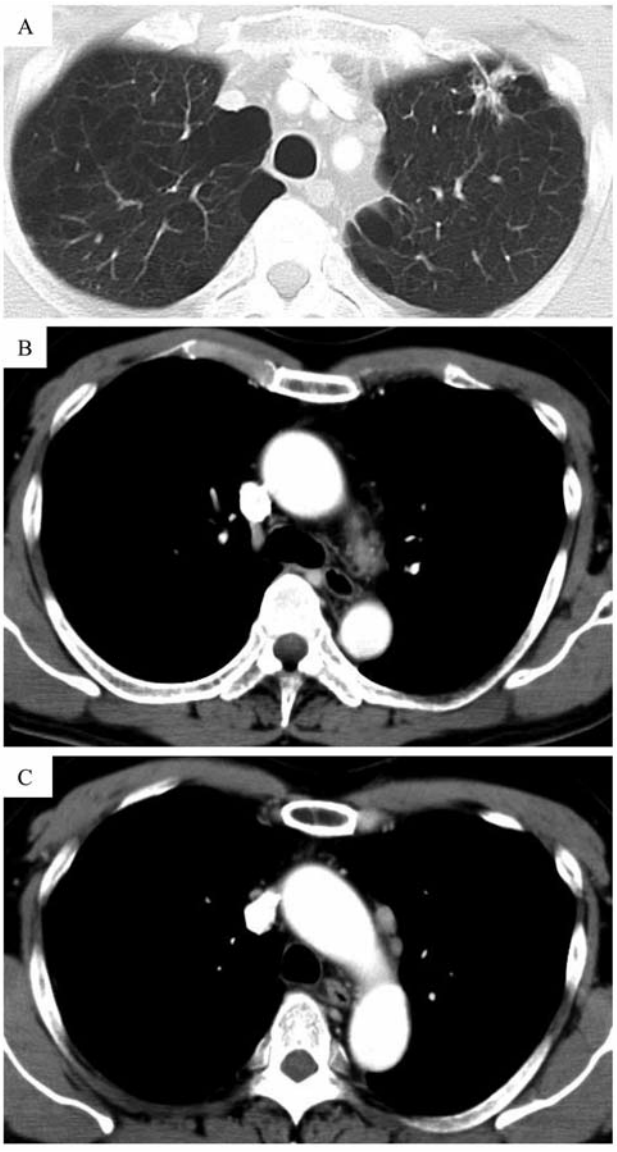

Figure 1. *A: Case 1: Case without recurrence: Chest computed tomography (CT) showing a tumor in the upper left lobe. B and C: Chest CT shows mediastinal lymph node swelling. Left upper lobectomy and and systematic lymphadenectomy were performed. The pathological findings showed papillary carcinoma (moderately differentiated adenocarcinoma), with ly3, v1, n2 (#4:1/5, #5:1/3, #6:4/5, #7:0/2, #10:0/3, #11:0/1, #12u:0/2, #12l:0/1). The pathological stage was judged to be T1N2M0 stage IIIA. EGFR was judged to be wild-type. Adjuvant therapy was administered. The patient has remained alive for five years without evidence of recurrence.*

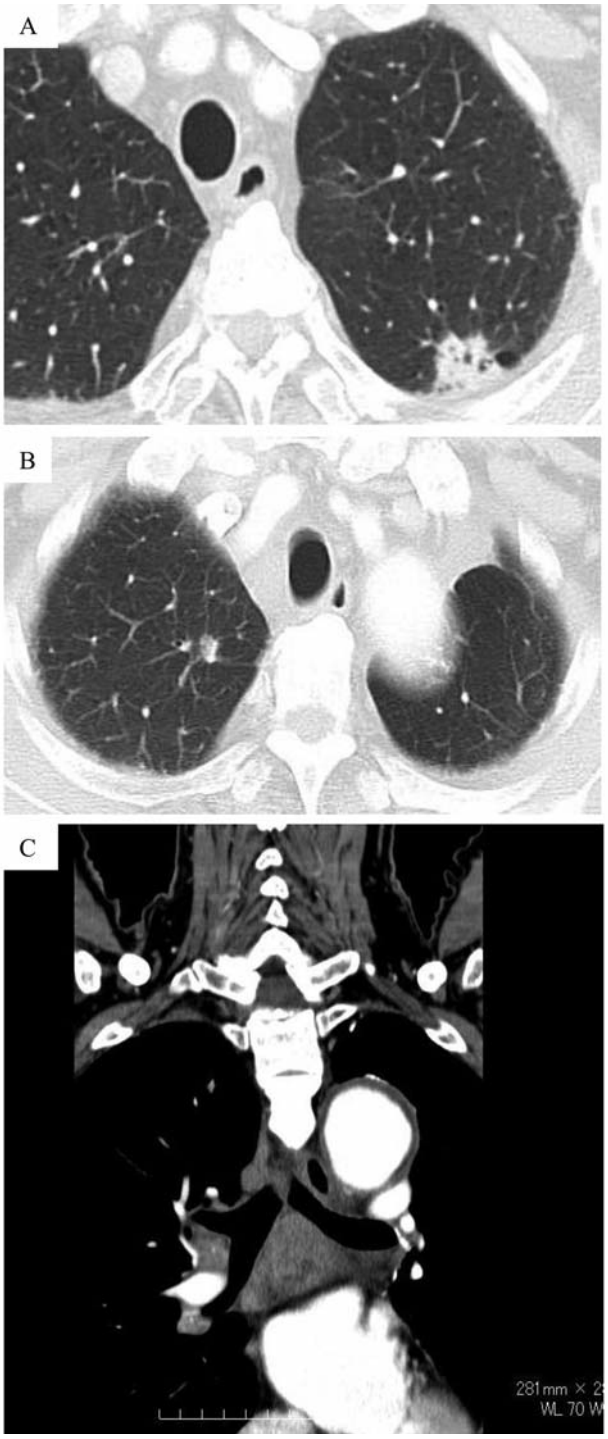

Figure 2. *Case 2: A: Recurrent case: Chest computed tomography (CT) showing a tumor in the upper left lobe, as in Case 1. The pathological findings showed squamous cell carcinoma. The pathological stage was judged to be T1bN0M0 stage IA. B and C: Pulmonary metastasis (B) and mediastinal lymph node metastases (C) were detected 29 months after complete resection. The pathological findings confirmed lymph node metastasis on mediastinoscopy. The disease showed rapid progression and no longer responded to chemoradiotherapy. As a result, the patient died due to multiple bone metastases and left adrenal metastasis.*

Results

Selection of a molecular marker of recurrence. We initially carried out mRNA profiling in the A925LPE3 cell line *versus* the A925L cell line and identified 93 genes commonly up-regulated by more than four-fold *versus* the A925L cell line. Next, 1,947 genes were identified in a case of recurrence

Table I. *Genes up-regulated by more than 4-fold in both the A925LPE3 cell line compared to the A925L cell line and the recurrent case compared to non-recurrent case.*

		Cell line		Tissue	
Symbol	Description	Fold difference	Log2	Fold difference	Log2
GUCY1A3	Guanylate cyclase soluble subunit alpha-3 (GCS-alpha-3)(EC 4.6.1.2) Soluble guanylate cyclase large subunit)(GCS-alpha-1) [Source:UniProtKB/Swiss-Prot;Acc:Q02108]	6.76	2.76	4.25	2.09
FABP6	Gastrotropin (GT)(Fatty acid-binding protein 6)(Ileal lipid-binding protein) (ILBP)(Intestinal 15 kDa protein)(I-15P)(Intestinal bile acid-binding protein) (I-BABP) [Source:UniProtKB/Swiss-Prot;Acc:P51161]	5.40	2.43	24.59	4.62
TMPRSS4	Transmembrane protease, serine 4 (EC 3.4.21.-)(Membrane-type serine protease 2)(MT-SP2) [Source:UniProtKB/Swiss-Prot;Acc:Q9NRS4]	43.89	5.46	21.20	4.41
SERPINA3	Alpha-1-antichymotrypsin Precursor (ACT)(Cell growth-inhibiting gene 24/25 protein) [Contains Alpha-1-antichymotrypsin His-Pro-less] [Source:UniProtKB/Swiss-Prot;Acc:P01011]	16.84	4.07	8.14	3.03
ACCN2	amiloride-sensitive cation channel 2, neuronal isoform a [Source:RefSeq peptide;Acc:NP_064423]	6.00	2.58	4.72	2.24
SERPINB5	Serpin B5 Precursor (Protease inhibitor 5)(Maspin) [Source:UniProtKB/Swiss-Prot;Acc:P36952]	7.52	2.91	229.63	7.84
PLCB1	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-1 (EC 3.1.4.11)(Phosphoinositide phospholipase C)(Phospholipase C-beta-1) (PLC-beta-1)(PLC-I)(PLC-154) [Source:UniProtKB/Swiss-Prot;Acc:Q9NQ66]	5.39	2.43	4.17	2.06
NPNT	Nephronectin Precursor (Preosteoblast EGF-like repeat protein with MAM domain)(Protein EGFL6-like) [Source:UniProtKB/Swiss-Prot;Acc:Q6UXI9]	5.47	2.45	9.77	3.29
SERPINB4	Serpin B4 (Squamous cell carcinoma antigen 2)(SCCA-2)(Leupin) [Source:UniProtKB/Swiss-Prot;Acc:P48594]	11.22	3.49	9.36	3.23
LEMD1	LEM domain-containing protein 1 (LEMP-1)(Cancer/testis antigen 50) (CT50) [Source:UniProtKB/Swiss-Prot;Acc:Q68G75]	4.68	2.23	7.13	2.83
CYP4F11	Cytochrome P450 4F11 (EC 1.14.14.1)(CYPIVF11) [Source:UniProtKB/Swiss-Prot;Acc:Q9HBI6]	4.65	2.22	55.83	5.80
CYP4F11	Cytochrome P450 4F11 (EC 1.14.14.1)(CYPIVF11) [Source:UniProtKB/Swiss-Prot;Acc:Q9HBI6]	4.62	2.21	9.88	3.30
P2RX7	P2X purinoceptor 7 (P2X7)(ATP receptor)(Purinergic receptor) (P2Z receptor) [Source:UniProtKB/Swiss-Prot;Acc:Q99572]	7.98	3.00	5.50	2.46
AL354993.1	Cell growth-inhibiting protein 7HCG1784586; [Source:UniProtKB/TrEMBL;Acc:B1H0U8]	4.55	2.19	14.64	3.87
AC024270.6	Hepatoma-derived growth factor-related protein 3 (HRP-3)(Hepatoma-derived growth factor 2) [Source:UniProtKB/Swiss-Prot;Acc:Q9Y3E1]	4.24	2.09	6.62	2.73
SERPINB3	Serpin B3 (Squamous cell carcinoma antigen 1)(SCCA-1)(Protein T4-A) [Source:UniProtKB/Swiss-Prot;Acc:P29508]	17.02	4.09	46.81	5.55
TSHZ2	Teashirt homolog 2 (Zinc finger protein 218)(Ovarian cancer-related protein 10-2)(OVC10-2) [Source:UniProtKB/Swiss-Prot;Acc:Q9NRE2]	4.04	2.02	10.23	3.35

compared to a case without recurrence. Then we prioritized 18 genes found to be up-regulated more than four-fold in both A925LPE3 cell line compared to the A925L cell line and the case of recurrence *versus* the non-recurrent case in order to find genes highly causative of metastasis (Table I). Among them, we selected *TMPRSS4*, the protein for which an antibody is commercially available to detect the expression in paraffin-embedded cancer tissues.

Detection of TMPRSS4 and correlation with clinicopathological factors. We examined the expression status of TMPRSS4 using an immunohistochemical analysis in 161 patients who underwent complete resection for lung adenocarcinoma. Positive reactions for TMPRSS4 (11) were mainly localized in the cytoplasm, and the expression of TMPRSS4 was observed in 93 (57.8%) patients (Figure 3A). A significant association was only seen between positive TMPRSS4 expression and positive N status (p=0.049), whereas other factors were not associated with the TMPRSS4 expression (Table II).

Relationship between TMPRSS4 expression and recurrence. At the last follow-up examination, 119 patients were alive and free of cancer, 10 patients had died of other causes

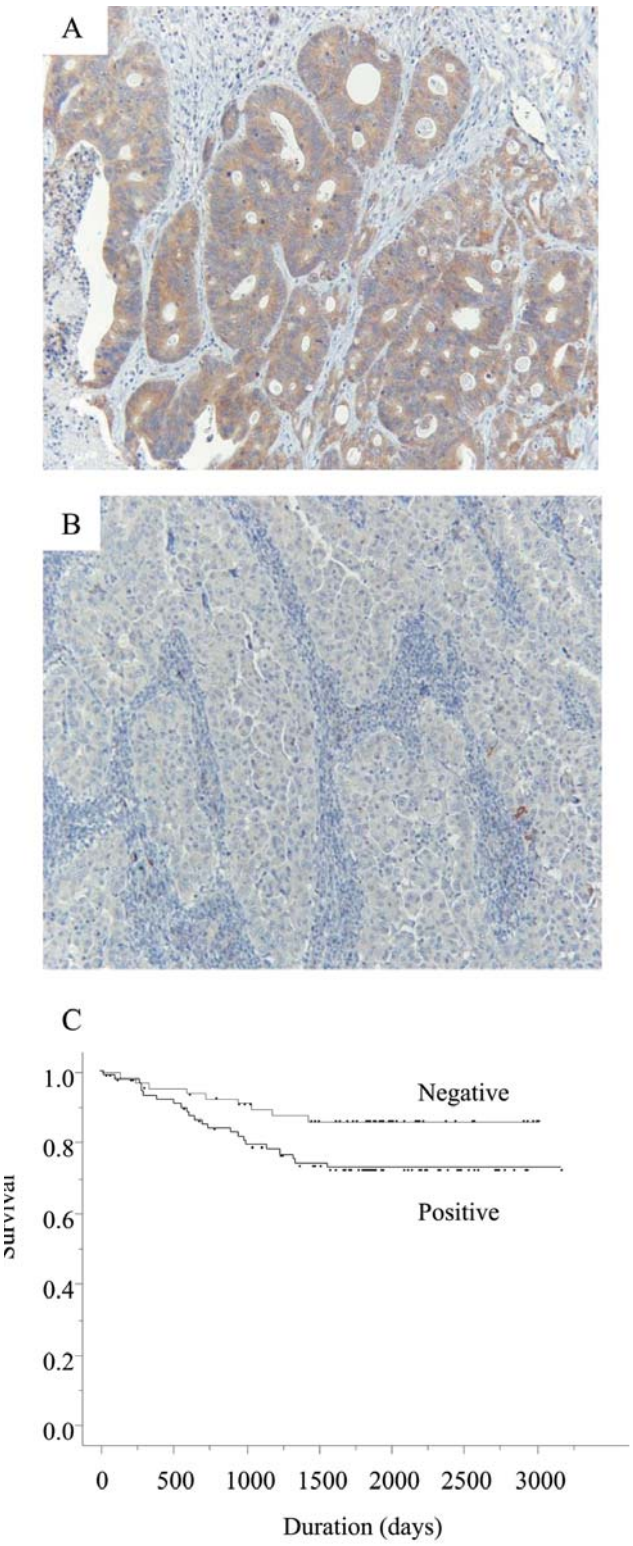

Figure 3. *A: Results of the immunohistochemical analysis of transmembrane protease, serine 4 (TMPRSS4) staining. A: Positive TMPRSS4 expression. B: Negative TMPRSS4 expression. C: Kaplan–Meier curves stratified based on TMPRSS4 staining. The heavy and narrow lines indicate a positive (n=93) and negative (n=68) TMPRSS4 expression, respectively.*

Table II. *Relationship between transmembrane protease, serine 4 (TMPRSS4) expression and clinicopathological characteristics in patients with lung cancer.*

Variable	No. of patients	TMPRSS4 expression		*p*-Value
	n=161	Positive n=93 (57.8%)	Negative n=68	
Gender				
Male	90	51	39	0.875
Female	71	42	29	
Age (years)				
<70	77	44	33	0.999
≥70	84	49	35	
Smoking status				
Never	67	36	31	0.475
Current/former	94	57	37	
p Stage				
IA	98	52	46	0.179
IB-III	63	41	22	
T Status				
T1	112	63	49	0.678
T2-4	49	30	19	
N Status				
Negative	132	71	61	**0.049**
Positive	29	22	7	

without evidence of cancer, 10 patients were alive with recurrent cancer and 22 patients had died of cancer. In total, 27 (16.8%) out of the 161 patients demonstrated disease recurrence after surgery. The majority of sites of tumor recurrence were hematogenous metastases. Twenty-four and six cases of recurrence were hematogenous (8 brain, 10 lung, 4 bone and 1 adrenal metastasis) and locoregional (four cases of lymph node metastasis and two cases of pleural dissemination), respectively. One patient of each group had recurrent tumors in both the brain and bone, brain and adrenal gland and bone and lymph nodes, respectively (12).

Positive expression of TMPRSS4 was identified in 24 (88.9%) out of 27 patients and 69 (51.5%) out of 134 patients with and without recurrence, respectively ($p<0.001$). The univariate logistic regression models indicated that positive TMPRSS4 expression in patients with adenocarcinoma was an independent predictor of recurrence, as was a high T and positive N status (Table III).

Influence of the TMPRSS4 status on overall survival (OS). The five-year OS rates among the patients positive and negative for TMPRSS4 were 72.4% and 86.2%, respectively ($p=0.43$). The Kaplan–Meier survival curves demonstrated that the TMPRSS4 expression was associated with statistically significant differences in survival in the lung adenocarcinoma patients (Figure 3C). A positive TMPRSS4

Table III. *Results of the univariate analysis using a proportional hazard model for overall survival in lung cancer.*

Variables	Characteristic		95% CI	HR	*p*-Value
	Unfavorable	Favorable			
Gender	Male	Female	1.237-7.194	3.115	0.008
Age (years)	<70	≥70	0.480-1.919	0.960	0.907
Smoking	Current/former	Never	1.431-8.454	3.478	<0.01
T Status	2- 4	1	1.866-7.576	3.759	<0.001
N Status	Positive	Negative	2.732-11.111	5.494	<0.001
TMPRSS4	Positive	Negative	1.004-4.694	2.170	0.049

HR: Hazard ratio; CI: confidence interval; TMPRSS4: transmembrane protease, serine 4.

expression was also found to be marginally negatively associated with the OS based on a univariate survival analysis (p=0.049).

Discussion

The present study demonstrated two major findings. Firstly, TMPRSS4, one of the type II transmembrane serine proteases, which have been recognized as a new subfamily of serine proteases (13), was identified as a marker of recurrence in cases of lung cancer. Hamamoto *et al*. also found expression of TMPRSS4 by screening surgically resected samples obtained from 90 Japanese patients with non-small cell lung cancer (NSCLC) patients using a cDNA microarray (10). Furthermore, siRNA knockdown of *TMPRSS4* has been shown to reduce cell invasion and migration (14). Positive TMPRSS4 expression is common in patients with lung adenocarcinoma. These data are consistent with previous results (15). Subsequent studies showed that TMPRSS4 is also highly expressed in other tumors, including thyroid neoplasms (16), and breast (15), gallbladder (17) and colon (18). Moreover, a positive expression of TMPRSS4 was found to be significantly correlated with lymph node metastasis in our study. This finding is also consistent with those for other tumor types, such as triple-negative breast cancer (15), prostate cancer (11) and gallbladder cancer (17). Interestingly, TMPRSS4 controls the metastatic potential of human cancer cells by facilitating the epithelial–mesenchymal transition (EMT) (19). In fact, the EMT is an important contributor to invasion and drug resistance in lung cancer according to our previous reports (20-22). Furthermore, TMPRSS4 expression has been reported to be increased under hypoxic culture conditions (14). These findings appear to be reasonable, as tumor angiogenesis is closely associated with tumor progression (23, 24). Hence, the tumor microenvironment and phenotypic alterations of cancer cells might be linked with each other.

Secondly, positive expression of TMPRSS4 was identified to be associated with a poorer OS. This phenomenon is seen for other tumor types, including triple-negative breast cancer (15) and gallbladder cancer (17). TTMPRSS4 is associated with a poor prognosis in patients with NSCLC with squamous cell histology based on PCR studies (25). However, the authors described finding no statistical relationships in patients with lung adenocarcinoma. This discrepancy may be due to disparities in sampling (mRNA *vs*. protein), experimental systems (PCR *vs*. IHC), and ethnic differences (Westerners *vs*. Japanese). In fact, tumor cell lines with high levels of *TMPRSS4* mRNA expression have been reported to fail to show detectable expression of TMPRSS4 protein on immunoblotting (26).

Ultimately, the current findings suggest that the expression of TMPRSS4 is likely to serve as a suitable biomarker for identifying candidate patients for a poor prognosis. Our findings are unique for several reasons: (i) a microarray expression analysis was performed using a combination of tissues and cell lines; (ii) the selected tumors were derived from a case without recurrence in a long-term survivor with advanced disease after resection and a case of recurrence despite early-stage disease; (iii) we used a comparatively large case series of 161 consecutive tumors; (iv) the analysis was limited to cases of adenocarcinoma, which is relatively homogeneous; and (v) the method was based on simple IHC staining, which has the advantage of maintaining the morphology of the tissues (12). However, the present study is associated with limitations that affect its interpretation: (i) the retrospective design, (ii) setting at a single Institution, and (iii) selection of only two albeit different cases.

Conclusion

We clearly demonstrate that the expression of TMPRSS4 is associated with a poor prognosis in patients with lung adenocarcinoma. While promising, this evidence is not yet sufficient to alter currently established clinical treatments. Future research is, therefore, needed to clarify the biological role of TMPRSS4 in order to determine its full clinical usefulness.

Conflicts of Interests

None declared.

Disclosure

The Authors reported no proprietary or commercial interest in any product mentioned or concept discussed in the article.

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