



# OPEN **TMPRSS4 as a prognostic biomarker after gastric cancer surgery in a multicenter retrospective study**

Hirofumi Tazawa<sup>1✉</sup>, Shinji Hato<sup>2</sup>, Shigefumi Yoshino<sup>3</sup>, Shinya Otsuka<sup>4</sup>, Atsusi Takeno<sup>5</sup>, Kazuhiro Toyota<sup>6</sup>, Hiromitsu Moriya<sup>7</sup>, Isao Nozaki<sup>8</sup>, Koji Tanakaya<sup>9</sup>, Hideaki Uchiyama<sup>10</sup>, Akihisa Saito<sup>11</sup>, Kazuya Kuraoka<sup>11</sup>, Takeshi Kato<sup>5</sup>, Takahisa Suzuki<sup>1</sup> & Hiroataka Tashiro<sup>1,12</sup>

Transmembrane protease serine 4 (TMPRSS4) is a member of the type II transmembrane serine protease family known to be upregulated in many malignancies. We previously showed that TMPRSS4 may be a prognostic biomarker and therapeutic target for gastric cancer (GC), especially in stage III. In this retrospective study conducted at 10 institutions, all 325 patients underwent R0 resection involving D2 lymph node dissection. TMPRSS4 expression was examined using immunohistochemical analysis. TMPRSS4 expression was upregulated in 44.9% of participants. The 5-year overall survival (OS) of the TMPRSS4-positive group was significantly lower than that of the TMPRSS4-negative group (62.4% vs. 76.4%, respectively;  $p = 0.0149$ ). Univariate analysis revealed that TMPRSS4 upregulation, tumor size, deeper tumor invasion, lymph node metastasis (N), lymphatic invasion, and tumor stage were significant prognostic factors for OS. Multivariate analysis revealed that N and TMPRSS4 upregulation were significant prognostic factors for OS. The 5-year OS rate was examined in two patient groups: the group with the receiver operating characteristic curve cut-off value  $\geq 45\%$  for TS-1 (cancer drug formulation) oral dosage and the group with TS-1 dosage cut-off value  $< 45\%$ . For the patients in the TS-1 dosage  $\geq 45\%$  group, there were significant differences in OS between the TMPRSS4-positive and -negative groups ( $p = 0.0284$ ): the 5-year OS rates of TMPRSS4-positive and -negative groups were 65.2% and 79.2%, respectively. Our findings suggest that TMPRSS4 overexpression is a useful biomarker for GC, and that an anti-TMPRSS4 antibody may have potential as a novel therapeutic agent.

**Keywords** Gastric cancer, Transmembrane serine protease 4, Biomarker, Overall survival, Progression free survival

Regarding Gastric Cancer (GC) prognosis by cancer stage, stage I GC survival is approximately 100%, while that for stage IV is 10%, despite advances in chemotherapy. For stage II/III GC, surgical treatment combined with perioperative chemotherapy is recommended in Japan<sup>1</sup>. In fact, in stage II, the 5-year survival rate was 84.2% in the S-1 (cancer drug formulation) treatment group with postoperative S-1 chemotherapy, showing

<sup>1</sup>Department of Surgery, NHO Kure Medical Center and Chugoku Cancer Center, 3-1, Aoyama, Kure, Hiroshima 737-0023, Japan. <sup>2</sup>Department of Gastroenterological Surgery, NHO National Hospital Organization Shikoku Cancer Center, Matsuyama, Ehime, Japan. <sup>3</sup>Department of Surgery, NHO National Hospital Organization Kanmon Medical Center, Shimonoseki, Yamaguchi, Japan. <sup>4</sup>Department of Surgery, NHO National Hospital Organization Fukuyama Medical Center, Fukuyama, Hiroshima, Japan. <sup>5</sup>Department of Surgery, NHO National Hospital Organization Osaka National Hospital, Osaka, Japan. <sup>6</sup>Department of Gastroenterological Surgery, NHO National Hospital Organization Higashihiroshima Medical Center, Higashihiroshima, Japan. <sup>7</sup>Department of Surgery, NHO National Hospital Organization Sagamiyama National Hospital, Sagamiyama, Kanagawa, Japan. <sup>8</sup>Department of Surgery, NHO National Hospital Organization Okayama Medical Center, Okayama, Japan. <sup>9</sup>Department of Surgery, NHO National Hospital Organization Iwakuni Clinical Center, Iwakuni, Yamaguchi, Japan. <sup>10</sup>Department of Surgery, NHO Fukuokahigashi Medical Center, National Hospital Organization Fukuokahigashi Medical Center, Koga, Fukuoka, Japan. <sup>11</sup>Department of Diagnostic Pathology, NHO Kure Medical Center and Chugoku Cancer Center, Kure, Hiroshima, Japan. <sup>12</sup>Department of Gastroenterological and Transplant Surgery, Graduate School of Biochemical and Health Sciences, Hiroshima University, Hiroshima, Japan. ✉email: thiroes@gmail.com

significantly better survival than 71.3% for the surgery alone group, indicating that the benefit of postoperative adjuvant S-1 therapy was favorable<sup>2,3</sup>. Conversely, the survival rate for stage III, especially stage IIIB, was 50.2% in the combined S-1 group and 44.1% in the surgery-alone group, indicating that the benefit of S-1 postoperative adjuvant therapy was insufficient and that there was room for improvement in postoperative chemotherapy in stage III<sup>2,3</sup>. Therefore, a clinical trial (JACCROGC-07) of adjuvant docetaxel and S-1 for stage III GC was conducted, and an interim analysis showed that adjuvant S-1 plus docetaxel significantly prevented GC recurrence compared with S-1 treatment alone<sup>4</sup>. With the results of this interim analysis, the trial was terminated and S-1 plus docetaxel was recommended as the new standard adjuvant chemotherapy for stage III GC in Japanese guidelines for the treatment of GC.

In parallel with these clinical trials, research on biomarkers for the prognosis and efficacy of chemotherapy is being vigorously pursued. Liquid biopsy (blood derived from cancer tissue) has recently been used as a noninvasive test to detect free DNA and non-coding RNA, such as exosome-derived microRNA, with high sensitivity using next-generation sequencers. However, there are many challenges to overcome, such as reading errors. In addition, in the biomarker studies associated with the clinical trials described above, the detection of messenger RNAs in tissues by immunostaining and reverse transcriptase-polymerase chain reaction (RT-PCR) has been investigated, and thymidylate synthase (TS)<sup>5</sup> and EphrinA2 receptors<sup>6</sup> have been identified as prognostic factors in stage II and III GC by immunostaining. However, the frequency of TS expressions in patients with GC is low (approximately 16%), and the breakdown of stage II and III GC tumors is unknown. Therefore, there are still issues to be addressed in clinical applications.

In this study, we focused on transmembrane protease serine 4 (TMPRSS4), a membrane serine protease belonging to the type II transmembrane serine protease family, as a new biomarker for GC<sup>7</sup>. It has been reported that TMPRSS4 is involved in invasive metastasis of cancer cells and is highly expressed in various cancer tissues, including GC<sup>8,9</sup>. Recent reports have also indicated that TMPRSS4 is useful as a biomarker for postoperative GC<sup>10,11</sup>, and that cancer cells exhibiting upregulated TMPRSS4 expression have increased resistance to anticancer drugs<sup>12</sup>. We have previously reported an analysis of postoperative GC specimens at a single institution where we observed a significantly poorer prognosis for patients with GC who showed upregulated TMPRSS4 expression, suggesting its usefulness as a biomarker<sup>13</sup>. However, our study was conducted at a single institution, and the sample size was small (105 cases overall and 39 cases in stage III GC). A verification of our reported results by employing a sufficiently high number of GC cases was needed. This report presents the results of data collected from 10 clinical facilities, and by increasing the number of cases in stages II and III, which more accurately reflect treatment effects, more reliable results were obtained.

## Patients and methods

### Study design

This was a multicenter retrospective study conducted at ten institutions including National Hospital Organization (NHO), Kure Medical Center and Chugoku Cancer Center (Kure, Hiroshima, Japan), NHO Shikoku Cancer Center (Matsuyama, Japan), NHO Kanmon Medical Center (Shimonoseki, Yamaguchi, Japan), NHO Fukuyama Medical Center (Fukuyama, Hiroshima, Japan), NHO Osaka National Hospital (Osaka, Japan), NHO Higashihiroshima Medical Center (Higashihiroshima, Japan), NHO Sagami National Hospital (Sagamihara, Kanagawa, Japan), NHO Okayama Medical Center (Okayama, Japan), NHO Iwakuni Clinical Center (Iwakuni, Yamaguchi, Japan), and NHO Fukuoka Medical Center (Koga, Fukuoka, Japan).

The major inclusion criteria were as follows: (a) Age between 20 and 85 years, (b) Patients with pathologically diagnosed GC stage II or III, (c) Patients who received postoperative adjuvant therapy with oral S-1, (d) Patients who underwent GC surgery with standard D2 or higher dissection, (e) Patients who have not received preoperative anticancer therapy for GC, (f) Patients who underwent surgery in 2015 or earlier. The study was conducted in accordance with the principles of the Declaration of Helsinki. This study was reviewed and approved by the NHO Central Ethics Committee [Approval Number: R3-NHO(Gastroenterology)-02]. All research was performed in accordance with relevant regulations and all patients signed a written informed consent form agreeing to their blood samples and data collected and used for research purposes.

### Patients

Between January 2011 and December 2015, 325 patients underwent resection for GC at the multicenter locations shown above. All patients received R0 resection according to the previously outlined guidelines<sup>14</sup>.

### List of patient assessments

- Pathological evaluation of post-resection GC specimens (GC Treatment Protocol, 15th Edition of the Japanese Classification of Gastric Carcinoma).
- Performance status (PS) must be 0 or 1.
- Size and number of primary lesions; site of occupancy; gross typing; depth (T); lymph node metastases (N); other metastases (M) or liver metastases (H); peritoneal metastases (P); cytology by peritoneal washing (CY); progression; resection margins and tumor remnants; histology; invasive cancer growth pattern; and vascular invasion.
- Expression of TMPRSS4 by immunostaining using resection specimens.
- Clinical data included patient sex, age, surgical technique (including the method of reconstruction), recurrence, and date of death.

## Endpoints assessment

The medical records of patients were reviewed retrospectively, and data were collected. Following this step, overall survival (OS) and progression-free survival (PFS) were analyzed. PFS was defined as the period between the day of gastrectomy and the day of initial disease progression (specifically, the date when the recurrence is recognized as a finding on imaging tests) or death from any cause and was censored on the last day of follow-up. OS was defined as the time from the initiation of the study treatment to death due to any cause. The survivors were censored at their last contact. Tumors were classified based on the Japanese Classification of Gastric Carcinoma, 14th edition<sup>15</sup>.

## Immunohistochemical analysis

Immunohistochemical analyses were performed according to the instructions of the manufacturer (DAKO). Tissue sections, 4 µm in thickness, were incubated with the primary rabbit anti-TMPRSS4 antibody (1:200; Cat. # ab150595, Abcam, Cambridge, UK) overnight at 4 °C followed by incubation with the secondary antibody. All immunohistochemistry results were determined by two independent pathologists (A.S. and K.K.) who were blind to the study. The TMPRSS4 expression status of the clinical specimens was scored as follows: specimens showing TMPRSS4 expression in ≥ 50% of all cells were deemed TMPRSS4-positive while those exhibiting TMPRSS4 expression in < 50% of all cells were denoted as TMPRSS4-negative.

## Quantifying TS-1 dosage as adjuvant therapy after surgery

Patients received S-1 (80–120 mg per day), depending on the body surface area of the patient, for 4 weeks, followed by 2 weeks of rest. This 6-week cycle was repeated for 1 year. For each patient, the actual oral dose was calculated by assuming that 100% of the dose was taken for 1 year without any dose reduction.

## Statistical analysis

Continuous variables were expressed as median (range) and were analyzed using the Mann–Whitney U-test. The categorical variables and postoperative courses were compared using  $\chi^2$  tests with the Yates correction. OS and PFS after surgical resection were compared in each group using the Kaplan–Meier method and the log-rank test. Univariate and multivariate analyses were performed using the Cox regression test. The differences were considered statistically significant if the *P* value was less than 0.05. Statistical analyses were performed using SPSS statistical software version 16 (Chicago, Illinois, USA).

## Results

### TMPRSS4 overexpression in GC tissues

Immunohistochemical analysis showed that TMPRSS4-positive staining was present in the cytoplasm of GC cells (Fig. 1). The frequency of upregulated TMPRSS4 expression was 44.9% (146/325 tissue specimens) among this GC cohort (Table 1).

### Association between clinico-pathological features and TMPRSS4 positivity

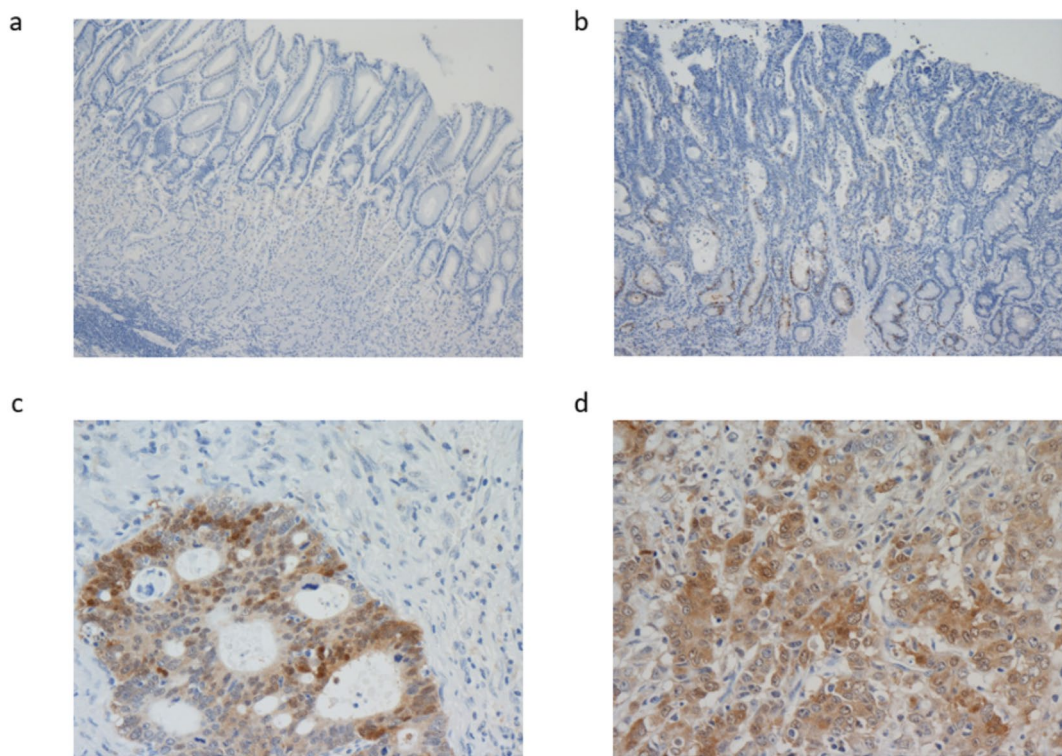
We evaluated the association between TMPRSS4 expression and clinicopathological features (Table 1). TMPRSS4 expression was positively and significantly correlated with patient age and depth of tumor invasion (T), but not with sex, tumor size, tumor location, lymph node metastasis (N), histological type, venous invasion, tumor stage, Lauren classification, tumor infiltrative pattern, and lymphatic invasion (LI).

The cases in this study were diagnosed before 2015, and there were differences in the number of cases registered between facilities in terms of clinical data and pathological specimens. However, all cases provided were included in the results of this study. In addition, we compared and examined the data on the oncology factors between facilities, but we found no significant differences (Supplementary Table S1).

### Association between TMPRSS4 expression and patient outcomes after gastrectomy

The five-year OS rate in the TMPRSS4-positive group was significantly lower than that in the TMPRSS4-negative group (62.4% vs. 76.4%, respectively;  $p=0.0149$ ). Moreover, the 5-year PFS rate was also significantly lower in the TMPRSS4-positive group compared with the TMPRSS4-negative group (57.1% vs. 74.5%, respectively;  $p=0.0046$ ) (Fig. 2). Univariate and multivariate analyses were conducted using the Cox proportional hazards model to evaluate the influence of TMPRSS4 expression and pathological factors on the prognosis of patients with GC who underwent gastrectomy. Univariate analysis revealed that TMPRSS4 expression, tumor size, T, N, LI, and tumor stage were significant prognostic factors for OS. Since tumor stage is a factor that is consistent with T and N, it was removed from the list of items for multivariate analysis. Moreover, multivariate analysis revealed that N and TMPRSS4 expression were significant prognostic factors for OS (Table 2). Considering PFS, univariate analysis revealed that, similar to OS, TMPRSS4 expression, tumor size, T, N, LI, and tumor stage were all significant prognostic factors for PFS. Moreover, multivariate analysis revealed that N and TMPRSS4 expression were significant prognostic factors for PFS (Table 3).

In terms of clinical stages of cancer, there were significant differences in the OS and PFS of patients with stage III GC between the TMPRSS4-positive and -negative groups ( $p=0.0324$  and  $p=0.0156$ , respectively): the 5-year OS rates of the TMPRSS4-positive and negative groups were 49.5% and 64.5%, respectively, and the 5-year PFS rates of the TMPRSS4-positive and -negative groups were 43.5% and 64.1%, respectively (Fig. 3a,b). For patients with stage II GC, the differences in OS and PFS between the TMPRSS4-positive and -negative groups were not statistically significant ( $p=0.0850$  and  $p=0.0676$ , respectively): the 5-year OS rates of TMPRSS4-positive and -negative groups were 82.3% and 95.5%, respectively, and the 5-year PFS rates of TMPRSS4-positive and -negative groups were 77.2% and 91.3%, respectively (Fig. 4a,b).



**Fig. 1.** Immunohistochemical staining for transmembrane protease serine 4 (TMPRSS4) in gastric cancer (GC) lesions and non-cancerous tissues. **(a)** TMPRSS4-negative non-cancerous gastric tissues (original magnification, 10X), **(b)** TMPRSS4-negative GC tissues with moderately differentiated adenocarcinoma (10X), **(c)** TMPRSS4-positive GC tissues with moderately differentiated adenocarcinoma (40X), **(d)** TMPRSS4-positive GC tissues with poorly differentiated adenocarcinoma (40X).

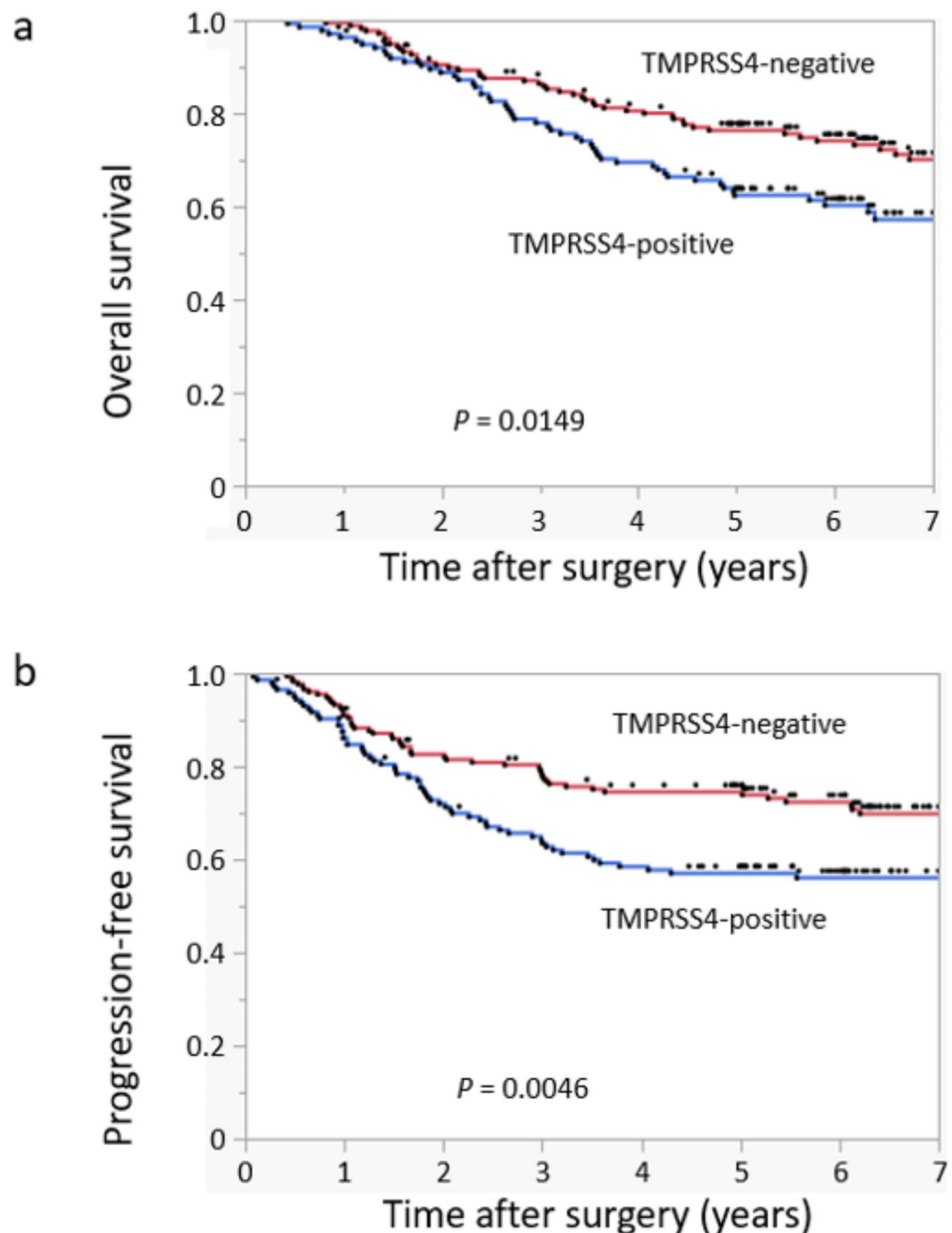
Variables	TMPRSS4 expression		P value
	Negativity	Positivity	
	N = 179	N = 146	
Age	64.8 (28–85)	67.5 (28–85)	0.0289
Gender (Male/Female)	113/66	99/47	0.3776
Location (J/U/M/L)	1/29/87/62	3/30/69/44	0.4137
Size	53.3 (20–420)	62.1 (13–450)	0.339
Lauren classification (i/m/d)	78/25/74	73/17/56	0.5435
Tumor infiltrative pattern (IFN) ( a/b/c)	25/74/78	10/63/73	0.0948
Histology (differentiated/undifferentiated)	63/114	58/88	0.4453
Depth of tumor invasion (T) (1/2/3/4)	11/22/69/77	3/9/68/66	0.0464
Lymph node metastasis (N) (0/1/2/3)	17/44/57/61	28/37/35/46	0.0605
Staging (II/III)	70/109	58/88	0.9094
Venous invasion (v) (0/1/2/3)	56/65/44/14	61/50/24/11	0.1611
lymphatic invasion (ly) (0/1/2/3)	25/65/53/36	29/47/37/33	0.4177

**Table 1.** Correlation between TMPRSS4 and clinical features. J, esophagogastric junction; U, upper third; M, middle third; L, lower third, i, intestinal type; m, mixed type; d, diffuse type.

**Association between S-1 dosage, patient outcomes after gastrectomy, and TMPRSS4 expression**

Receiver operating characteristic (ROC) curve analysis was used to calculate the optimal cutoff value of S-1 oral dose as adjuvant chemotherapy in positive or negative TMPRSS4. Thus, the optimal dose of S-1 oral dose was evaluated by ROC curve analysis (Supplementary Fig. S1). The ROC curve showed that the best cut-off value for quantifying the S-1 dosage was  $\geq 45\%$ . The 5-year OS rate was examined by dividing the patients into two groups: the group with an ROC curve cut-off value  $\geq 45\%$  for S-1 dosage (n = 254) and the group with the S-1 dosage ROC curve cut-off value  $< 45\%$  (n = 71). For patients in the S-1 dosage  $< 45\%$  group, there were no differences in





**Fig. 2.** Kaplan–Meier curves for all patients with GC classified on the basis of TMPRSS4 expression. **(a)** Overall survival (OS) curves of all patients with GC. **(b)** Progression-free survival (PFS) curves of all patients with GC. The OS and PFS outcomes were compared using the log-rank test.

OS between the two groups ( $p=0.8769$ ), and the 5-year OS rates of the TMPRSS4-positive and -negative groups were 55.7% and 58.4%, respectively (Fig. 5a). Conversely, for patients in the S-1 dosage  $\geq 45\%$  group, there were significant differences in OS between the TMPRSS4-positive and -negative groups ( $p=0.0284$ ); the 5-year OS rates of the TMPRSS4-positive and negative groups were 65.2% and 79.2%, respectively (Fig. 5b).

## Discussion

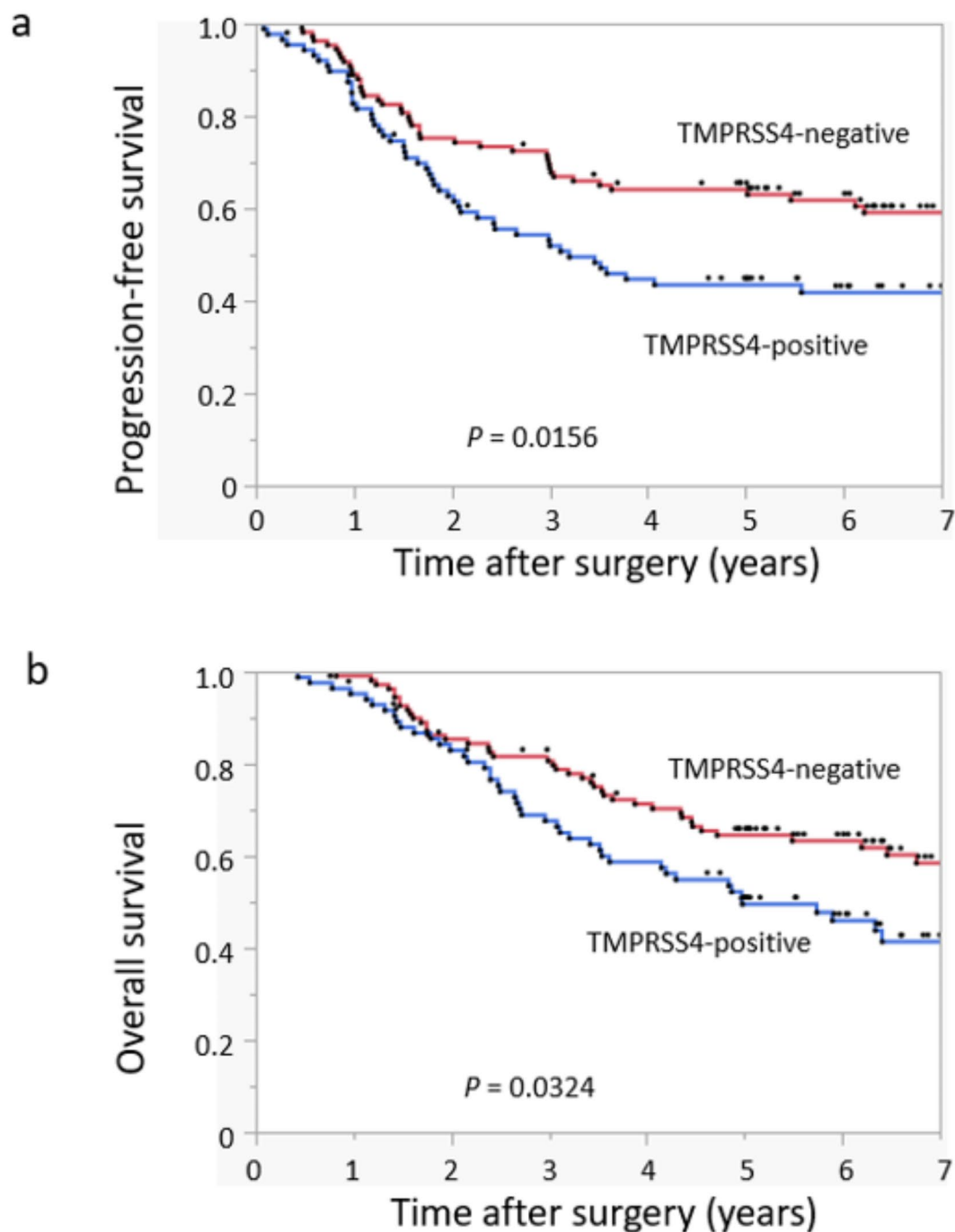
This report summarizes the data collected from 10 clinical facilities. By going further than the results presented for a single facility and increasing the number of cases by focusing on stages II and III, we were able to obtain results that lend more credibility to the involvement of TMPRSS4 in the progression of GC. Even when looking

Variables	Number of patients	OS	p value	PFS	p value
		5-year survival rate (%)		5-year survival rate (%)	
Age					
70 <	141	66.4	0.247	62.1	0.092
70 ≥	183	73.2		70.2	
Gender					
Male	212	66.2	0.0423	61.9	0.045
Female	113	77.9		75.7	
TMPRSS4					
Positivity	179	62.4	0.0149	57.1	0.0046
Negativity	145	76.4		74.5	
Tumor location					
Junction	4	50.0	0.2071	50.0	0.2732
Upper	59	70.6		61.0	
Middle	155	71.1		69.0	
Lower	106	70.2		67.4	
Tumor size					
97 <	263	53.1	0.0029	52.1	0.0164
97 ≥	61	74.3		70.1	
Stage					
II	128	89.8	< 0.0001	84.8	< 0.0001
III	196	58.2		55.1	
Depth of tumor invasion(T)					
T1	14	85.7	< 0.0001	78.6	0.0142
T2	31	89.7		89.7	
T3	137	77.8		71.0	
T4	143	58.3		56.9	
Lymph node metastasis (N)					
0	45	85.0	< 0.0001	75.1	< 0.0001
1	81	89.3		86.0	
2	92	72.0		67.3	
3	106	49.2		48.4	
Lymphatic invasion (Ly)					
0	54	77.2	0.0014	71.3	0.0278
1	112	82.3		75.4	
2	89	60.1		58.3	
3	69	58.9		60.3	
Venous invasion (v)					
0	117	70.2	0.7067	67.5	0.5548
1	114	72.2		65.6	
2	68	63.9		62.2	
3	25	80.0		80.0	
Histology					
Differentiated	120	72.3	0.3084	0.4445	
Undifferentiated	212	69.0		68.8	
				65.2	
Tumor infiltrative type (IFN)					
a	35	81.4	0.0872	65.7	0.3692
b	136	73.4		70.7	
c	151	65.3		63.0	
Lauren type					
i	150	74.9	0.1621	71.9	0.0904
m	42	70.7		58.0	
d	130	64.9		63.0	

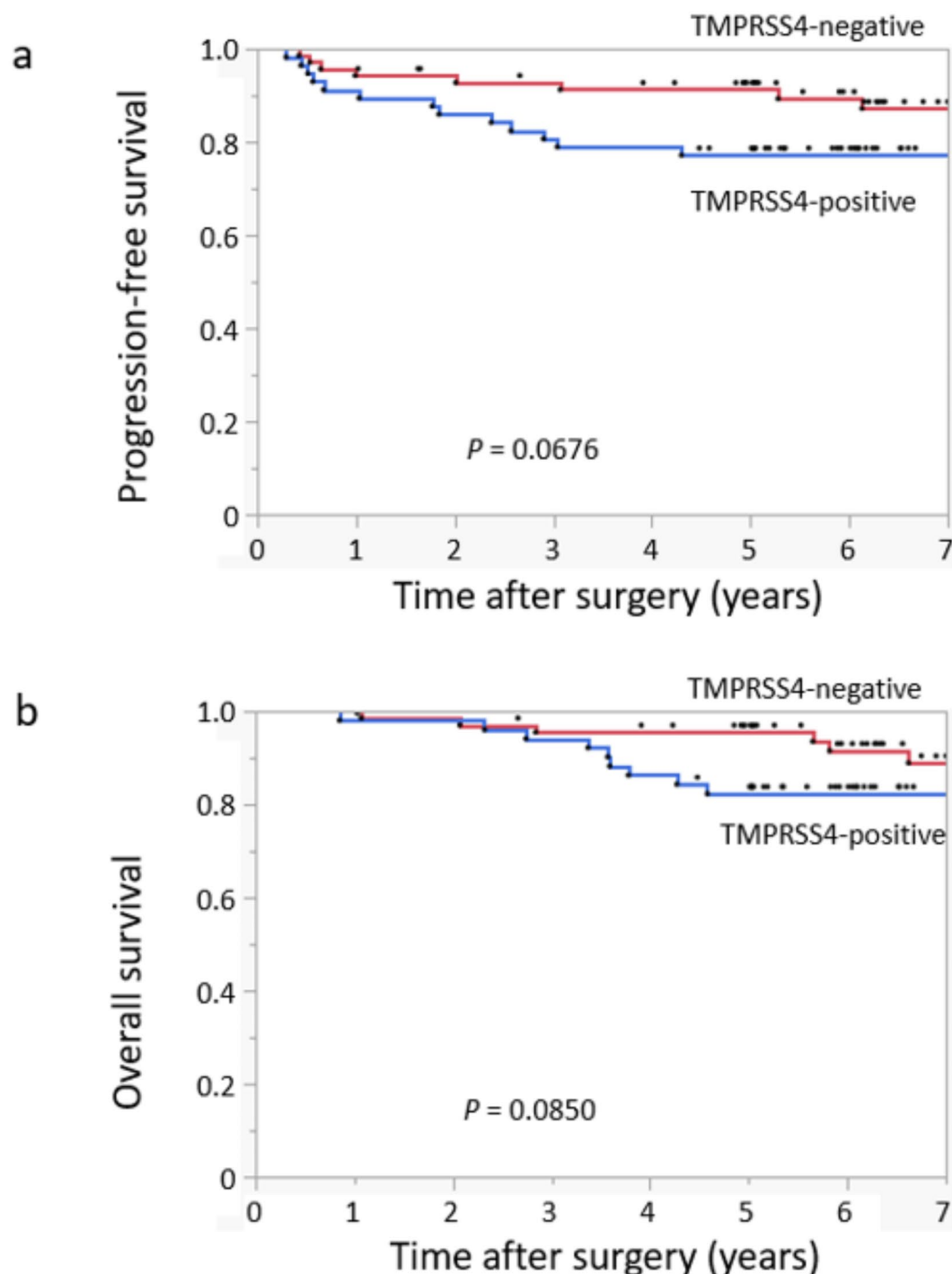
**Table 2.** Univariate analyses of OS and RFS in patients with gastric cancer. OS, overall survival; PFS, progression-free survival.

	OS			PFS		
	HR	95% CI	<i>p</i> value	HR	95% CI	<i>p</i> value
Lymph node metastasis (N)	6.64	– 1.198–0.060	0.0001	5.411	– 1.411–1.540	0.0001
TMPRSS4	2.26	0.062–0.367	0.0059	2.498	0.112–0.556	0.0032

**Table 3.** Multivariate analyses of OS and RFS in patients with gastric cancer. OS, overall survival; PFS, progression-free survival; HR, hazard ratio; CI, confidence interval.



**Fig. 3.** Kaplan–Meier curves are categorized on stage III for patients with GC classified on the basis of TMPRSS4 expression. (a) PFS curves for patients with GC in stage III. (b) OS curves of patients with GC in stage III.

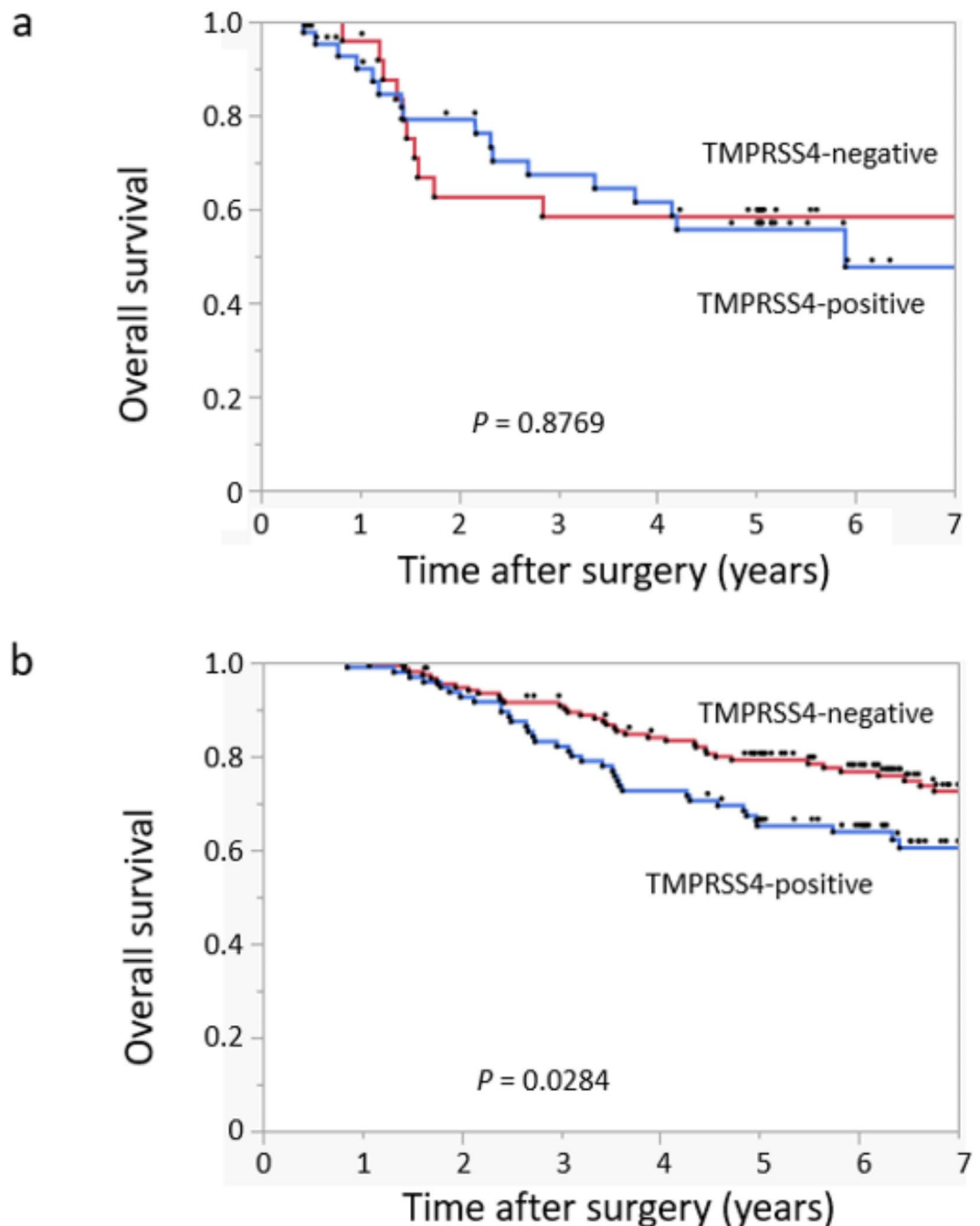


**Fig. 4.** Kaplan–Meier curves are categorized on stage II for patients with GC classified on the basis of TMPRSS4 expression. (a) PFS curves for patients with GC in stage II. (b) OS curves for patients with GC in stage II.

at the disease separately, a significant difference in TMPRSS4 was observed in both OS and PFS for stage III GC. Unfortunately, no significant difference was observed for stage II GC, but such a trend was observed.

These groundbreaking results are based on pathological immunostaining of TMPRSS4. The criteria for TMPRSS4 immunostaining were defined by us, and there are some questions about these criteria. First, staining intensity was not used as a criterion because it varies depending on the condition of the staining preparation, and second, there is no clear criterion for staining intensity as Human epidermal growth factor receptor 2 (HER2) staining<sup>16</sup>. Regarding the staining area, there is no clear standard here either. However, reports on TMPRSS4 in lung cancer, pancreatic cancer, and breast cancer all include a 50% staining area line as a criterion<sup>17–19</sup>, and we





**Fig. 5.** Kaplan–Meier curves are categorized on S-1 dosage for patients with GC classified on the basis of TMPRSS4 expression. (a) OS curves of the TS-1 oral < 45% group (n = 71), (b) OS curves of the TS-1 oral  $\geq 45\%$  group (n = 254).

set the criterion for this study according to these papers. Of course, this setting is not necessarily correct, but it is at least sufficient to prove that this biomarker is involved in the progression of gastric cancer.

Biomarkers for stage II and III GC have been studied in Japan from patients who participated in the ACTS-GC trial<sup>2</sup>. Terashima et al. examined the expression of 63 genes by RT-PCR from 829 cases and performed a prognostic analysis. In their report, they showed that TOP2A, GGH, and PECAM1 gene expression correlated with hematogenous metastasis, lymph node metastasis, and peritoneal recurrence, but only PECAM1 gene expression correlated with peritoneal recurrence in the S-1 postoperative adjuvant therapy group, while there was insufficient analysis by individual GC stage<sup>20</sup>. However, in the adjuvant S-1 group, only PECAM1 gene expression correlated with peritoneal dissemination recurrence, and the analysis by individual GC stage was insufficient<sup>20</sup>. Similarly, in a follow-up study of the ACTS-GC trial, the expression of protein enzymes related to 5-FU metabolism was examined by immunostaining; however, the GC stage-specific analysis was insufficient in the group with high expression of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD)<sup>5</sup>.

Conversely, in a non-Japanese study, TS levels, excision repair cross-complementation group ((ERCC1), programmed death-ligand 1 (PDL), and S-1 were detected by immunostaining in patients with stage II/III GC who also underwent Xelox postoperative chemotherapy for 6 months. In patients who received postoperative chemotherapy (66 patients), high TS expression was associated with significantly poorer RFS, but the high TS group was about 15% and was not examined by stage as well<sup>21</sup>. In our previous study, we demonstrated that TMPRSS4-silenced NUGC-3 and MKN-45 cells were significantly sensitive to 5-FU treatment compared to the corresponding non-specific control siRNA-transfected cells. Furthermore, in the mouse model low TMPRSS4 expression suppressed the migration of GC cells and enhanced their sensitivity to 5-FU treatment<sup>13</sup>. The pharmacological compounds that inhibit TMPRSS4 in combination with chemotherapy, such as S-1, may promote the sensitivity to chemotherapy through inhibiting the function of TMPRSS4. In this study, we showed that patients in the S-1 dose  $\geq 45\%$  group with TMPRSS4-negative tumors had significantly better OS than those with TMPRSS4-positive tumors. This result suggests that TMPRSS4-positive GC cells are resistant to S-1. However, there was not enough of a difference to show a result of S-1 resistance in the stage II TMPRSS4-positive group, that is the reason why there was no significant difference at OS and PFS.

Recently, Claudin 18.2 has also attracted attention as a new biomarker for gastric cancer. Claudin 18.2 is a protein involved in the formation of tight junctions and is selectively expressed in normal gastric mucosa<sup>22</sup>. Zolbetuximab is an anti-Claudin 18.2 monoclonal antibody that targets and binds to Claudin 18.2, inducing tumor cell death via antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) that induces cell death<sup>23</sup>. The higher the expression of Claudin 18.2, the better the effect<sup>24</sup>. More recently, randomized phase III trials (SPOTLIGHT, GLOW) have shown that zolbetuximab significantly prolonged OS and PFS when added to pyrimidine fluoride and platinum-based combination chemotherapy<sup>25,26</sup>. Both TMPRSS4 and Claudin are transmembrane proteins and have been reported to be involved in signal transduction with each other<sup>27</sup>. Furthermore, recent research on genetically modified mice and mice administered protease inhibitors has shown that abnormalities in the expression of TTSPs are causally related to the development and progression of cancer<sup>28–30</sup>. In colon, prostate, and lung cancer cells, TMPRSS4 promotes the downregulation of E-cadherin. This is often accompanied by morphological changes and actin reorganization, leading to EMT events and cancer cell invasion. TMPRSS4 also regulates cell–matrix adhesion and cell extension by regulating integrins such as  $\alpha 5\beta 1$  and  $\alpha 4\beta 1$ , which are involved in EMT, cell motility, and/or cell survival<sup>31,32</sup>. In line with this, TMPRSS4 promotes the invasion of GC cells by activating NF- $\kappa$ B signaling, but the exact mechanism needs to be elucidated<sup>33</sup>. Furthermore, TMPRSS4 may be involved in the regulation of the tumor microenvironment (e.g., regulation of the immune state and angiogenesis) via NF- $\kappa$ B, and may contribute to malignancy. Further investigation is needed to confirm these possibilities. Recently, 2-hydroxydiarylamide derivatives, IMD-0354 and KRT1853, were developed as TMPRSS4 serine protease inhibitors<sup>34</sup>. These new compounds have been shown to inhibit cancer cell invasion, migration, and proliferation; however, their sensitivity for GC chemotherapy was not determined.

This study is a multicenter retrospective study. It has the limitation of not examination the sample size in advance and not enough for it. Furthermore, regional differences between facilities and differences in treatment methods have not been examined. However, in Japan, there is a universal health insurance system, and treatment guidelines have been created for each disease, so there are few differences in treatment policies between hospitals. Our data suggest that TMPRSS4 is involved in the progression of GC and might be a new treatment method.

We look forward to the potential of anti-TMPRSS4 antibodies as a new therapeutic agent.

## Data availability

The datasets used and/or analyzed in this study are available upon reasonable request. If someone wants to request the data from this study, he/she may request them from the corresponding author.

Received: 20 September 2024; Accepted: 6 March 2025

Published online: 11 March 2025

## References

1. GASTRIC (Global Advanced/Adjuvant Stomach Tumor Research International Collaboration) Group et al. Benefit of adjuvant chemotherapy for resectable gastric cancer: a meta-analysis. *JAMA* **303**, 1729–1737. <https://doi.org/10.1001/jama.2010.534> (2010).
2. Sakuramoto, S. et al. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl. J. Med.* **357**, 1810–1820. <https://doi.org/10.1056/NEJMoa072252> (2007).
3. Sasako, M. et al. Five-year outcomes of a randomized phase III trial comparing adjuvant chemotherapy with S-1 versus surgery alone in stage II or III gastric cancer. *J. Clin. Oncol.* **29**, 4387–4393. <https://doi.org/10.1200/JCO.2011.36.5908> (2011).
4. Yoshida, K. et al. Addition of docetaxel to oral fluoropyrimidine improves efficacy in patients with stage III gastric cancer: interim analysis of JACCRO GC-07, a randomized controlled trial. *J. Clin. Oncol.* **37**, 1296–1304. <https://doi.org/10.1200/JCO.18.01138> (2019).
5. Sasako, M. et al. Impact of the expression of thymidylate synthase and dihydropyrimidine dehydrogenase genes on survival in stage II/III gastric cancer. *Gastric Cancer*. **18**, 538–548. <https://doi.org/10.1007/s10120-014-0413-8> (2015).
6. Kikuchi, S. et al. Over expression of Ephrin A2 receptors in cancer stromal cells is a prognostic factor for the relapse of gastric cancer. *Gastric Cancer*. **18**, 485–494. <https://doi.org/10.1007/s10120-014-0390-y> (2015).
7. Tanabe, L. M. & List, K. The role of type II transmembrane Serine protease-mediated signaling in cancer. *FEBS* **284**, 1421–1436. <https://doi.org/10.1111/febs.13971> (2017).
8. Martin, C. E. & List, K. Cell-surface anchored Serine proteases in cancer progression and metastasis. *Cancer Metastasis Rev.* **38**, 357–387. <https://doi.org/10.1007/s10555-019-09811-7> (2019).
9. Larzabal, L. et al. Over expression of TMPRSS4 in non-small cell lung cancer is associated with poor prognosis in patients with squamous histology. *Br. J. Cancer*. **105**, 1608–1614. <https://doi.org/10.1038/bjc.2011.432> (2011).
10. Sheng, H., Shen, W., Zeng, J., Xi, L. & Deng, L. Prognostic significance of TMPRSS4 in gastric cancer. *Neoplasma* **61**, 213–217. [https://doi.org/10.4149/neo\\_2014\\_027](https://doi.org/10.4149/neo_2014_027) (2014).

11. Luo, Z. Y., Wang, Y. Y., Zhao, Z. S., Li, B. & Chen, J. F. The expression of TMPRSS4 and Erk1 correlates with metastasis and poor prognosis in Chinese patients with gastric cancer. *PLOS ONE*. **8**, e70311. <https://doi.org/10.1371/journal.pone.0070311> (2013).
12. Exposito, F. et al. Targeting of TMPRSS4 sensitizes lung cancer cells to chemotherapy by impairing the proliferation machinery. *Cancer Lett.* **453**, 21–33. <https://doi.org/10.1016/j.canlet.2019.03.013> (2019).
13. Tazawa, H. et al. Utility of TMPRSS4 as a prognostic biomarker and potential therapeutic target in patients with gastric cancer. *J. Gastrointest. Surg.* **26**, 305–313. <https://doi.org/10.1007/s11605-021-05101-2> (2022).
14. Japanese gastric cancer society. Guideline for diagnosis and treatment of carcinoma of the stomach <https://link.springer.com/article/10.1007/s10120-022-01331-8>
15. Kanehara, T. Japanese Research Society for gastric cancer: Japanese classification of gastric carcinoma. 14th ed., pp. 1–88; (in Japanese) (2010).
16. Antonio, C. et al. Human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical oncology/college of American pathologists clinical practice guideline focused update. *J. Clin. Oncol.* **36**, 2105–2122. <https://doi.org/10.1200/jco.2018.77.8738> (2018).
17. Ruben, P. J. et al. Data-Driven identification of targets for Fluorescence-Guided surgery in Non-Small cell lung Cancer. *Mol. Imaging Biol.* **25**, 228–239. <https://doi.org/10.1007/s11307-022-01791-5> (2023).
18. Gu, J. et al. TMPRSS4 promotes cell proliferation and inhibits apoptosis in pancreatic ductal adenocarcinoma by activating ERK1/2 signaling pathway. *Front. Oncol.* **18**, 11. <https://doi.org/10.3389/fonc.2021.628353> (2021).
19. Daye Cheng, H. & Kong Yunhui Li TMPRSS4 as a poor prognostic factor for Triple-Negative breast Cancer. *Int. J. Mol. Sci.* **14**, 14659–14668. <https://doi.org/10.3390/ijms140714659> (2013).
20. Terashima, M. et al. TOP2A, GGH, and PECAM1 are associated with hematogeneous, lymph node, and peritoneal recurrence in stage II/III gastric cancer patients enrolled in the ACRS-GC study. *Oncotarget* **8**, 57574–57582. <https://doi.org/10.18632/oncotarget.15895> (2017).
21. Kim, M. H. et al. Immunohistochemistry biomarkers predict survival in stage II/III gastric cancer patients: from a prospective clinical trial. *Cancer Res. Treat.* **51**, 819–831. <https://doi.org/10.4143/crt.2018.331> (2019).
22. Mahmud, H. et al. Preclinical characterization of an mRNA-encoded anti-claudin 18.2 antibody. *Oncoimmunology* **16**, 2255041. <https://doi.org/10.1080/2162402x.2023.2255041> (2023).
23. Tureci, O., Mitnacht-Kraus, R., Woll, S., Yamada, T. & Sahin, U. Characterization of Zolbetuximab in pancreatic cancer models. *Oncoimmunology* **10**, e1523096. <https://doi.org/10.1080/2162402x.2018.1523096> (2018).
24. Sahin, U. et al. FAST: a randomised phase II study of Zolbetuximab (IMAB362) plus EOX versus EOX alone for first-line treatment of advanced CLDN18.2-positive gastric and gastro-oesophageal adenocarcinoma. *Ann. Oncol.* **32**, 609–619. <https://doi.org/10.1016/j.annonc.2021.02.005> (2021).
25. Shah, M. A. et al. Zolbetuximab plus CAPOX in CLDN18.2-positive gastric or gastroesophageal junction adenocarcinoma: the randomized, phase 3 GLOW trial. *Nat. Med.* **29**, 2133–2141. <https://doi.org/10.1038/s41591-023-02465-7> (2023).
26. Shitara, K. et al. Zolbetuximab plus mFOLFOX6 in patients with CLDN18.2-positive, HER2-negative, untreated, locally advanced unresectable or metastatic gastric or gastro-oesophageal junction adenocarcinoma (SPOTLIGHT): a multicentre, randomised, double-blind, phase 3 trial. *Lancet* **401**, 1655–1668. [https://doi.org/10.1016/S0140-6736\(23\)00620-7](https://doi.org/10.1016/S0140-6736(23)00620-7) (2023).
27. Shaya Mahati, D. et al. TMPRSS4 promotes cancer stem cell traits by regulating CLDN1 in hepatocellular carcinoma. *Biochem. Biophys. Res. Commun.* **490**, 906–912. <https://doi.org/10.1016/j.bbrc.2017.06.139> (2017).
28. Carly, E. & Martin, K. Cell surface-anchored Serine proteases in cancer progression and metastasis. *Cancer Metastasis Rev.* **38**, 357–387. <https://doi.org/10.1007/s10555-019-09811-7> (2019).
29. Lauren, M. & Tanabe, K. The role of type II transmembrane Serine protease-mediated signaling in cancer. *FEBS J.* **284**, 1421–1436. <https://doi.org/10.1111/febs.13971> (2017).
30. Andrew, S., Murray, F. A., Varela, K. & List Type II transmembrane Serine proteases as potential targets for cancer therapy. *Biol. Chem.* **397**, 815–826. <https://doi.org/10.1515/hsz-2016-0131> (2016).
31. Jung, H. et al. TMPRSS4 promotes invasion, migration and metastasis of human tumor cells by facilitating an epithelial-mesenchymal transition. *Oncogene* **27**, 2635–2647. <https://doi.org/10.1038/sj.onc.1210914> (2008).
32. Lee, Y. et al. TMPRSS4 promotes cancer stem-like properties in prostate cancer cells through upregulation of SOX2 by SLUG and TWIST1. *J. Exp. Clin. Cancer Res.* **40**, 372. <https://doi.org/10.1186/s13046-021-02147-7> (2021).
33. Jin, J. et al. TMPRSS4 promotes invasiveness of human gastric cancer cells through activation of NF-kappaB/MMP-9 signaling. *Biomed. Pharmacother. Biomed. Pharmacother.* **77**, 30–36. <https://doi.org/10.1016/j.biopha.2015.11.002> (2016).
34. Kim, S. et al. Anti-cancer activity of the novel 2-hydroxydiarylamide derivatives IMD-0354 and KRT1853 through suppression of cancer cell invasion, proliferation, and survival mediated by TMPRSS4. *Sci. Rep.* **9**, 10003. <https://doi.org/10.1038/s41598-019-46447-7> (2019).

## Acknowledgements

We thank Mrs. Ishida for her contribution in maintaining the cell cultures and for her contribution in conducting statistical analyses.

## Author contributions

H. Tazawa and H. Tashiro wrote the manuscript. A. Saito and K. Kuraoka performed immunohistochemical analyses. All authors conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

## Funding

This research received no specific grants from any funding agency in the public, commercial, or not-for-profit sectors.

## Declarations

## Competing interests

The authors declare no competing interests.

## Ethics statements

This study was reviewed and approved by the NHO Central Ethics Committee [Approval Number: R3-NHO(Gastroenterology)-02].

### Informed consent

Informed consent was obtained from all participants or their legal guardians.

### Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-93422-6>.

**Correspondence** and requests for materials should be addressed to H.T.

**Reprints and permissions information** is available at [www.nature.com/reprints](http://www.nature.com/reprints).

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2025