Table 2 Univariate analysis of OS and RFS: expression of each gene was categorized as low or high at the 66.7th percentile

Marker	Group	Status	Number of patients	OS			RFS		
				5-Year survival (%)	HR (95 % CI)	*P* value (log-rank)	5-Year survival (%)	HR (95 % CI)	*P* value (log-rank)
TS	All	Low	540	66.5	1	0.222	58.1	1	0.085
		High	268	71.8	0.844 (0.642–1.109)		66.2	0.805 (0.629–1.031)	
	S-1	Low	275	69.9	1	0.008	62.2	1	0.003
		High	127	83.9	0.521 (0.319–0.850)		78.9	0.530 (0.344–0.816)	
	Surgery only	Low	265	63.0	1	0.623	53.8	1	0.923
		High	141	60.8	1.088 (0.777–1.522)		54.9	1.015 (0.747–1.380)	
DPD	All	Low	539	68.9	1	0.589	60.8	1	0.941
		High	268	66.9	1.075 (0.828–1.395)		60.6	1.009 (0.796–1.279)	
	S-1	Low	271	73.2	1	0.522	66.0	1	0.444
		High	130	76.3	0.870 (0.568–1.333)		70.1	0.862 (0.590–1.261)	
	Surgery only	Low	268	64.5	1	0.230	55.5	1	0.486
		High	138	58.0	1.225 (0.879–1.708)		51.6	1.114 (0.822–1.509)	
TP	All	Low	539	66.7	1	0.233	59.2	1	0.209
		High	268	71.1	0.848 (0.647–1.112)		63.8	0.856 (0.672–1.091)	
	S-1	Low	260	72.3	1	0.317	65.8	1	0.368
		High	141	77.6	0.806 (0.528–1.230)		70.2	0.843 (0.581–1.223)	
	Surgery only	Low	279	61.5	1	0.585	53.1	1	0.512
		High	127	63.8	0.907 (0.637–1.290)		56.6	0.898 (0.652–1.238)	
OPRT	All	Low	540	66.2	1	0.120	58.4	1	0.108
		High	267	72.2	0.805 (0.612–1.059)		65.7	0.818 (0.639–1.046)	
	S-1	Low	260	71.6	1	0.125	64.9	1	0.196
		High	141	78.9	0.715 (0.465–1.100)		72.0	0.779 (0.533–1.139)	
	Surgery only	Low	280	61.2	1	0.635	52.3	1	0.436
		High	126	64.7	0.918 (0.644–1.309)		58.7	0.879 (0.636–1.216)	

Predictive value of biomarker analysis

Kaplan–Meier plots of OS showed that S-1 treatment improved survival irrespective of TS or DPD expression (Fig. 2a–d). The HR for OS of the S-1 to surgery-only groups was lower in the high TS expressing population (>66.7th percentile; HR = 0.370; 95 % CI 0.221–0.619) than in the low TS expressing population (<66.7th percentile; HR = 0.757; 95 % CI 0.563–1.018). This interaction between TS expression and OS was statistically significant ($P = 0.015$). Similarly, the HR for OS of the S-1 to surgery only groups was lower in the high DPD expressing population (>33.3rd percentile; HR = 0.520; 95 % CI 0.376–0.720) than in the low DPD expressing group (<33.3rd percentile; HR = 0.848; 95 % CI 0.563–1.276). This interaction was also statistically significant ($P = 0.065$).

Analysis of OS in the biomarker study population found no interactions with gender, age, cancer stage, or histological type (data not shown), but did find an interaction with TS and DPD expression (Fig. 2e). No interaction was found between TP or OPRT expression and S-1 treatment (data not shown).

Prognostic impact of TS and DPD

Since univariate analysis had shown a significant association between both high TS and high DPD expression and a good outcome in the S-1 group, we also assessed the prognostic relevance of TS and DPD using a multivariate proportional hazards model adjusted for age, cancer stage (Japanese Classification of Gastric Carcinoma, second English edition) [18], and histological type. We found that cancer stage and TS expression were independent prognostic factors (Table 4).

Discussion

This study retrospectively evaluated the influence of TS, DPD, TP, and OPRT expression on the outcome for patients enrolled in the ACTS-GC. We found an

Table 3 Univariate analysis of OS and RFS: expression of each gene was categorized as low or high at the 33.3rd percentile

Marker	Group	Status	Number of patients	OS			RFS		
				5 year survival (%)	HR (95 % CI)	*P* value (log-rank)	5 year survival (%)	HR (95 % CI)	*P* value (log-rank)
TS	All	Low	272	67.9	1	0.969	57.1	1	0.292
		High	536	68.4	0.995 (0.766–1.293)		62.7	0.883 (0.700–1.113)	
	S-1	Low	138	72.3	1	0.595	62.9	1	0.270
		High	264	75.3	0.897 (0.599–1.341)		70.0	0.819 (0.574–1.169)	
	Surgery only	Low	134	63.2	1	0.769	51.1	1	0.559
		High	272	61.8	1.053 (0.745–1.488)		55.7	0.913 (0.672–1.240)	
DPD	All	Low	269	64.7	1	0.137	57.9	1	0.180
		High	538	69.9	0.823 (0.636–1.064)		62.2	0.853 (0.676–1.076)	
	S-1	Low	136	66.8	1	0.015	60.8	1	0.039
		High	265	78.0	0.616 (0.416–0.914)		70.8	0.690 (0.485–0.983)	
	Surgery only	Low	133	62.6	1	0.942	55.1	1	0.978
		High	273	62.1	1.013 (0.718–1.429)		53.8	0.996 (0.730–1.359)	
TP	All	Low	269	64.4	1	0.148	56.8	1	0.168
		High	538	70.0	0.827 (0.640–1.070)		62.7	0.850 (0.673–1.072)	
	S-1	Low	129	67.7	1	0.067	62.0	1	0.116
		High	272	77.2	0.690 (0.463–1.029)		69.8	0.750 (0.523–1.075)	
	Surgery only	Low	140	61.5	1	0.831	52.0	1	0.776
		High	266	62.6	0.964 (0.688–1.351)		55.3	0.957 (0.706–1.296)	
OPRT	All	Low	269	67.1	1	0.838	57.0	1	0.246
		High	538	68.7	0.973 (0.749–1.264)		62.6	0.872 (0.691–1.099)	
	S-1	Low	129	74.3	1	0.907	66.4	1	0.877
		High	272	74.1	1.025 (0.674–1.559)		67.8	0.971 (0.671–1.406)	
	Surgery only	Low	140	60.5	1	0.807	48.4	1	0.191
		High	266	63.2	0.959 (0.685–1.342)		57.3	0.819 (0.608–1.105)	

association between high TS and high DPD expression, a positive prognosis in the S-1 group only, and an enhanced benefit from S-1 treatment. This was unexpected, as it contradicted many previous studies.

Many studies have evaluated the correlation between TS and DPD expression levels in tumors and clinical outcomes for gastrointestinal cancer patients [12, 19–23]. Ichikawa reviewed these studies for gastric cancer and noted that most had found that TS expression was a prognostic marker for survival regardless of whether therapy was given in an adjuvant or metastatic setting [9]. Similarly, high temporal DPD gene expression has been correlated with a lack of response to fluoropyrimidine-based therapy and an adverse outcome for gastric cancer patients in many studies [9]. The majority of published studies concern retrospective analyses of data derived from mainly nonrandomized and relatively small studies, often from a single institution, so they may have some limitations with respect to power and bias. We believe this ACTS-GC biomarker study overcomes these disadvantages, since the biomarker population used was representative of the total study population in terms of survival analysis and clinicopathological factors, and gene expression values were well balanced in each treatment group (Table 1).

However, we have to consider reasons for the difference between our results and previous reports. First, we discuss a methodological issue. Since no methodology has yet been validated for measuring TS and DPD, and only a few studies have compared IHC with RT-PCR, we used both methods. Although IHC scores for TS correlated with RT-PCR results, those for DPD did not (see Fig. S3 of the ESM). The gene expression of DPD had a greater variability among the cases with an IHC score of 3+ ($N = 434$), comprising the majority of cases. We also observed considerable overlap in gene expression between the four groups used to score TS expression in IHC, which may result from the heterogeneous immunostaining frequently seen in different randomly selected areas of slides. We consider RT-PCR to be a more quantifiable method than IHC, at least in this study, as almost all tumor cells in FFPE sections were dissected for RT-PCR.

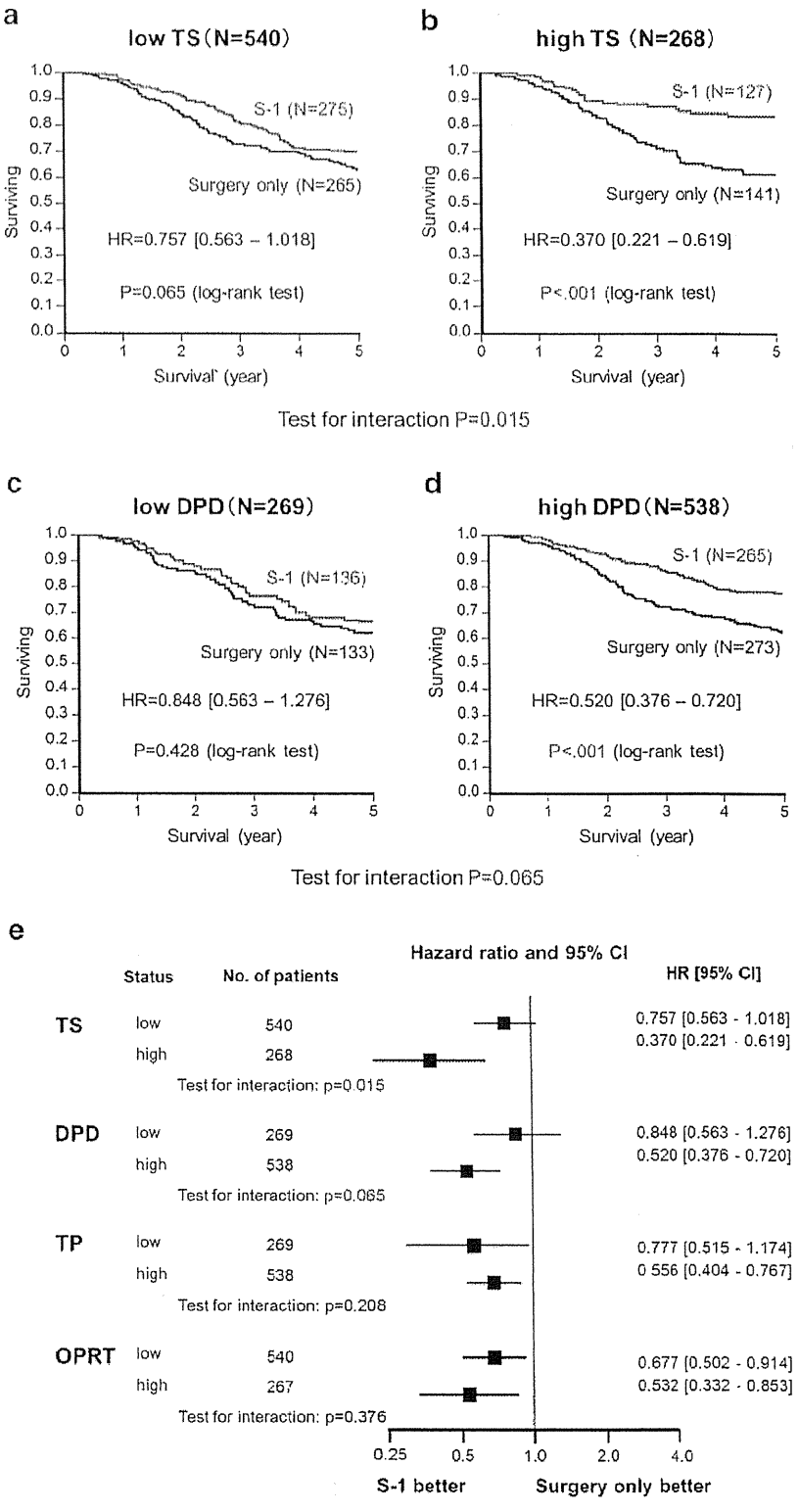

Fig. 2a–e Kaplan–Meier curves showing overall survival for patients in the S-1-treated (*red*) and surgery-only (*blue*) groups for tumors with **a** low TS expression (<66.7th percentile), **b** high TS expression(>66.7th percentile), **c** low DPD expression (<33.3rd percentile), **d** high DPD expression (>33.3rd percentile). **e** Subgroup analysis of hazard ratios and overall survival

A second issue is the cutoff value used for RT-PCR, as an optimal value has not yet been defined and the median has been used in several previous studies [19, 22]. We planned to use three cutoff points in this study, and the significant cutoff points were found to be different for TS and DPD. Furthermore, we explored this issue by analyzing

Table 4 Cox regression multivariate analysis of prognostic factors for OS in the S-1 group

Factor	Group	Number of patients	5-Year survival (%)	HR (95 % CI)	*P* value
Age	<60	157	76.6	1	
	60–69	146	78.1	1.288 (0.995–1.665)	
	70–80	98	64.5	1.659 (0.990–2.773)	0.055
Cancer stage (Japanese classification)	II	177	82.8	1	<0.001
	IIIa	153	72.5	1.746 (1.345–2.267)	
	IIIb	71	55.3	3.047 (1.809–5.141)	
Histologic type	Differentiated	242	76.2	1	0.250
	Undifferentiated[a]	159	71.0	1.283 (0.838–1.956)	
TS (66.7th percentile)	Low	275	69.9	1	0.011
	High	126	83.7	0.537 (0.317–0.87)	
DPD (33.3rd percentile)	Low	136	66.8	1	0.053
	High	265	78.0	0.663 (0.44–1.005)	

the relationship between using different cutoff values for stratification and the *P* values from log-rank tests for TS and DPD gene expression. As shown in Fig. S4 of the ESM, the lowest *P* values were observed at the 66.7th percentile for TS but the 33.3rd for DPD in the S-1 group. This indicated that the tertile was the optimal cutoff value for TS and DPD gene expression in this cohort.

High TS and high DPD expression have been thought to result in lower sensitivity to 5FU-based chemotherapy. In contrast, Fujiwara et al. reported that S-1 showed better antitumor activity than 5FU in GT3TKB human gastric tumor xenografts with high TS and DPD activity [24]. In GT3TKB xenografts, the 5FU incorporated into RNA was significantly higher in the S-1 group than in the 5FU group. They speculated that the increase in the 5-fluoro-2′deoxyuridine-5′-monophosphate level was insufficient to enhance TS inhibition, and blocking of RNA function by the increased level of 5-fluorouridine-5′-triphosphate (another mechanism of action of 5FU) may have predominated. It was also suggested that a potent DPD inhibitor such as gimeracil could be used to circumvent the resistance to 5FU that occurs at high levels of DPD activity [24, 25]. The unexpected results observed in this study may be explained by noting that S-1 showed some effects not presented by other fluoropyrimidines.

For colorectal cancer, conflicting results have been published on TS expression in metastases versus primary tumors, and on the response to 5FU chemotherapy in advanced colorectal cancer versus the survival benefit of adjuvant 5FU therapy [26–28]. Kormann et al. reported that adjuvant 5FU chemotherapy prolonged the survival of patients with high TS mRNA levels, based on archival FFPE colorectal tumor tissue from 309 patients [28]. Their suggested explanation for their results was that the major effect of adjuvant therapy is the eradication of circulating cancer cells before they become established, and the milieu of circulating cells is clearly different from that of an established tumor in many respects. Thus, the mechanism by which S-1 suppresses recurrence after surgery could differ from the mechanism it uses to inhibit the growth of advanced tumors. Furthermore, gastric tumor tissue is known to be highly heterogeneous and complex. Therefore, a small tumor cell population (e.g., HER2-positive cells) could play an important role in tumor recurrence, and surrounding stromal cells that may have roles in tumor angiogenesis and immunity could also contribute to tumor recurrence [29–31]. To understand the roles of TS and DPD in the suppression of recurrence by S-1, their expression in both tumors and the surrounding normal cells in a micrometastatic tumor model needs to be investigated.

The most critical limitation of this study is that the results were obtained from a single cohort, even though the ACTS-GC was a large, randomized, phase III trial. To confirm the reproducibility of our results, further retrospective and prospective biomarker studies using FFPE samples from gastric cancer patients treated with adjuvant S-1 will be needed, using the same RT-PCR method and cutoff point.

Recently, the CLASSIC study—another prospective, randomized, phase III trial—demonstrated that adjuvant capecitabine plus oxaliplatin treatment after curative D2 gastrectomy was also more effective than surgery alone in East Asian patients with stage II/III gastric cancer [32]. A subgroup analysis suggested that adjuvant capecitabine and oxaliplatin was beneficial for all subgroups, although relatively high HR (0.90) was observed in node-negative patients. Adverse events were observed more frequently in the CLASSIC study than in the ACTS-GC study [2]. At present, we have two standard treatments for gastric cancer in Asia, and determining which patients would derive most

benefit from these treatments remains a clinical problem for the future. The present study suggests that the tumoral expression levels of TS and DPD could provide useful information for selecting adjuvant treatment, either S-1 monotherapy or doublet treatment. Gastric tumors with high expression levels of TS or DPD are thought to be capable of responding to S-1 alone, whereas doublet treatment (such as capecitabine with oxaliplatin) would be required for patients with low tumoral expression levels of TS or DPD, since these individuals have a poor prognosis after S-1 treatment alone. Additionally, our results may provide some insight into the molecular characteristics of relapsed tumors after adjuvant S-1 treatment. As the majority would be expected to have relatively low TS and DPD expression, 5FU-based therapy would still benefit patients with relapsed tumors. Further understanding of the molecular biological and pathology of gastric cancer is needed to improve treatment for this disease.

In conclusion, this study provided evidence that high TS and DPD expression were associated with a positive prognosis in S-1 treated patients only, and with an enhanced benefit from S-1 therapy. Stratification by TS, DPD, TP, and OPRT gene expression levels did not suggest the existence of a subgroup of stage II/III gastric cancer patients who should not be offered adjuvant S-1 therapy.

Acknowledgments This study was funded by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). We did not have any grant to support this study. We dedicate this manuscript to the memory of Prof. Tetsuro Kubota, who made a great contribution to the conception and design of this study. We thank the patients and their families as well as the investigators from the 65 institutions (as shown in the "Appendix") for their cooperation.

Appendix

The following Japanese institutions participated in this study as ACTS-GC Biomarker Study Group: Iwate Medical University Hospital (Morioka), Iwate Prefectural Central Hospital (Morioka), National Hospital Organization Sendai Medical Center (Sendai), Miyagi Prefectural Cancer Center (Natori), Yamagata Prefectural Central Hospital (Yamagata), Tsuboi Cancer Center Hospital (Koriyama), Niigata Cancer Center Hospital (Niigata), Gunma University Hospital (Maehashi), Gunma Prefectural Cancer Center (Ota), Tsuchiura Kyodo General Hospital (Tsuchiura), Tsukuba University Hospital (Tsukuba), Dokkyo Medical University Hospital (Mibu), Tochigi Cancer Center (Utsunomiya), Saitama Medical University Hospital (Moroyama, Hidaka), Chiba University Hospital (Chiba), National Cancer Center Hospital East (Kashiwa), Chiba Cancer Center (Chiba), National Center for Global Health and Medicine (Tokyo), Showa University Toyosu Hospital (Tokyo), National Cancer Center Hospital (Tokyo), Tokyo Metropolitan Bokutoh Hospital (Tokyo), Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital (Tokyo), Cancer Institute Hospital (Tokyo), Nihon University Itabashi Hospital (Tokyo), Tokyo Metropolitan Tama Medical Center (Fuchu), Showa General Hospital (Kodaira), Kitasato University East Hospital (Sagamihara), Shizuoka General Hospital (Shizuoka), Fujieda Municipal General Hospital (Fujieda), Showa Inan General Hospital (Komagane), Gifu Municipal Hospital (Gifu), Ogaki Municipal Hospital (Ogaki), Social Insurance Chukyo Hospital (Nagoya), Aichi Cancer Center Hospital (Nagoya), National Hospital Organization Nagoya Medical Center (Nagoya), Aichi Cancer Center Aichi Hospital (Okazaki), Fukui-ken Saiseikai Hospital (Fukui), Fukui Red Cross Hospital (Fukui), Toyama Prefectural Central Hospital (Toyama), Kyoto Second Red Cross Hospital (Kyoto), NTT West Osaka Hospital (Osaka), Osaka City General Hospital (Osaka), National Hospital Organization Osaka National Hospital (Osaka), Osaka Medical Center for Cancer and Cardiovascular Diseases (Osaka), Sakai Municipal Hospital (Sakai), Kinki University Hospital (Osaka-Sayama), Hyogo Cancer Center (Akashi), Kansai Rosai Hospital (Amagasaki), Hiroshima University Hospital (Hiroshima), Hiroshima City Asa Hospital (Hiroshima), Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital (Hiroshima), Shimane Prefectural Central Hospital (Izumo), Tottori University Hospital (Yonago), Yamaguchi University Hospital (Ube), National Hospital Organization Shikoku Cancer Center (Matsuyama), National Kyushu Cancer Center (Fukuoka), National Hospital Organization Kyushu Medical Center (Fukuoka), Kitakyushu Municipal Medical Center (Kitakyusyu), Kokura Memorial Hospital (Kitakyushu), Social Insurance Tagawa Hospital (Tagawa), Saga Prefectural Hospital Koseikan (Saga), Sasebo City General Hospital (Sasebo), Oita Prefectural Hospital (Oita), Saiseikai Kumamoto Hospital (Kumamoto), Japanese Red Cross Kumamoto Hospital (Kumamoto).

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ANTICANCER RESEARCH *34*: 5169-5174 (2014)

Optimal Treatment Change Criteria for Advanced Gastric Cancer with Non-measurable Peritoneal Metastasis: Symptom/Tumor Marker-based *Versus* CT-based

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Abstract. *Background: For advanced gastric cancer (AGC) with peritoneal metastasis, decision-making regarding treatment change is often challenging because of the absence of measurable lesions. We attempted to clarify which criterion for treatment change contributes more to longer survival. Patients and Methods: We retrospectively reviewed 50 patients with non-measurable peritoneal metastasis in whom first-line chemotherapy for AGC was changed based on aggravated clinical symptoms or tumor markers (TMs), or radiologically-confirmed disease progression. Prognostic factors for overall survival (OS) were investigated. Results: Patients whose treatment was changed based on symptoms/TMs had significantly longer OS than patients with computed tomographic-based treatment change (p=0.04). On multivariate analysis, treatment change based on symptoms/TMs was identified as an independent prognostic factor for favorable OS (hazard ratio=0.321, 95% confidence interval=0.154–0.668, p=0.002). Conclusion: The present study suggests that aggravated clinical symptoms/elevated TMs could be a sensitive predictor for disease progression in patients with AGC with non-measurable peritoneal metastasis.*

Gastric cancer is the second most common cause of cancer-related death worldwide, although its global incidence has been declining for several decades (1-3). The current mainstay treatment for advanced gastric cancer (AGC) is systemic chemotherapy with fluoropyrimidine plus platinum, although the prognosis of patients with AGC remains poor, with a median survival time of 10-13 months (4-6).

Peritoneal metastasis, which commonly occurs along with diffuse-type adenocarcinoma, causes many serious complications such as uncontrollable ascites, intestinal obstruction, obstructive jaundice, and hydronephrosis. These complications usually result in complaints such as abdominal fullness, nausea, anorexia, and abdominal pain, and sometimes progress rapidly. Although two clinical trials have been conducted so far (7, 8), no standard treatment has been established for patients with AGC with peritoneal metastasis owing to the absence of measurable lesions that would otherwise enable treatment evaluation by the standard response evaluation criteria in solid tumors (RECIST) (9). In addition, a lack of measurable lesions makes it difficult to determine the optimal timing for treatment change. In these patients, clinical symptoms or tumor markers (TMs) instead of radiological findings are often used to evaluate disease progression. It remains uncertain whether long-term survival is better-achieved by basing treatment change on symptoms and TMs, or on radiological recognition of disease progression.

We attempted to clarify the appropriate criteria for treatment change and evaluated the prognostic significance of various clinicopathological parameters in patients with non-measurable peritoneal metastasis of AGC.

Patients and Methods

Study population. A total of 217 patients with primary unresectable or recurrent gastric cancer were treated at the Osaka National Hospital between April 2005 and March 2012. Out of these, 50 patients fulfilled the following criteria and were enrolled in this retrospective study: histologically proven unresectable or recurrent gastric adenocarcinoma with non-measurable lesions; histologically confirmed peritoneal metastasis or cancer cells on peritoneal lavage cytology without any bowel stenosis or ascites beyond the pelvic cavity; absence of other distant metastatic lesions such as in the liver, lung, bone, lymph nodes, or central nervous system;

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Key Words: Treatment change, peritoneal metastasis, tumor marker, CT, advanced gastric cancer.

performance status (PS) of 2 or less on the Eastern Cooperative Oncology Group (ECOG) scale at the initiation of first-line chemotherapy; adequate oral intake; commencement of second-line chemotherapy after the failure of first-line chemotherapy; adequate bone marrow function (WBC count 3,000-12,000/mm^3, platelet count ≤100,000/mm^3, and hemoglobin ≥8.0 g/dl), hepatic function (total bilirubin ≤1.5 mg/dl, serum transaminases ≤100 U/l), and renal function (serum creatinine greater than the upper institutional limit) at the initiation of first-line chemotherapy; no other severe medical conditions; and no concurrent active malignancy.

Criteria for disease progression. While receiving first-line chemotherapy, patients underwent physiological assessments that included digital rectal examination and measurements of three TMs namely carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9, and CA125, every month, and abdominal computed tomographic (CT) scans every 2-3 months. The cut-off values for CEA, CA19-9, and CA125 were 5 ng/ml, 37 U/ml, and 35 U/ml, respectively.

Chemotherapy regimens were changed if progressive disease (PD) developed, as defined by either of the following criteria: aggravated clinical symptoms or elevated TMs, or radiologically confirmed disease progression.

In terms of clinical symptoms, abdominal pain and abdominal distension were assessed according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 (10). Disease progression was defined as the appearance of abdominal pain or distension of grade 2 or more, or a growing mass in Douglas' pouch by digital rectal examination.

For TMs, a rise of more than 50% in the initial value of any of the evaluated TMs was defined as disease progression.

Radiological disease progression was defined as peritoneal or mesenteric thickening, new-onset bowel wall thickening, a significant increase in ascites, or the appearance of one or more new lesions.

Statistics. Overall survival (OS) was defined as the time from initiation of first-line chemotherapy to the date of death from any cause or the last follow-up. Time-to-treatment failure (TTF) was defined as the interval between initiation of first-line chemotherapy and treatment discontinuation for any reason, including disease progression, treatment toxicity, patient preference, or death. Both OS and TTF were calculated using the Kaplan–Meier method and compared with the log-rank test. Differences in proportions were evaluated with the Chi-square test, and the significance of age differences was estimated by the Mann–Whitney test. Univariate and multivariate analyses were performed using the Cox proportional hazards model to identify variables independently associated with OS. Statistical results with a p-value of less than 0.05 were considered significant.

Results

Patients' characteristics. Clinicopathological characteristics of the 50 patients at the initiation of first-line chemotherapy are shown in Table I. There were 20 males and 30 females with a median age of 62 (range=5-78) years. Forty-five patients had a PS of 0 or 1, and the remaining 5 patients had a PS of 2. Primary gastric cancer was intestinal-type adenocarcinoma in two patients and diffuse-type adenocarcinoma in the remaining 48. Ascites limited to the pelvic cavity was confirmed in 21 patients by CT scan. While 14 patients showed no elevation in pre-treatment serum levels of TMs, the remaining patients demonstrated TM elevations as follows: CEA in 19 patients with a median value of 20.0 (range=0.4-317.0) ng/ml, CA19-9 in 16 patients with a median value of 70.0 (range=1.0-1299.0) U/ml, and CA125 in 11 patients with a median value of 47.3 (range=21.0-412.0) U/ml. In terms of clinical signs or symptoms, abdominal pain of grade 1 occurred in five patients and abdominal distension of grade 1 was observed in seven, whereas the remaining 38 patients had no abdominal findings prior to the initiation of first-line chemotherapy.

Chemotherapeutic regimens. Table II summarizes the chemotherapy regimens administered. As first-line treatment, 34 patients received S-1 combined doublet/triplet chemotherapy (6, 11-15), while 16 patients received S-1 monotherapy (11). The majority of patients were participants in clinical trials and were treated according to trial protocols. For non-trial participants, chemotherapy regimens were chosen at their physicians' discretion. As second-line treatment, an S-1 based regimen (6, 11-14, 16) was administered to 15 patients, taxane monotherapy (17, 18) to 23, and irinotecan-based therapy (17, 19) to 12, which was partly in accordance with the recent global consensus identifying taxanes and irinotecan as standard second-line treatments (20, 21).

Treatment change. As shown in Table III, 24 patients underwent treatment changes based only on aggravated symptoms or elevated TMs, while treatment changes were made in 26 patients after confirmed PD on CT scan. In the 24 patients in the first group, aggravated symptoms included abdominal pain in nine, abdominal distension in five and a growing mass in Douglas' pouch in four, with symptoms overlapped in 2 patients. Of note, only five out of these 24 patients were confirmed as having PD on CT scan even after the decision to change treatments. In contrast, in the 26 patients whose treatments were changed based on CT scan findings, elevated TMs and aggravated symptoms were observed in 14 and 20 patients, respectively, but none underwent treatment change before confirmed PD on CT scan.

Survival according to treatment change criteria. The median OS of all patients was 16.8 months with a median follow-up time of 18.5 months (18.1 months in 41 patients who died and 28.5 months in nine living patients). Twenty-four patients undergoing treatment change based on symptoms or TMs had significantly longer OS than the 26 patients with treatment change based on CT (25.5 months *vs.* 14.3 months, p=0.04) (Figure 1). Median TTF for first-line chemotherapy did not differ between these two cohorts (7.8 months in the former 24 patients, and 6.4 months in the latter 26 patients, p=0.48) (Figure 2).

Table I. *Patient characteristics at the initiation of first-line chemotherapy*

		n=50
Gender	Male/Female	20/30
Age (years)	60>/60≤	24/26
	Median (range)	62 (25-78)
ECOG PS	0-1/2	45/5
Disease status	Primary/Recurrent	28/22
Histology (Lauren's)	Intestinal/Diffuse	2/48
Ascites	Present/Absent	21/29
Alb (g/dl)	3.5>/3.5≤	21/29
Hb (g/dl)	10>/10≤	21/29
Pretreatment elevated TMs	CEA/CA19-9/CA125	19/16/11
Pretreatment symptoms abdominal pain/abdominal distension/ palpable mass in Douglas' pouch	5/7/0	

ECOG, Eastern Cooperative Oncology Group; PS, performance status; Alb, albumin; Hb, hemoglobin; TMs, tumor markers.

Table II. *Chemotherapy regimens.*

First-line	n=50
S-1 alone	16
S-1 + cisplatin	20
S-1 + irinotecan	2
S-1 + paclitaxel	5
S-1 + docetaxel	4
S-1 + cisplatin + paclitaxel	2
S-1 + cisplatin+ docetaxel	1
Second-line	**n=50**
S-1 alone	8
S-1 + irinotecan	2
S-1 + paclitaxel	2
S-1 + docetaxel	3
Paclitaxel	19
Docetaxel	4
Irinotecan alone	10
Irinotecan + cisplatin	2

Table III. *Reasons for treatment change.*

Based on	n
Symptoms/TMs	24
Elevated TMs (50%)	18
Aggravated symptoms	16
Both elevated TMs and aggravated symptoms	10
CT scan	26

CT: Computed tomography; TMs: tumor markers.

Table IV. *Univariate analysis of prognostic factors for overall survival.*

Prognostic factor	MST (months)	*p*-Value
Gender		
Male	18.4	0.3170
Female	22.3	
PS		
1	19.8	0.2282
2	10.3	
Age (years)		
≤60	18.4	0.4331
>60	17.7	
Histology		
Intestinal	18.4	0.5266
Diffuse	15.7	
Disease status		
Primary	18.4	0.7211
Recurrent	21.8	
Ascites		
Absent	22.3	0.1800
Present	17.7	
Hemoglobin		
<10 g/dl	17.7	0.8936
≥10 g/dl	22.6	
Albumin		
<3.5 g/dl	25.5	0.0275
≥3.5 g/dl	14.3	
Treatment change criteria		
Based on symptoms/TMs	25.5	0.0396
Based on CT scan	14.3	

CT: Computed tomography; MST: median survival time; PS: performance status;
TMs: tumor markers.

Prognostic factors. The results of univariate and multivariate analyses on the impact on OS of various factors such as gender, PS, age, histology, presence of primary tumor and ascites, serum albumin levels, and hemoglobin levels at the initiation of first-line chemotherapy, as well as the treatment change criteria are summarized in Tables IV and V, respectively. When incorporating the potential prognosticators with *p*-values ≤0.15 in univariate analysis, multivariate analysis identified treatment change, based on symptoms or TMs [hazard ratio (HR)=0.321, 95% confidence interval (CI)=0.154-0.668, p=0.002), as an independent prognostic factor for favorable OS.

Discussion

Peritoneal metastasis presents a diagnostic and treatment challenge in patients with AGC. It is often noted initially based on clinical symptoms such as ascites, bowel hypomotility, and bowel obstruction, because radiological tests cannot always detect the spread of malignant cells within the

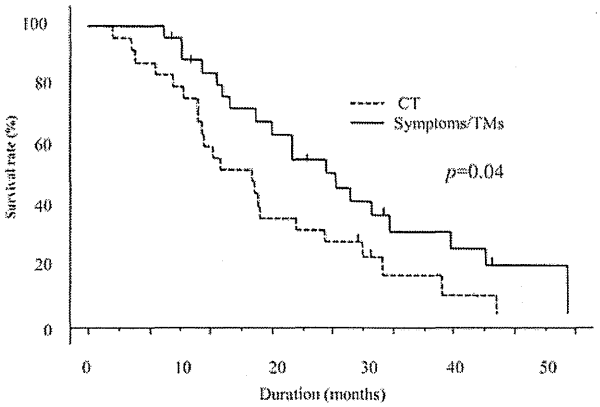

Figure 1. *Overall survival (OS) in patients undergoing treatment change based on symptoms or tumor markers (TMs) compared with that based on computed tomography (CT).*

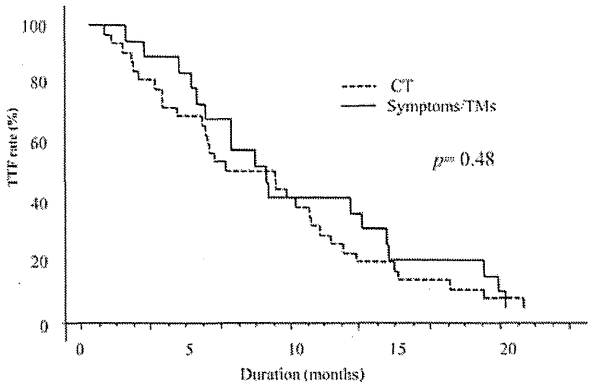

Figure 2. *Time to treatment failure (TTF) on first-line chemotherapy in patients undergoing treatment change based on symptoms or tumor markers (TMs) compared with that based on computed tomography (CT).*

Table V. *Multivariate analysis of prognostic factors for overall survival.*

Prognostic factor	HR	95% CI	*p*-Value
Albumin			
<3.5 g/dl	1.134	0.578-2.225	0.715
≥3.5 g/dl	1		
Treatment change criteria			
Based on symptoms/TMs	0.321	0.154-0.668	0.002
Based on CT scan	1		

HR, Hazard ratio; CI, confidence interval.

peritoneal cavity and no radiological methods have demonstrated a high predictive value for this condition. Therefore, exploratory laparoscopic examination plays a key role in the diagnosis of peritoneal metastasis by direct observation of the peritoneal cavity (22). Chemotherapy is the mainstay of treatment for alleviating symptoms and improving survival in these patients. However, these patients do not usually have measurable lesions according to the RECIST criteria, which makes it difficult to assess the efficacy of chemotherapy based on radiological findings. Therefore, some delay in the diagnosis of PD is unavoidable in patients with only non-measurable lesions and physicians often have to evaluate disease progression by integrating clinical symptoms and changes in TMs.

With respect to the relationship between clinical symptoms and survival, asymptomatic patients generally have a favorable prognosis because those with a higher tumor burden experience symptoms caused by tumor growth (23, 24). However, in this study, OS was better in patients undergoing treatment changes based on symptoms or TMs than in those receiving treatment alterations after PD was proven on CT scan. In cases of peritoneal metastasis, especially non-measurable peritoneal metastasis alone, aggravated clinical symptoms could become a more sensitive predictor for disease progression, while PD detected by CT scan might reflect a comparatively higher tumor burden. Similarly, symptom alleviation was able to sensitively predict disease control by systemic chemotherapy in patients with advanced pancreatic cancer (25).

Among 18 patients in whom elevated TMs led to treatment change (Table III), a rise of more than 50% in the initial values of CEA, CA19-9, and CA125 was observed in 12, 10, and 5 patients, respectively (data not shown). A previous study found that an increase in initial values of TMs greater than 50% correlated well with disease progression in patients with AGC under first-line chemotherapy (26). CEA and CA19-9 have been shown to be the most useful markers for monitoring PD in gastric cancer (27-29), and the biological relevance of CA125 in the progression or reduction of peritoneal metastasis from AGC has been recently demonstrated (30, 31). In the present study, a more than 50% rise in initial values of TMs showed promise for detecting PD in patients with non-measurable peritoneal metastasis. For other TMs, CA72-4 or the combination of CA72-4 and CA125 are expected to be highly specific for peritoneal metastasis from AGC (31-35), thereby sensitively reflecting disease progression (27, 30).

When comparing the OS and TTF shown in Figures 1 and 2, post-progression survival, defined as the time from recognition of disease progression on first-line chemotherapy to death from any cause or last follow-up, was shorter in patients undergoing treatment change after confirmed PD on CT scan, which suggests a higher tumor burden in these patients at the point when the decision is made to switch to second-line treatment.

Regarding other modalities for diagnosing progression of non-measurable peritoneal metastasis, ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG-PET) would be unreliable because of its low sensitivity for diffuse-type gastric adenocarcinoma (36).

Although the prognostic factors shown in Tables IV and V have been identified for patients with AGC undergoing first-line chemotherapy (37-41), treatment change based on symptoms or TMs was chosen as an independent prognostic factor in our patient cohort with non-measurable peritoneal metastasis. This was partly due to our unique approach of noting treatment change irrespectively of the presence of radiologically-confirmed PD.

The limitations of this study, which include its retrospective, single-Institution nature, and the relatively small sample size of 50 patients, need to be taken into account before generalizing the results to daily clinical practice until prospective, multi-center validation is available. However, we believe that our findings will help physicians prognosticate disease course and facilitate decision making on switching to second-line treatments.

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Received May 31, 2014
Revised June 20, 2014
Accepted June 24, 2014

Gastric Cancer
DOI 10.1007/s10120-014-0426-3

ORIGINAL ARTICLE

A novel splice variant of XIAP-associated factor 1 (XAF1) is expressed in peripheral blood containing gastric cancer-derived circulating tumor cells

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Received: 12 April 2014 / Accepted: 23 August 2014

Abstract
Background XIAP-associated factor 1 (XAF1) is ubiquitously expressed in normal tissues, but its suppression in cancer cells is strongly associated with tumor progression. Although downregulation of XAF1 is observed in tumors, its expression profile in the peripheral blood of cancer patients has not yet been investigated. Here, we identified a novel *XAF1* splice variant in cancer cells and then investigated the expression level of this variant in peripheral blood containing gastric cancer-derived circulating tumor cells (CTCs).

Methods To identify splice variants, RT-PCR and DNA sequencing were performed in mRNAs extracted from many cancer cells. We then carried out quantitative RT-PCR to investigate expression in peripheral blood from all 96 gastric cancer patients and 22 healthy volunteers.
Results The XAF1 variant harbored a premature termination codon (PTC) and was differentially expressed in highly metastatic cancer cells versus the parental cells, and that nonsense-mediated mRNA decay (NMD) was suppressed in the variant-expressing cells. Furthermore, splice variants of *XAF1* were upregulated in peripheral blood containing CTCs. In *XAF1* variant-expressing patients, the expression levels of other NMD-targeted genes also increased, suggesting that the NMD pathway was suppressed in CTCs.
Conclusions Our study identified a novel splice variant of *XAF1* in cancer cells. This variant was regulated through the NMD pathway and accumulated in NMD-suppressed metastatic cancer cells and peripheral blood containing CTCs. The presence of XAF1 transcripts harboring the PTC in the peripheral blood may be useful as an indicator of NMD inhibition in CTCs.

Keywords Alternative splicing · Circulating tumor cells · Gastric cancer · Nonsense-mediated mRNA decay · Quantitative real-time polymerase chain reaction (qRT-PCR)

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Electronic supplementary material The online version of this article (doi:10.1007/s10120-014-0426-3) contains supplementary material, which is available to authorized users.

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Introduction

XIAP-associated factor 1 (XAF1) has been identified as a nuclear protein and a binding partner that directly interacts with endogenous X-linked inhibitor of apoptosis (XIAP) [1]. XAF1 overexpression induces apoptosis and inhibits

Published online: 13 September 2014

tumor growth in multiple types of cancer including gastric, colorectal, and pancreatic cancers [2–7]. *XAF1* is ubiquitously expressed in normal cells but expressed at extremely low levels in several types of cancer cells [8]. Lower expression of this gene in tumor tissues is strongly associated with tumor stage [2, 5, 9, 10]. Splice variants of *XAF1* have been detected in various cancer cell lines [11–13]. Fang et al. [14] found a switch from full-length to short XAF1 transcripts in prostate cancer cells, suggesting differential function of the short variant in apoptosis regulation. The production of these transcripts is regulated through aberrant epigenetic modification [12–14]. However, the expression profile of *XAF1* splice variants in human cancer remains unclear.

Nonsense-mediated RNA decay (NMD) helps the cell to maintain mRNA quality [15]. Abnormal transcripts generated by alternative splicing often harbor premature termination codons (PTC), leading to degradation of these transcripts via the NMD pathway. NMD is suppressed by cellular stresses in the tumor microenvironment, the inhibition of which promotes stabilization of NMD-targeted mRNA and tumorigenesis [16–19]. Recently, Tani et al. [20] reported that accumulation of noncoding RNA growth-arrest-specific 5 (GAS5) by NMD inhibition through cellular stress (such as serum starvation) leads to the downregulation of apoptosis-related genes. Thus, aberrant RNAs that accumulate through NMD inhibition are considered to be potential tumor markers or biomarkers.

Although a meta-analysis of the published literature revealed that circulating tumor cells (CTCs) are involved in the poor prognosis of gastric cancer patients [21], the associated gene expression profiles remain unclear. Epithelial-mesenchymal transition (EMT)-related genes have been shown to be often expressed in CTCs [22–24], and several genes are abnormally spliced in the EMT [25–27]. RNA-Seq analysis revealed that alternative splicing can induce critical aspects of EMT-associated phenotypic changes, suggesting that the EMT is closely related to RNA splicing [28]. Recently, Valacca et al. [29] reported that aberrantly spliced transcripts accumulated as a result of NMD inhibition in an in vitro model of the EMT. Thus, CTCs in which the EMT is occurring may demonstrate alternative splicing that generates transcripts which would normally be targeted by NMD.

Quantitative real-time polymerase chain reaction (qRT-PCR) is one of the most sensitive methods for evaluation of gene expression and is utilized to detect mRNA tumor markers in peripheral blood, such as mRNA encoding cytokeratin 19 (*CK19*), cytokeratin 20 (*CK20*), and carcinoembryonic antigen (CEA; synonym, *CEACAM5*) [30–32]. The PAXgene qRT-PCR assay can detect stabilized RNA in the peripheral blood, thus reflecting the expression level of transcripts in CTCs, which showed strong concordance with the results of CTC counting by immunomagnetic separation (CellSearch; Janssen Diagnostics, Raritan, NJ, USA) [33, 34]. However, several markers detected in peripheral blood are frequently expressed in normal epithelial cells, resulting in decreased sensitivity and specificity of qRT-PCR [35]. To maintain the performance of this method using peripheral blood samples, cancer-specific transcripts must be selected [34, 36].

In this study, to evaluate the potential utility of a splice variant harboring PTC as a biomarker or tumor marker, we investigated the presence of aberrant *XAF1* transcripts in cancer cell lines and in the peripheral blood of patients with gastric cancer using qRT-PCR. The RT-PCR analysis and DNA sequencing revealed that a novel splice variant of *XAF1* was expressed in gastric, pancreatic, colorectal, and breast cancer cell lines. This splice variant harboring PTC accumulated in NMD-suppressed cells. Furthermore, the *XAF1* variant in peripheral blood containing CTCs obtained from patients with gastric cancer was significantly upregulated relative to samples from healthy volunteers. These findings suggest that the novel *XAF1* variant identified in this study is a potential blood biomarker.

Materials and methods

Patients and specimens

From April 2010 to August 2012, PAXgene (PreAnalytiX; Hombrechtikon, Switzerland) was used to collect peripheral blood samples (2.5 ml) from 96 patients [65 men, 31 women; median age, 67 (30–85) years] with gastric cancer at Shizuoka Cancer Center Hospital and from 22 healthy volunteers [16 men, 6 women; median age, 36 (26–70) years] who were coworkers at the hospital. Informed consent was obtained from all patients, and the Institutional Review Board at Shizuoka Cancer Center approved all aspects of this study.

Cell cultures, RNA sample preparation, and RT-PCR

The cell lines used in this study are listed in Table S1 in the Supplementary Material. cDNA for different splice variants of *XAF1* was screened using the intron-spanning exonic primers listed in Table S2 in the Supplementary Material. The methods are described in detail in the Supplementary Material.

DNA sequencing analysis

To determine the sequence of the novel *XAF1* transcript, rapid amplification of cDNA ends (RACE) was conducted

using the GeneRacer Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The primer sequences are shown in Table S2. The methods used for DNA sequencing analysis are provided in detail in the Supplementary Material.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using the SYBR Green dye technique and the ABI PRISM 7900HT Fast Real-Time PCR System (Life Technologies). The methods and the validation results are described in detail in the Supplementary Material.

NMD inhibition assay with caffeine

The method for inhibiting NMD was previously described in detail [37]. Briefly, cells were seeded in two culture plates, and caffeine (10 mM) was added to one plate. Following 4 h of incubation, the medium was removed from both plates and the cells were washed twice with phosphate-buffered saline. Both actinomycin D (actD, 2 mg/ml) and caffeine (10 mM) were added to one plate (pretreated with caffeine), and actD alone was added to the other plate. After a further 4 h incubation, total RNA was obtained from both plates (see details in the Supplementary Material).

Statistical analysis

The coefficient of determination in the qRT-PCR standard curve was derived by Pearson's correlation analysis. *XAF1* and survivin (Baculoviral IAP Repeat Containing 5, *BIRC5*) expression levels in gastric cancer patients and healthy volunteers were depicted as box plots containing outliers and extreme outliers. The inner and outer fences for the plotting of the outliers were calculated using the 1.5× interquartile range (IQR) and 3.0× IQR, respectively. Because of the difference in sample size between patients and volunteers, the p value of the qRT-PCR analysis was calculated based on Welch's t test. Receiver operating characteristic (ROC) curves were computed using the R software and the associated pROC package [38]. The area under the curve (AUC) and optimal threshold (cutoff value), i.e., the point closest to the top left in the plot, were calculated in R. The 95 % confidence interval (CI) was computed to assess the variability of the measure using 10,000 bootstrap replicates [39]. Gene expression and clinicopathological characteristics according to the Japanese Classification of Gastric Carcinoma, 14th edition, were compared using Fisher's exact test. Significance was defined as $p < 0.05$.

Results

Identification of novel *XAF1* exons

Several splice variants of *XAF1* are known to be expressed in cancer cell lines [11–14]. To analyze the variants, we initially characterized the expression pattern of *XAF1* in the highly metastatic gastric cancer cell MKN45P (Fig. 1a). RT-PCR was conducted using previously reported primers [12] and newly designed primers to detect known transcripts and novel variants, respectively. Electrophoresis of the RT-PCR amplicons revealed that an unknown transcript candidate (*XAF1F*) was coexpressed with *XAF1A* and *XAF1C* in MKN45P. PCR-based cloning and DNA sequencing analysis using RACE subsequently showed that *XAF1F* was a novel splice variant (Fig. 1b). Specifically, this variant lacked exon 5 and possessed a unique exon 3 that contained exon 3-ext derived from the intronic region, resulting in seven exons. The sequence of the novel exonic region had a stop codon that was regarded as a PTC.

XAF1F expression in cancer cell lines

XAF1 transcripts are downregulated or absent in colorectal cancer cells [12]. We thus investigated the expression profile of *XAF1F* transcripts in gastrointestinal (colorectal, gastric, and pancreatic) and breast cancer cell lines using RT-PCR analysis (Fig. 2). The *XAF1F* transcript was expressed in 20 cell lines (20/45, 44 %) and also coexisted with other XAF1 transcripts (*XAF1A/C*) in 20 cell lines (20/20, 100 %). However, 10 of 32 *XAF1A*-expressing cell lines did not express the XAF1F transcript (10/32, 31 %). These results suggest that although *XAF1F* harboring PTC was often degraded by the NMD pathway, this transcript was coexpressed with other *XAF1*s in many cancer cells.

mRNA expression of NMD-related genes

Although *XAF1F* possessed a PTC in its mature mRNA sequence, it was expressed in 20 cancer cell lines. Among them, comparison of two pairs of cell lines (MKN45P vs. MKN45 and KP-3L vs. KP-3) in a metastatic model obtained by xenografting (for details, see Table S1) revealed that XAF1 expression, including that of *XAF1F*, in highly metastatic cells was higher than that in the parental cells (Fig. 2 and S2 in the Supplementary Material). To investigate NMD activity in variant-expressing cells, qRT-PCR for NMD-related genes was performed using these pairs (Fig. 3a). In MKN45P cells, the NMD target transcripts *ATF3* [16, 40] and *MAFF*

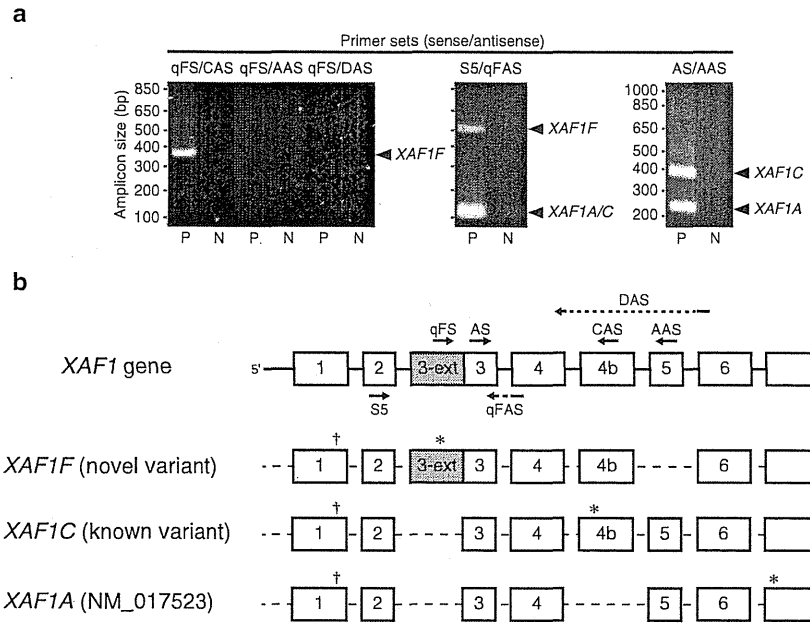

Fig. 1 Identification of a novel XIAP-associated factor 1 (*XAF1*) transcript. **a** Detection of *XAF1* transcripts expressed in a gastric cancer cell line (MKN45P). The novel transcript (*XAF1F*) was detected by RT-PCR using the primer sets qFS/CAS and S5/qFAS (sense/antisense). *P* and *N* under the *individual lanes* indicate the positive control (RT-PCR with MKN45P template) and negative control (without template), respectively. **b** Schematic representation of the exon structures of the *XAF1* gene and transcripts identified in this study. The *gray boxes* show a novel exonic region extending from known exon 3 in the *XAF1* gene, resulting in 8 exons. The primer positions for RT-PCR are indicated by *arrows*. Primers qFAS and DAS are designed to step over the intron between exons 3 and 4 and exons 4b and 5, respectively. The *dagger* and *asterisk* symbols indicate the locations of the start and stop codon, respectively

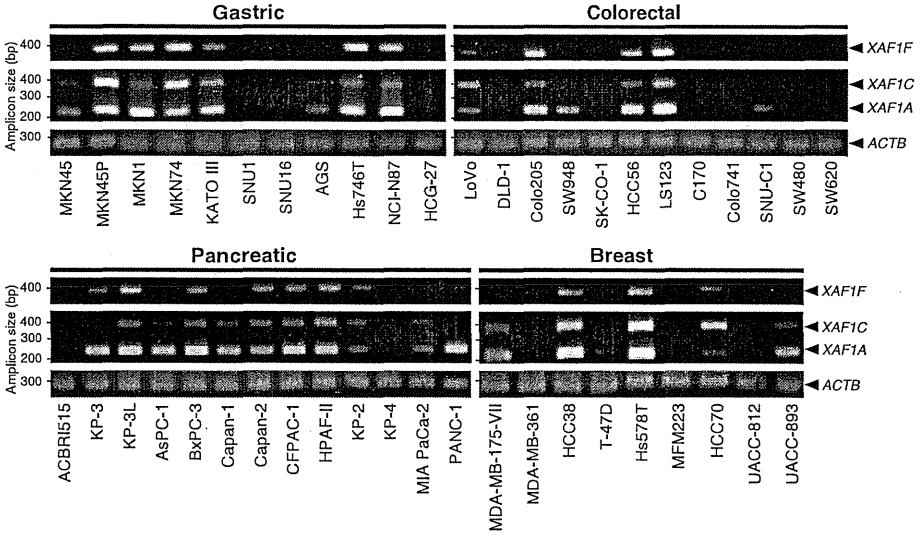

Fig. 2 Expression profile of *XAF1* transcripts in gastric, colorectal, pancreatic, and breast cancer cell lines. The transcripts of *XAF1F* and *XAF1A/C* were detected by RT-PCR using specific primer sets qFS/CAS and AS/AAS (sense/antisense), respectively. RT-PCR for beta-actin (*ACTB*) was conducted as a positive control experiment. Agarose gels were used at a concentration of 2 %

[41] were upregulated but the NMD factor *UPF1* [16, 41] was downregulated, indicating that the NMD pathway was inhibited. In KP-3L cells, mRNA expression of *ATF3*, *GADD45B* [16], and *UPF1* was significantly increased.

XAF1 expression in NMD-inhibited cells

To determine whether the transcript was degraded through the NMD pathway, we performed an NMD inhibition assay using caffeine and actD (Fig. 3b). The combination of

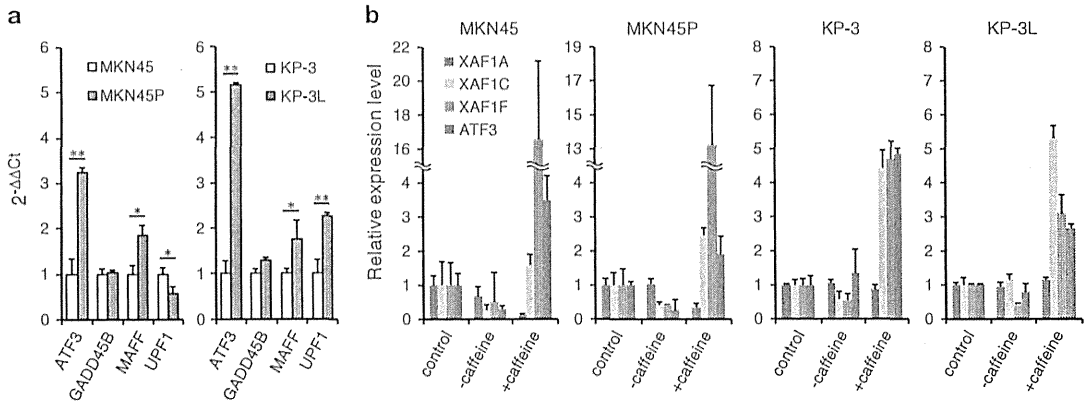

Fig. 3 Expression analysis of nonsense-mediated mRNA decay (NMD)-related and *XAF1* transcripts in pairs of cell lines used as a metastatic model. **a** Comparison of NMD-related gene expression between highly metastatic cells and their parental cells. The pairs of gastric cells (MKN45 vs. MKN45P) and pancreatic cells (KP-3 vs. KP-3L) are shown in *left* and *right panels*, respectively. The expression levels in the parental cells (MKN45 or KP-3) are shown as 1.0. SD was calculated from the relative expression level of triplicates. Statistical significance ($*p < 0.05$ and $**p < 0.01$) was evaluated by Student's *t* test. **b** Expression levels of *XAF1* transcripts in NMD-inhibited cancer cells. NMD was inhibited by caffeine and actD treatment (+caffeine). Treatment with actD alone (−caffeine) was used to observe RNA stability after blockade of transcription. Cancer cells that were not treated with reagents are presented as the control. The expression levels of *XAF1A* and *XAF1C* were estimated based on previous reports [12]. qRT-PCR of *ATF3* was conducted to determine NMD inhibition

these reagents was adopted to block increased transcription resulting from a stress response to NMD inhibition [37]. The NMD target transcript *ATF3* was upregulated in caffeine-treated cancer cells, indicating that the NMD pathway was inhibited by this treatment. Furthermore, mRNA expression of *XAF1C* and *XAF1F* harboring PTC was significantly increased in NMD-inhibited cancer cells. These upregulations were observed in other colorectal and breast cancer cells expressing *XAF1F* (Fig. S3 in the Supplementary Material).

Quantification of *XAF1F* in the peripheral blood of gastric cancer patients

Peripheral blood samples from all 96 patients and the 22 healthy volunteers were collected using PAXgene to stabilize whole RNA in the blood and subsequently evaluated using our qRT-PCR assay and semiquantitative RT-PCR as previously reported [12] (Fig. 4). To minimize the influence of RNA degradation on PCR, the RNA from all the samples was confirmed to have an RNA integrity number (RIN) [42] of at least 6.5 [43] (data not shown). *XAF1F* and *XAF1C* expression levels were significantly increased in patients relative to healthy volunteers. To investigate the relationship between age and mRNA expression, we calculated Spearman's rank correlation coefficient (ρ) for the samples (Table S3 in the Supplementary Material). The ρ value in the targeted genes was low, indicating a very weak to negligible correlation, suggesting that the expression was independent of age. These results indicate that *XAF1* splice variants were upregulated in the peripheral blood of gastric cancer patients.

mRNA expression of NMD-related genes in peripheral blood containing CTCs

qRT-PCR performed using peripheral blood collected with PAXgene can detect transcripts in CTCs [33, 34]. Recently, several research groups reported that CTC-derived survivin (*BIRC5*) was frequently detected in the peripheral blood of gastrointestinal cancer patients and that the mRNA expression level of this gene is useful as a prognostic factor [36, 44–47]. Therefore, to isolate CTC-positive samples, we adopted the *BIRC5* gene as a marker and performed a qRT-PCR assay for the quantification of *BIRC5* and *XAF1F* transcripts (Fig. S4 in the Supplementary Material). The prediction performance for CTC detection was evaluated using a ROC curve (Fig. 5). The AUC and cutoff values were 0.8299 (95 % CI, 0.7311–0.9048) and 180.75 (sensitivity, 82.99 %; specificity, 77.27 %), respectively. These data indicate that *BIRC5* expression, as measured by our qRT-PCR assay, is an efficient predictor for CTCs in the peripheral blood of gastric cancer patients. Using the calculated cutoff value, 79 CTC-positive samples were identified from 96 patients with gastric cancer.

To investigate the relationship between *XAF1* splice variants and the NMD target gene *ATF3* in the CTC-positive population, we also performed a ROC curve analysis for *XAF1F* and *XAF1C* (Fig. S5 in the Supplementary Material). Although the AUC of the variants was lower

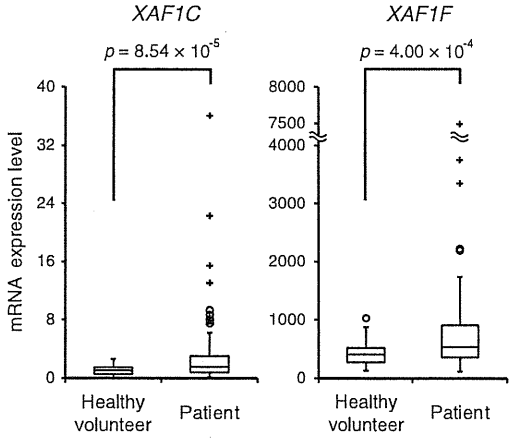

Fig. 4 Quantification of *XAF1F* and *XAF1C* transcripts in peripheral blood. *XAF1F* and *XAF1C* expression in gastric cancer patients ($n = 96$) and healthy volunteers ($n = 22$) was quantified using the qRT-PCR assay described in Figure S1 in the Supplementary Material. The normal and extreme outliers are indicated by *circle* and *plus symbols*, respectively

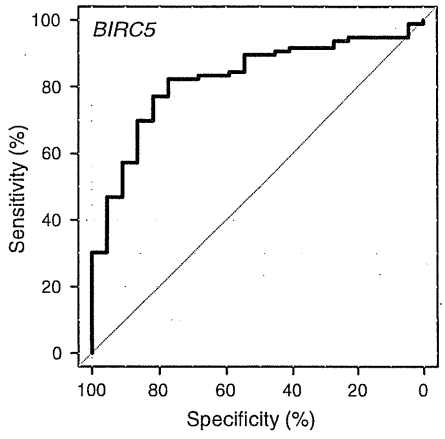

Fig. 5 ROC curve of *BIRC5* expression in peripheral blood. The *black solid line* indicates the curve for *BIRC5* in all peripheral blood samples ($n = 118$)

than that of *BIRC5*, the cutoff values were determined from the ROC curve. Table 1 shows *ATF3* expression in *XAF1C/F*-positive and *XAF1C/F*-negative populations distinguished by these values. The *XAF1F*- and *XAF1C*-positive patients accounted for 62.0 % (49/79) and 45.6 % (36/79), respectively, of the patients with CTCs. The population that was positive for both variants expressed the *ATF3* transcript. The expression level of *ATF3* in the positive patients was higher than that in the negative patients.

To further evaluate the differences in the patients with CTCs in the *XAF1C/F*-positive and *XAF1C/F*-negative populations, we correlated clinicopathological characteristics with the expression level of the splice variants

Table 1 mRNA expression level of *XAF1* variants and *ATF3* in gastric cancer patients

	Sample number	*ATF3*	
		Expression level (mean ± SD)	*p* value
In all patients			
BIRC5+	79	1.06 ± 0.51	NS
BIRC5−	17	0.87 ± 0.47	
In *BIRC5*-positive patients			
XAF1F+	49	1.15 ± 0.54	0.032
XAF1F−	30	0.92 ± 0.42	
XAF1C+	36	1.27 ± 0.61	0.0013
XAF1C−	43	0.89 ± 0.31	
XAF1F/C+	26	1.33 ± 0.62	0.0040
XAF1F/C−	53	0.93 ± 0.38	

Test used: Welch's *t* test

NS not significant

(Table 2). Among tumors invading into the subserosa (SS) or further, 74 % (29/39) expressed the *XAF1F* transcript, whereas 50 % (20/40) of tumors invading the muscularis propria (MP) were *XAF1F* negative ($p = 0.0368$). Comparison of patients with *XAF1F* expression demonstrated that 59 % (29/49) of the tumors in those patients invaded as far as the SS or further. Among patients with venous invasion, 80 % (32/40) were *XAF1F* positive, whereas 56 % (22/39) of patients without venous invasion did not show expression of *XAF1F* ($p = 0.0011$). In the *XAF1F*-positive population, 65 % (32/49) of the patients had evidence of venous invasion. Furthermore, 76 % (32/42) of patients with lymph node metastasis were *XAF1F* positive ($p = 0.0101$). To investigate the relationship between this metastasis and lymphatic invasion, we further analyzed the frequency of invasion in *XAF1F*-positive patients with lymph node metastasis. Although this splice variant was not correlated with lymphatic invasion in 79 patients with CTCs, 27 of 32 patients with metastasis (N1, 2, 3) had lymphatic invasion (84 %), whereas 82 % of patients without metastasis (N0) had no lymphatic invasion (14/17, $p = 7.528 \times 10^{-6}$ in Fisher's exact test). This result indicates that the lymph node metastasis associated with *XAF1F*-expressing CTCs accompanies lymphatic invasion. These CTCs may therefore easily metastasize to a lymph node rather than a lymphatic vessel.

Discussion

Alternative splicing allows a single gene to generate multiple mRNAs that can be translated into diverse proteins

Table 2 Expression of XAF1F and XAF1C in clinicopathological characteristics of circulating tumor cell (CTC)-containing patients

	XAF1F			*XAF1C*			*XAF1F/C*		
	Positive ($n = 49$)	Negative ($n = 30$)	p value	Positive ($n = 36$)	Negative ($n = 43$)	p value	Positive ($n = 26$)	Negative ($n = 53$)	p value
Gender			NS			NS			NS
Male	33	20		25	28		16	37	
Female	16	10		11	15		10	16	
Depth of tumor invasion			0.0368			NS			NS
≤MP[a] (T2)	20	20		16	24		11	29	
≥SS[b] (T3)	29	10		20	19		15	24	
Lymph node metastasis			0.0101			NS			NS
N0	17	20		17	20		11	26	
N1, 2, 3	32	10		19	23		15	27	
Peritoneal cytology			NS			NS			NS
CY0	7	1		2	6		2	6	
CY1	42	29		34	37		24	47	
Stage			NS			NS			NS
I, II	27	23		25	26		17	33	
III, IV	22	7		11	17		9	20	
Histological typing			NS			NS			NS
Differentiated	19	15		17	17		10	24	
Undifferentiated	30	15		19	26		16	29	
Lymphatic invasion			NS			NS			NS
ly0	19	17		17	19		13	23	
ly1, 2, 3	30	13		19	24		13	30	
Venous invasion			0.0011			NS			NS
v0	17	22		18	21		10	29	
v1, 2, 3	32	8		18	22		16	24	
Recurrence			NS			NS			NS
Yes	3	0		1	2		1	2	
No	46	30		35	41		25	51	

Test used: Fisher's exact test

NS not significant

[a] Muscularis propria

[b] Subserosa

[48]. Many transcripts have been predicted by in silico approaches and registered in public databases (e.g., Ensembl, http://www.ensembl.org) as candidate splice variants [49, 50]. Recently, Furuta et al. [51, 52] and our research group independently found that aberrant alternative splicing in cancer cells results in the insertion of intronic regions as extended exons, which results in the generation of new splice variants. Thus, it is important to explore the intronic regions of target genes to find novel splice variants, and here, we investigated the exons and introns of *XAF1* simultaneously.

In agreement with previous reports [2, 12], *XAF1* was downregulated in more than half the cell lines tested. However, the *XAF1F* transcript harboring a PTC was not often coexpressed with other *XAF1* transcripts. Comparative analysis of metastatic models obtained by xenografting (MKN45P vs. MKN45 and KP-3L vs. KP-3) revealed that the *XAF1F* and NMD target genes were upregulated in MKN45P and KP-3L cells. These data suggest that the NMD pathway in *XAF1F*-expressing cells with metastatic potential is suppressed relative to the parent cells. Recent studies have shown that tumor growth and metastasis are facilitated by NMD inhibition [53, 54]. The suppression of the NMD pathway in MKN45P and KP-3L cells may therefore be associated with cancer metastasis.

We found that the *XAF1F* transcript harboring PTC was upregulated in NMD-suppressed cancer cells and presumably subjected to NMD. To elucidate the degradation of the

XAF1F transcript through the NMD pathway, an NMD inhibition assay was performed. The NMD factor *UPF1* and NMD target genes (Fig. 3a) were significantly upregulated in KP-3L cells, which raises the possibility that NMD inhibition in *XAF1F*-expressed cells is regulated by other NMD factors. Therefore, we used caffeine as an NMD inhibitor [37] rather than depletion of *UPF1* by RNAi [16]. In cancer cells treated with only actD, which blocks transcription, *XAF1F* expression was decreased, indicating that this transcript is especially unstable in cells with active NMD. In contrast, *XAF1F* accumulated in cells in which NMD was inhibited with caffeine. These results suggest that the PTC-harboring *XAF1F* is degraded through the NMD pathway. Therefore, we concluded that expression analysis of this splice variant is valuable to evaluate NMD inhibition in cancer cells.

Splice variants of *XAF1* have been found to be significantly upregulated in the peripheral blood of gastric cancer patients. Furthermore, survivin, which is considered to be expressed in CTCs, is also detected in many patients. Therefore, *XAF1* variants are likely to be derived from CTCs. However, use of the qRT-PCR assay and PAXgene cannot eliminate the influence of circulating cell-free RNA [55] (including mRNA in microvesicles or exosomes [56, 57]) and lymphocytes or other nucleated cells because of the stabilization of whole RNA in the peripheral blood. Further studies should thus investigate the expression of *XAF1* variants in CTCs isolated using immunomagnetic separation systems (such as CellSearch). However, Chi et al. reported that *XAF1* transcripts are significantly downregulated in gastric and colorectal tumors [2, 12]. If the upregulated *XAF1* transcripts that we observed are derived from CTCs, then the expression levels of these transcripts would thus be inconsistent between the tumor tissue and CTCs. Several reports described such a difference in the gene expression profiles of primary tumors and CTCs [58, 59]. This discrepancy should be also examined using isolated CTCs in further studies.

We further attempted to discriminate survivin-positive patients using the cutoff value calculated from the ROC curve to identify the population who had CTCs. Among the patients, 82 % (79/96) were categorized as survivin-expressing CTC-positive patients, which was similar to the discriminative performance in a previous study using RT-PCR ELISA [35]. Furthermore, the *XAF1F/C*-positive population accounted for approximately half the CTC-positive patients and showed significant expression of the *ATF3* transcript. These results suggest that the NMD pathway is often suppressed in peripheral blood containing survivin-expressing CTCs derived from gastric cancer. Recently, several research groups have reported that heterogeneity may lead to differences in protein expression or cellular adhesion in CTCs [60–62]. The NMD-suppressed population identified in this study may also contribute to the heterogeneity of CTCs.

XAF1 in cancer cells acts as a tumor suppressor because of its pro-apoptotic function (Fig. 6) [13]. *XAF1* expression in various cancer cell types was found to be transcriptionally inactivated by the methylation of CpG sites in the promoter region [9, 10]. Heat shock factor (HSF)1 and p53 can also negatively regulate the *XAF1* gene via the binding of these binding elements [63, 64]. Several studies have demonstrated that *XAF1* is upregulated by interferon (IFN), resulting in the sensitization of cells to apoptosis [65–69]. To our knowledge, this study is the first to show that *XAF1F* is generated by aberrant pre-mRNA processing through NMD inhibition, which is often induced by cellular stress in the tumor microenvironment [17–19]. The *XAF1F*-expressing cells with inhibition of NMD may be stressed by external stimuli or have an amplified cellular stress response. Our qRT-PCR assay revealed that the NMD pathway tends to be inhibited in some CTCs from gastric cancer. Several studies have demonstrated that CTCs exposed to blood flow undergo physiological shear stress, leading to a change in the gene expression pattern [70, 71]. These findings suggest that *XAF1F*-expressing CTCs with inhibition of NMD may be strongly stressed by the external environment in comparison with the *XAF1F*-negative population or that they may be highly sensitive to stress.

Recent studies detected CTCs from gastric or hepatocellular cancer in patients with vascular invasion [72, 73], whereas CTCs derived from head and neck cancer were associated with lymph node metastasis [74, 75], indicating that these characteristics depend on the primary tumor. In our study, the mRNA expression level of *XAF1F* in CTCs was significantly correlated with venous invasion, lymph node metastasis, and tumor invasion that reached the SS. Additionally, the lymph node metastasis associated with *XAF1F*-expressing CTCs accompanied lymphatic invasion. Metastatic cells in which the EMT has occurred have been shown to penetrate local tissue and blood or lymphatic vessels [76]. These findings raise the possibility that *XAF1F*-positive CTCs in gastric cancer have the characteristics of EMT. In several cancer cell types, the EMT is closely related with RNA splicing [28] or NMD inhibition that can generate aberrant transcripts [29]. Therefore, CTCs expressing splice variants via NMD suppression may have been phenotypically converted by the EMT. At the minimum, these findings suggest that expression of both *XAF1F* and survivin in the peripheral blood of gastric cancer patients is a predictor of venous invasion, lymph node metastasis, and depth of tumor invasion.

A significant correlation was found between *XAF1F* in CTCs and the depth of tumor invasion/lymph node metastasis, whereas *XAF1F* expression tended to be associated with the stage of gastric cancer ($p = 0.05985$ in