Original Article

Thymidine Phosphorylase Expression in Gastric Cancer Tissues is Associated with Thrombocytosis but not with Serum Thymidine Phosphorylase Concentration

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ABSTRACT

Background: The relationship of thymidine phosphorylase (TP) expression and patient outcomes has been investigated in various cancers. This study investigated the association of TP expression, serum TP, and platelet counts in patients with gastric cancer.

Methods: A series of 77 patients with gastric adenocarcinoma were enrolled in the study. Pretreatment serum TP was determined by enzyme-linked immunosorbent assay and the patient characteristics, including platelet count, gender, age, tumor stage and histological grade, and immunoreactivity of surgically resected tissue specimens, were recorded.

Results: The serum TP level was significantly higher in poorly differentiated and signet ring cell adenocarcinomas than in more differentiated types. TP expression was observed within well- and moderately differentiated adenocarcinoma cancer cells, but was significantly weaker in poorly differentiated adenocarcinomas and signet ring cell carcinomas. There were no significant differences in the survival of patients with different histological types and serum TP levels. Although the differences were not statistically significant, high TP expression and high platelet counts were poor prognostic factors. TP expression was not correlated with serum TP levels, but was slightly correlated with the platelet count.

Conclusions: TP expression in gastric cancer tissue was slightly related to the platelet count rather than to the serum TP concentration. Therefore, a larger number of patients should be evaluated to assess the relationship between platelet count and capecitabine treatment response in gastric cancer patients.

Toho J Med 4 (2): 66–73, 2018

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DOI: 10.14994/tohojmed.2017-017

Received Dec. 7, 2017: Accepted Jan. 24, 2018
Toho Journal of Medicine 4 (2), June 1, 2018.
ISSN 2189–1990, CODEN: TJMOA2

KEYWORDS: gastric cancer, thymidine phosphorylase, ELISA, immunohistochemistry, platelet count

Introduction

Thymidine phosphorylase (TP) and the platelet-derived endothelial cell growth factor are identical protein encoding the same gene.[1] Tumors with high TP expression tend to have a high blood vessel density,[2–4] and TP has been associated with prognosis in carcinomas.[5–7] TP expression has also been associated with the depth of tumor invasion,[8, 9] lymph node metastasis,[8] and lymphatic[8] and venous invasion.[10] However, these reports evaluated only the TP expression in the tumor tissue and not the serum TP.

We previously reported that the serum TP level was significantly associated with tumor TP expression and platelet count as well as with poor prognosis in esophageal cancer.[11–13] A significant correlation of serum and tumor TP levels has also been reported in gastric cancer.[14] In clinical studies of neoadjuvant chemotherapy for gastric cancer, TP was found to be associated with resistance to 5-FU[15] and tegafur/uracil, but gastric cancer cells expressing TP were shown to be sensitive to capecitabine.[16, 17] Capecitabine was first recommended for treating gastric cancer in the 2014 Gastric Cancer Treatment Guidelines.[18, 19] Serum biomarker, rather than tissue protein expression, may be useful in clinical practice. Serum TP level is one of the candidates for such convenient biomarkers.

This study evaluated the relationships between serum TP, tumor TP expression, and platelet count with the aim of identifying capecitabine-sensitive gastric cancer.

Patients and Methods

Patients and serum samples

Serum samples were obtained from 77 consecutive patients before surgical treatment of gastric adenocarcinoma at the Toho University Hospital between April 2011 and July 2013. Forty-eight patients were men and 29 were women, and the median age was 70 years (Table 1). Of the

Table 1 Characteristics and median serum TP concentration of 77 patients with gastric cancer.

		Number	serum TP level (mg/dl) Median (IQR)	*P* value
Gender	Female	29	5.66 (3.88–9.10)	
	Male	48	5.36 (3.84–7.75)	0.966[a]
Age	<65 years old	27	5.20 (3.81–6.43)	
	≧65 years old	50	5.66 (3.88–9.25)	0.308[a]
Pathological stage	I	43	5.20 (3.74–6.44)	
	II	19	6.99 (4.12–8.92)	
	III	5	4.68 (4.27–4.69)	
	IV	10	5.28 (4.66–17.80)	0.541[b]
Histology	Tub1 or Tub2	37	4.69 (3.67–5.93)	
	Por or sig	40	6.40 (4.49–10.53)	0.008[a]
Proportion of TP staining	Proportion 0	31	5.20 (3.66–7.27)	
	Proportion 1	25	5.79 (4.62–8.50)	
	Proportion 2	13	4.52 (3.67–7.65)	
	Proportion 3	8	5.51 (4.77–7.37)	0.702[b]
Intensity of TP staining	Intensity 0	31	5.20 (3.66–7.27)	
	Intensity 1	29	5.52 (3.83–8.54)	
	Intensity 2	14	5.41 (4.72–6.88)	
	Intensity 3	3	6.22 (4.72–7.76)	0.405[b]

The TP level of poorly differentiated adenocarcinoma or signet ring cell carcinoma was significantly higher than that of tubular adenocarcinoma (median ± quartile deviation; 6.40 ± 3.02 ng/ml vs. 4.69 ± 1.12 ng/ml, $P = 0.008$*)

TP: thymidine phosphorylase, Tub1: well differentiated adenocarcinoma, Por: poorly differentiated adenocarcinoma, IQR: inter quartile range, Tub2: moderately differentiated adenocarcinoma, Sig: signet-ring cell carcinoma, a) Mann-Whitney *U* test, b) Kruskal-Wallis test

77 patients, 43 had TNM stage I disease, 19 had stage II disease, five had stage III disease, and 10 had stage IV disease based on the Japanese Classification of Gastric Cancer, 13th Edition.[19] Patients were followed until October 31, 2017. Pathological evaluation found that 37 tumors were well- or moderately differentiated adenocarcinomas and 40 were poorly differentiated adenocarcinomas or signet ring cell carcinomas. This study was approved by the institutional review board of the Toho University School of Medicine (#22-112, #22-047, #24-045). Informed consent was obtained from all patients for whom identifying information is included in this article.

Serum sampling and TP assay

Venous blood samples were collected before surgery; serum was separated by centrifugation at 3,000 × *g* for 5 minutes and then frozen at −80℃ until assayed. Repeated sample thawing and freezing was avoided. TP was determined using an enzyme-linked immunosorbent assay (ELISA) kit (USCN Life Science, cat. no. MBS889092) following the manufacturer's instructions. This sandwich ELISA included a monoclonal anti-TP antibody and streptavidin conjugated to horseradish peroxidase (HRP). Briefly, 40 µl of each serum sample was added to 10 µl of anti-TP antibody and 50 µl of streptavidin-HRP and incubated for 60 min at 37℃. After incubation, 50 µl of each chromogen solution was added, the mixture was incubated 10 min at 37℃ in the dark, and a stop solution was added to halt the reaction. The optical density was read at 450 nm within 15 min of adding the stop solution, and the TP sample concentration was calculated by comparison to a curve generated by the assay of five serial dilutions of known concentrations of a TP standard supplied with the kit.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissues were sectioned at 4 µm for immunoperoxidase staining of TP using a rabbit polyclonal primary antibody (HPA001072, SIGMA-Aldrich, St. Louis, MO, USA). A Ventana Benchmark XT (Ventana Medical Systems, Inc. Tucson, AZ, USA) automated slide staining system was used. Sections were deparaffinized, rehydrated, and incubated with the primary antibody (1:100 dilution) for 32 min at room temperature. The Ventana system used an endogenous biotin blocking kit to reduce nonspecific staining. The antibody binding was visualized by diaminobenzidine detection, and sections were counterstained with hematoxylin II and Bluing Reagent (Ventana Medical Systems). Human appendix tissue was used as a positive control, and the antibody vehicle (Agilent Technologies Company, Santa Clara, CA, USA) was used as the negative control. The stained sections were analyzed at high magnification. Staining was positive for TP expression if >10% of the tumor cells were stained; proportion 0 indicating no staining, proportion 1 indicated 10%-30% stained cells; proportion 2 indicated 31%-60%, and proportion 3 indicated >60%. The intensity of the TP staining was intensity 0, no staining; intensity 1, weak staining; intensity 2, medium staining; and intensity 3 strong staining.

TP expression in gastric cancer tissue

Gastric cancer tissues were characterized by proportion and intensity of TP expression, as shown in Fig. 1. The left panel shows a moderately differentiated adenocarcinoma with strong, intensity 3 staining (Fig. 1a, c). The right panel, shows a poorly differentiated adenocarcinoma with medium, intensity 2 staining (Fig. 1b, d). Patients with TP-negative tumors were classified as proportion/intensity 0 and those with TP-positive tumors were classified as proportion/intensity 1, 2, and 3.

Statistical analysis

Differences in the serum TP concentration were tested for significance using the Mann-Whitney *U* and the Kruskal-Wallis tests. Differences in the grade of the immunohistochemical staining were tested for significance using the G-test. The Kaplan-Meier product limit method was used to estimate survival following surgery. Differences in survival were valuated using the log-rank test. The association of clinicopathological variables and overall survival was evaluated by univariate analysis. Comparisons of blood platelet counts and serum TP levels were analyzed using the Spearman's rank correlation coefficient. Comparisons of platelet counts and histology, and TP-immunoreactivities were analyzed using the Welch's *t*- and the Dunnett's tests. All statistical analyses were performed with StatMate V for Win&Mac Hybrid (ATMS Co., Ltd., Tokyo, Japan). *P*-values <0.05 were considered statistically significant.

Results

Clinicopathological variables, serum TP concentration, and TP expression in tumor tissue

The serum TP concentration and patient clinicopathological characteristics are shown in Table 1. Sex, age, and disease stage were not associated with differences in serum TP concentration, but tumor histology was. TP con-

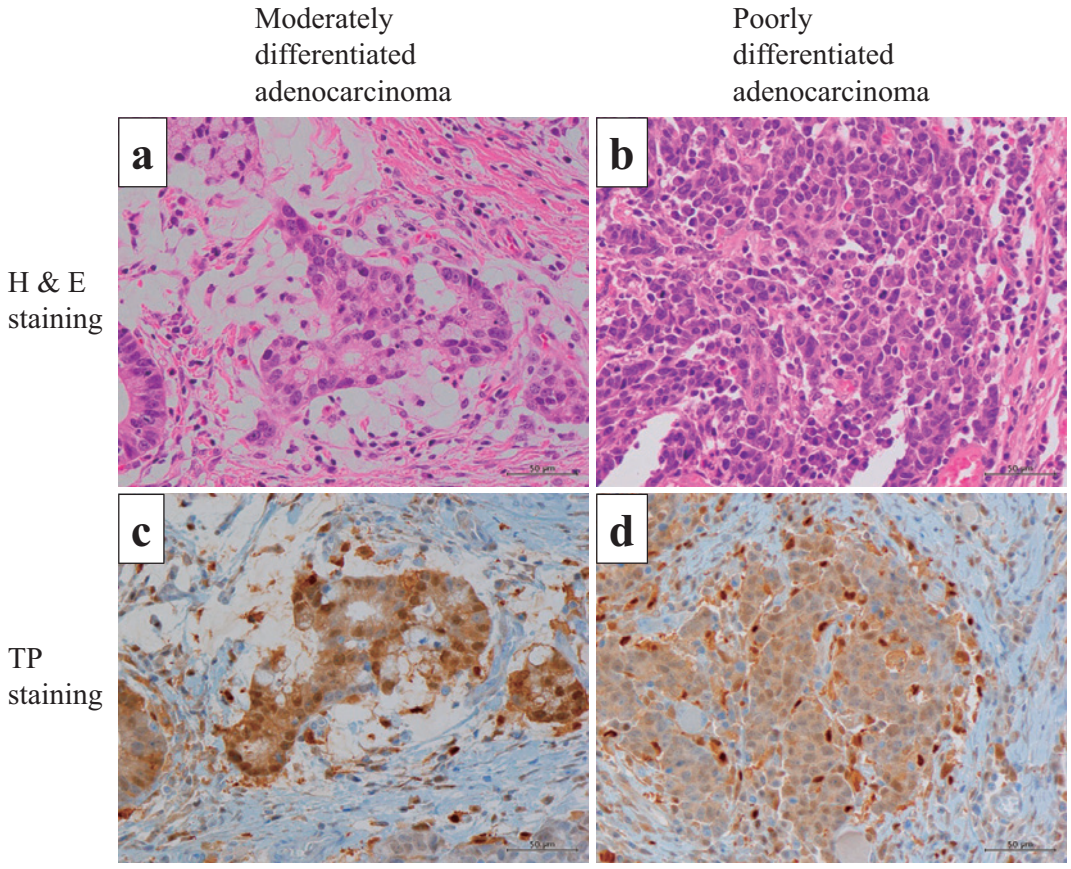


Fig. 1 Representative histological staining of gastric adenocarcinoma (a) HE staining of moderately differentiated adenocarcinoma. (b) HE staining of poorly differentiated adenocarcinoma. (c) TP staining of moderately differentiated adenocarcinoma. (d) TP staining of poorly differentiated adenocarcinoma (200×).

centration was significantly higher in poorly differentiated adenocarcinoma or signet ring cell carcinoma (6.40 ± 3.02 ng/ml) than in tubular adenocarcinoma (4.69 ± 1.12 ng/ml, P = 0.008*). TP expression in tumor tissue indicated by either proportion of stained cells or staining intensity was not associated with differences in serum TP concentration (P = 0.702 and 0.405, respectively, Table 1).

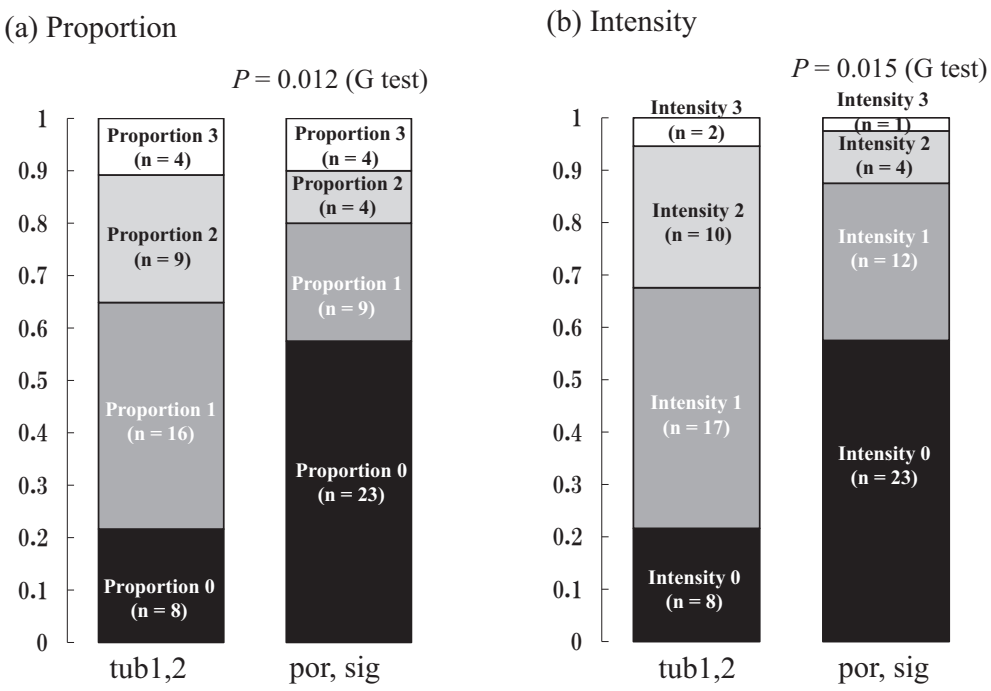
When TP expression in each histological type was evaluated, significantly fewer TP-positive cells were observed in poorly differentiated adenocarcinomas or signet ring cell carcinomas than in well- or moderately differentiated adenocarcinomas (P = 0.012, Fig. 2a). Staining intensity of TP-positive cells was significantly weaker in poorly differentiated adenocarcinomas or signet ring cell carcinomas than in well- or moderately differentiated adenocarcinomas (P = 0.015, Fig. 2b).

A case of poorly differentiated gastric adenocarcinoma with high serum TP concentration

A 71-year-old man had the highest serum TP concentration (41.891 mg/dl) in this patient series. Gastrointestinal endoscopy showed a 0-IIc lesion in the upper posterior stomach wall (Fig. 3a). There was no significant swelling of the lymph nodes (Fig. 3b). The pathological diagnosis was poorly differentiated adenocarcinoma, pT1b, pN0 (pStage I). Although the interstitial cells, including inflammatory cells, were TP-positive, the tumor cells were TP-negative (Fig. 3c).

Serum TP concentration, tumor TP expression, and survival

Patients were stratified by serum TP concentration using the median value (5.524 mg/dl, IQR 3.845-8.149 mg/dl) as the cutoff and by platelet count using the median value (210×10^3/μl, IQR 189×10^3-257×10^3/μl) as the cutoff. A univariate analysis did not find a significant association of



tub: tubular adenocarcinoma por: poorly differentiated adenocarcinoma sig: signet-ring cell carcinoma

Fig. 2 Immunoreactivity and TP expression (a) Proportion of TP-stained cells and (b) intensity of TP staining.

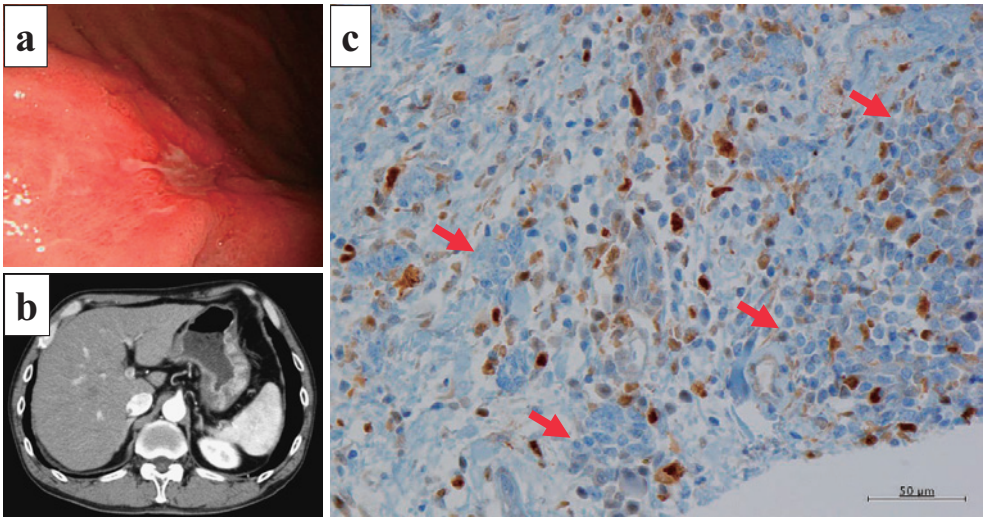


Fig. 3 Gastric cancer case with the highest serum TP level (a) Upper gastrointestinal endoscopic findings, (b) abdominal enhanced CT findings, and (c) TP staining.

histological type, serum TP concentration, TP expression, or platelet count with survival (Fig. 4). Although the difference was not statistically significant, the TP-positive group showed poorer survival than the TP-negative group (Fig. 4c). Similarly, the high platelet count group also showed poorer survival than the low platelet count group (Fig. 4d).

Platelet count, serum TP concentration, and tumor TP expression

The association of platelet count and serum TP concentration was not significant (Fig. 5a). Platelet counts of differentiated type seemed to be higher than those of poorly differentiated type; however, this difference was not statistically significant (Fig. 5b). The platelet count was slightly

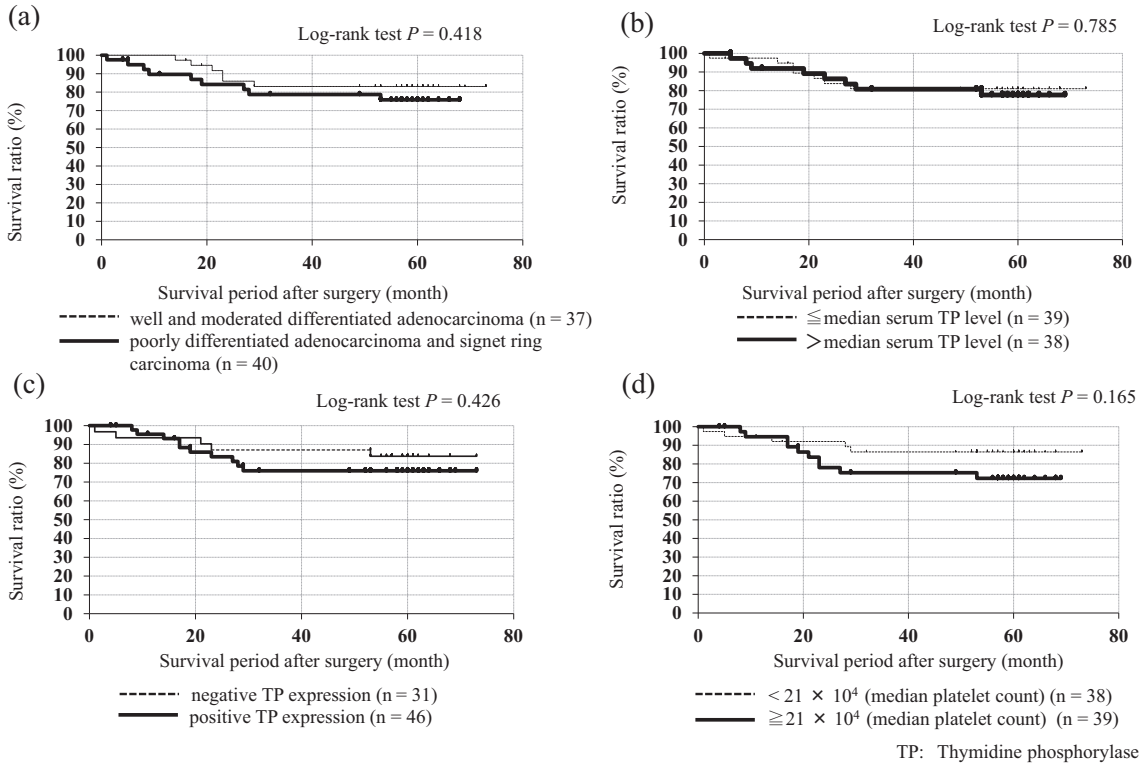


Fig. 4 Overall survival curves (a) Solid line shows the overall survival of patients with poorly differentiated adenocarcinoma and signet ring cell carcinoma. The dotted line represents the overall survival of patients with well- and moderately differentiated adenocarcinoma. (b) The dotted line shows the overall survival of patients with lower than median serum TP level. The solid line represents the overall survival of patients with greater than median serum TP level. (c) The dotted line shows the overall survival of patients negative for TP expression. The solid line represents the overall survival of patients positive for TP expression. (d) The dotted line shows the overall survival of patients with lower than median platelet count. The solid line represents the overall survival of patients with higher than median platelet count.

associated with the proportion of TP-expressing cells (Fig. 5c) and intensity of the TP staining (Fig. 5d).

Discussion

The platelet count was significantly associated with the proportion of TP-expressing cells and the intensity of TP staining. There was no statistically significant difference because there were only three cases in the intensity 3 group. An association between serum TP concentration and TP expression in tumor tissue and an increase with pathological staging had been expected. However, no correlation of serum TP with tumor TP expression or gastric cancer stage was observed.

Previous reports also failed to demonstrate a correlation between TP expression and progression of pathological stage.[10, 20, 21] Although the serum TP level was significantly higher in poorly differentiated adenocarcinoma and signet ring cell carcinoma than in differentiated adenocarcinoma, a few TP-expressing cells were present in the latter tumors. Shimaoka et al. reported finding more TP-expressing cells in differentiated adenocarcinomas than in undifferentiated adenocarcinomas,[22] which is consistent with the results in this study, in which we found an association between differentiated adenocarcinoma and platelet counts. Wang et al. reported that high TP expression was positively correlated with thrombocytosis.[23] This discrepancy may be partly explained by the high immunoreactivity of TP in stromal cells, including fibroblasts, lymphocytes, and macrophages.[24, 25] Liakakos et al. reported that TP expression in tumor-associated stromal cells was observed only in poorly differentiated carcinomas.[24] Han et al. reported that TP expression in tumor-associated stromal cells was not associated with TP expression in cancer cells.[26] The serum TP concentration might have been influenced by the high TP expression in tumor-associated stromal cells of poorly differentiated adenocar-

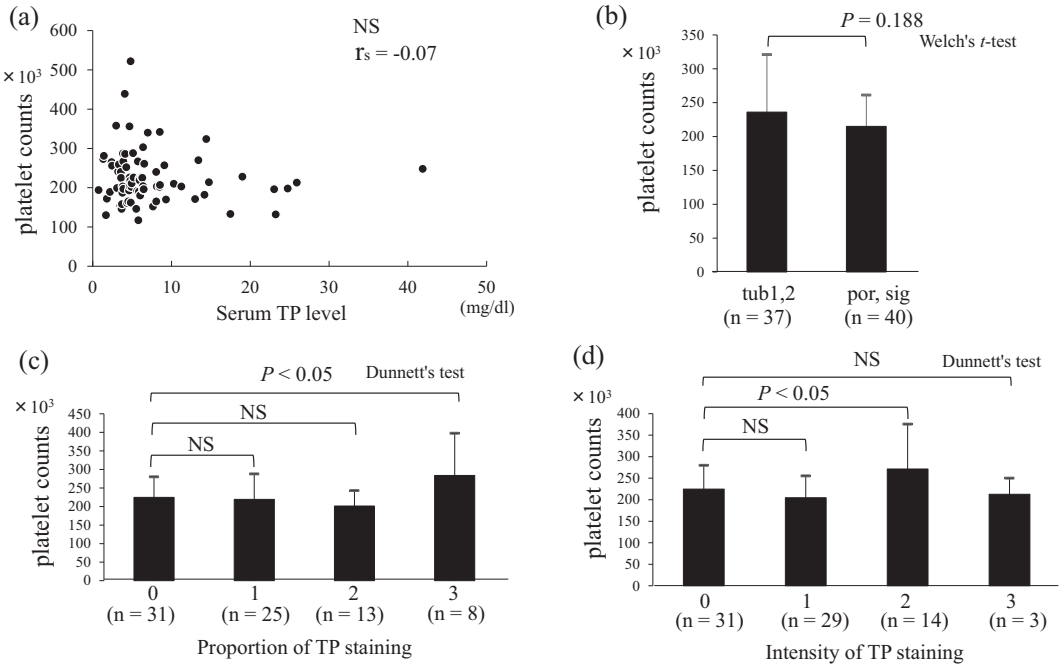


Fig. 5 Relationships of platelet count, serum TP, pathology, and TP expression (a) Correlation of platelet count with serum TP level. (b) Comparison of the platelet count and pathology. (c) Comparison of the platelet count and proportion of TP-staining cells. (d) Comparison of platelet count and TP staining intensity.

cinoma and signet ring cell carcinomas. Serum TP concentration cannot be considered a biomarker of TP expression in tumor cells. To test this, preoperative and postoperative serum TP concentrations should have been compared. However, surgically removed stromal cells along with the tumor tissue could infuluence postoperative serum TP levels.

Although high TP expression and platelet counts did decrease survival, as previously reported,[27] the effect was not statistically significant. A similar tendency was reported by Konno et al.,[28] who showed that TP-positive gastric cancer was more likely to recur than TP-negative gastric cancer. TP expression has been associated with hematogenous metastasis involving the liver.[19, 21, 29] The CLASSIC trial found that capecitabine plus oxaliplatin combination therapy strongly reduced the recurrence in the liver, but weakly reduced peritoneal recurrence.[30, 31] The ACTGC trial found that tegafur/gimeracil/oteracil reduced peritoneal and/or lymph node recurrence.[32] Therefore, capecitabine may be more effective than tegafur/gimeracil/oteracil against high TP-expressing differentiated adenocarcinoma at liver metastasis.

In summary, we found that TP expression in gastric cancer tissue was slightly related to the platelet count rather than to the serum TP concentration. Therefore, a larger number of patients should be evaluated to assess the relationship between platelet count and capecitabine treatment response in gastric cancer patients.

This research was partly supported by a Grant-in-Aid for Scientific Research (nos. 26460951) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflicts of interest: Non declared.

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