Use of *ANGPTL2* mRNA levels in formalin-fixed paraffin-embedded tissues as a biomarker to diagnose gastric cancer and to evaluate the extent of vascular invasion

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Abstract. With the recent advances in medical technologies, gastric cancer can often be removed with minimally invasive surgical techniques when identified early. Surgery must remove all gastric cancer, since residual cancerous tissue may lead to recurrence. Resected cancerous tissues are pathologically evaluated to determine whether all cancerous areas have been removed, but such assessments are rarely straightforward, and cancer markers could inform such pathological evaluations of cancer. An ideal marker would be identifiable in formalin-fixed paraffin-embedded (FFPE) tumor tissue. The first objective of the present study was to compare levels of angiopoietin-like protein 2 (ANGPTL2) in cancerous and noncancerous areas of FFPE tissues to determine whether ANGPTL2 is a marker relevant to the pathological diagnosis of cancer. The second objective was to evaluate whether *ANGPTL2* mRNA is useful as a marker of the extent of vascular invasion of gastric cancer. Out of the 15 patients studied, 12 had a higher *ANGPTL2* mRNA levels in cancerous areas compared with noncancerous areas. This finding indicated that *ANGPTL2* mRNA is useful as a biomarker for identifying cancerous areas in FFPE tissues, at least for male patients. Spearman's rank correlation analysis showed a significant correlation between the *ANGPTL2* mRNA level and the degree of vascular invasion of cancer (r=0.66; P=0.01). In receiver operating characteristic curve analysis of the association between the *ANGPTL2* mRNA level and the degree of vascular invasion, the area under the curve was 0.92 (95% confidence interval, 0.78-1.00; P=0.01), indicating a significant association. The present study demonstrates that *ANGPTL2* mRNA in FFPE tissues is a potential biomarker that informs the pathological diagnosis of gastric cancer and that *ANGPTL2* mRNA may be predictive of vascular invasion, which is an indicator of metastasis in gastric cancer.

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Introduction

Gastric cancer is prevalent worldwide, with ~800,000 gastric cancer-associated mortalities occurring each year, making it the second most common cause of cancer-associated mortality (1). With the recent advances in medical technologies, gastric cancer can often be removed with minimally invasive surgical techniques if identified early (2). Endoscopic submucosal dissection is the least invasive and, therefore, the most widely used of these surgical procedures (2). It is important for all gastric cancer tissue to be removed by the endoscopic submucosal dissection, since residual cancerous tissue can lead to recurrence (3). Resected cancerous tissues are pathologically evaluated to determine whether all cancerous areas have been removed; however, pathological assessment is rarely straightforward. Interpretations of stained cancerous tissues are, however, not consistent between pathologists, meaning that different conclusions may be drawn by different pathologists (4).

Cancer markers could inform pathological evaluations of cancer. An ideal marker would be one that is identifiable in formalin-fixed paraffin-embedded (FFPE) tissue samples, which a high proportion of medical institutions prefer as these tissues store well and are easy to handle (5).

The first objective of the present study was to quantitatively determine the levels of angiopoietin-like protein 2 (ANGPTL2) in cancerous and noncancerous areas of FFPE tissues to evaluate whether ANGPTL2 is a marker relevant to pathological diagnoses of gastric cancer.

ANGPTL2, a member of the ANGPTL family, contributes to the onset and progression of chronic inflammation and to the associated diseases caused by this (6-10). ANGPTL2 also regulates angiogenesis in the body (11). Endo *et al* (9) considered ANGPTL2 to be a potential biomarker for diagnosing lung and breast cancer in humans.

Our previous studies showed ANGPTL2 to be widely expressed in gastric cancer cell lines and patients with gastric or colon cancer (12,13). These findings highlight the potential of ANGPTL2 as a biomarker for identifying gastric and colon cancer in clinical settings.

In the present study, mRNA levels of *ANGPTL2* were determined in FFPE tumor tissues collected from patients with mucosal (M), submucosal (SM), tunica muscularis propria (MP), serosa-exposed (SE) and subserosal (SS) gastric cancer. A second objective was to evaluate whether *ANGPTL2* mRNA is useful as a marker of the extent of vascular invasion of gastric cancer, which portends hematogenous metastasis (14).

Materials and methods

Patients and tissue samples. Serum samples were obtained from 15 patients who attended the clinic between May 2013 and November 2014 at the Nanpuh Hospital (Kagoshima, Japan). Patient characteristics, including sex, age, body mass index (BMI), serum carcinoembryonic antigen (CEA) levels and serum carbohydrate antigen 19-9 (CA19-9) levels, are summarized in Table I. Serum concentrations of CEA and CA19-9 were determined using an electro-chemiluminescence immunoassay using the LUMIPULSE G1200® (Fujirebio Diagnostics, Inc., Tokyo, Japan) according to the manufacturer's protocol.

Tissues removed from the patients were immersed in 10%-formaldehyde neutral buffer solution. FFPE tissues were prepared using the Tissue-Tek VIP6® (Sakura Finetek Japan Co., Ltd., Nagano, Japan).

Table II shows the sex, diagnosis, levels of *ANGPTL2* mRNA, degree of differentiation, tumor invasion depth, lymph node metastasis, distant metastasis, tumor stage and degrees of lympho-vascular and vascular invasion for each patient. Lympho-vascular invasion was classified into four grades according to Japanese Classification of Gastric Carcinoma (15): ly0, no lymphatic invasion; ly1, minimal lymphatic invasion; ly2, moderate lymphatic invasion; and ly3, extensive lymphatic invasion. Vascular invasion was also classified into four grades according to Japanese Classification of Gastric Carcinoma (15): v0, no venous invasion; v1, minimal venous invasion; v2, moderate venous invasion; and v3, extensive venous invasion. Of the 15 patients with gastric cancer, 9 were diagnosed with adenocarcinoma, 5 with tubular adenocarcinoma and 1 with signet-ring cell carcinoma. The patient group included 3 patients diagnosed with M, 3 patients with SM, 4 patients with MP and 5 patients with SE or SS cancer.

Among the patient group, 5 patients were diagnosed with clinical stage IA, 1 with stage IB, 4 with stage IIA, 2 with stage IIIA, 1 with stage IIIB, 1 with stage IIIC and 1 with stage IV. Cancer staging was based on routine histopathological analysis and clinical assessment, according to the Tumor Node Metastasis (TNM) classification (16). Tumors were classified according to the recommendations of the International Union Against Cancer/TNM system (17). The characteristics of the subjects are summarized in Table III.

Six consecutive slices, each 3-μm thick, were prepared from the FFPE tissues. Hematoxylin and eosin (H&E) staining was performed on one slice to identify the cancerous areas. Tissue slices were stained with hematoxylin (for 10 min) and eosin (1 min) at room temperature. A pathologist observing the H&E-stained slices classified the cancerous and noncancerous areas. For patients with M cancer, the cancerous and noncancerous areas in the M layer of the remaining five slices were obtained using microdissection. For patients with SM, MP or SE/SS cancer, the cancerous and noncancerous areas in the SM layer were obtained using microdissection. Tissues were also stained with Victoria blue (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 12 h at room temperature to determine venous invasion.

Written informed consent was obtained from all patients. The study design was approved by the Ethics Committee of Nanpuh Hospital (Kagoshima Kyosaikai, Public Interest Inc. Association, Japan). Clinical examinations were performed according to the principles of the Declaration of Helsinki.

RNA extraction from FFPE tissue. Total RNA was extracted from FFPE tissue slices using the PureLink FFPE Total RNA Isolation kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed with equipment of the Division of Gene Research, Kagoshima University. The RT reaction was performed using random primers (Toyobo Co., Ltd., Osaka, Japan) and ReverTra Ace® (Toyobo Co., Ltd.), according to the manufacturer's protocol, using 100 ng RNA. Cycle conditions were 95˚C for 1 min, followed by 45 cycles of denaturation for 15 sec at 95˚C, annealing and extension steps for 30 sec at 60˚C each.

The amplification was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosciences; Thermo Fisher Scientific, Inc.) using a SYBR Green Realtime PCR Master Mix kit (Toyobo Co., Ltd.) according to the manufacturer's protocol. The specific primers for human *ANGPTL2* (purchased from Thermo Fisher Scientific, Inc.) were 5'-GCCACCAAGTGT CAGCCTCA-3' (forward) and 5'-TGGACAGTACCAAAC ATCCAACATC-3' (reverse). Human β-actin, used as a control, was amplified using the following specific primers: Forward, 5'-AAGCCACCCCACTTCTCTCTAA-3'; and reverse, 5'-AAT GCTATCACCTCCCCTGTGT-3' (Thermo Fisher Scientific, Inc.). With the *ANGPTL2* mRNA level in the noncancerous areas taken to be 1.0, *ANGPTL2* mRNA levels in the cancerous areas were calculated with the $2^{-\Delta\Delta Cq}$ method (18).

Statistical analysis. Data are presented as the mean ± standard deviation. Data were analyzed using SPSS version 23 (IBM SPSS, Armonk, NY, USA). The correlations of the *ANGPTL2* mRNA concentration with the patient age, BMI and serum CEA and CA19-9 levels were analyzed using Pearson's correlation analysis. The correlations of the *ANGPTL2* mRNA concentration with the degree of differentiation, tumor invasion depth, lymph node metastasis, distant metastasis, tumor stage, degree of lympho-vascular invasion and degree of vascular invasion were analyzed using Spearman's rank correlation analysis. A receiver operating characteristic (ROC) curve was plotted to evaluate the ability of *ANGPTL2* mRNA level to predict vascular invasion. Youden's index method (19) was used to determine the optimal cutoff for the *ANGPTL2* mRNA level for assessing the presence of vascular invasion in

Table I. Characteristics of patients.

Variable	Mucosal (n=3)	Submucosal (n=3)	MP (n=4)	SE/SS (n=5)	Total (n=15)
Sex					
Male	2	3	3	4	12
Female	1	0	1	1	3
Age, years					
Mean ± SD	66.0±7.9	80.0±9.8	56.8±15.4	65.0±17.6	66.0±15.1
Range	60-75	69-88	43-75	36-78	36-88
BMI, kg/m^2					
Mean ± SD	19.8±2.6	20.2±0.7	22.8±2.1	21.4±1.4	21.2±2.0
Range	17.8-22.7	19.4-20.7	19.9-24.5	19.3-23.3	17.8-24.5
CEA level, ng/ml					
Mean ± SD	1.4±0.1	4.6±4.2	6.4±6.2	28.7±58.0	12.5±33.4
Range	1.3-1.4	2.0-9.4	2.0-15.5	0.6-132.4	0.6-132.4
CA19-9 level, U/ml					
Mean ± SD	14.3±4.1	13.0±8.3	18.0±10.7	99.4±209.2	43.4±119.3
Range	9.6-16.9	6.5-22.4	6.9-30.3	0.1-473.6	0.1-473.6

MP, tunica muscularis propria; SE/SS, serosa exposed/subserosal; BMI, body mass index; SD, standard deviation; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

gastric cancer. P<0.05 was considered to indicate a statistically significant difference.

Results and Discussion

To the best of our knowledge, this is the first study to investigate whether ANGPTL2 in FFPE tissues is a useful biomarker for diagnosing gastric cancer. To prepare FFPE tissues, a tissue specimen removed from a patient was fixed in a formalin solution to cross-link biological molecules, and then embedded in paraffin. This procedure may denature mRNA and other biological components. von Ahlfen *et al* (20), however, successfully extracted the mRNA for telomere-binding protein from FFPE tissues (21), which indicated that *ANGPTL2* mRNA may be extractable from FFPE tissues. In the present study, the cancerous and noncancerous areas were distinguished from one another by pathological diagnosis using H&E-stained cross-sections.

Of the 15 patients studied, 12 (80%) had a higher *ANGPTL2* mRNA level in cancerous areas compared with the reference level (set as 1.0) in noncancerous areas (Fig. 1). In total, 2 of the 3 patients with M cancer, 2 of the 3 patients with SM cancer, 3 of the 4 patients with MP cancer and all 5 patients with SE/SS cancer had an *ANGPTL2* mRNA level >1.0 (Fig. 1). This finding indicated that *ANGPTL2* mRNA is useful as a biomarker for identifying cancerous areas in FFPE tissues, at least for male patients (owing to the small female sample size). Furthermore, the results indicated that the *ANGPTL2* mRNA level may have higher diagnostic precision in advanced cancer.

The association between *ANGPTL2* mRNA expression and BMI, as well as other factors, was also evaluated. As shown in Table IV, *ANGPTL2* mRNA levels in FFPE tissues were not correlated with age (correlation coefficient, r=-0.31; P=0.26), BMI (r=0.09; P=0.75), CEA level (r=0.04; P=0.90), CA19-9 level (r=0.03; P=0.91), degree of tumor differentiation (r=0.21; P=0.46), depth of tumor invasion (r=0.35; P=0.21), degree of lymph node metastasis (r=0.31; P=0.26), degree of distant metastasis (r=0.00; P=1.00), tumor stage (r=0.33; P=0.23) or degree of lympho-vascular invasion (r=0.07; P=0.80). *ANGPTL2* mRNA levels were, however, correlated with the degree of vascular invasion (r=0.66; P=0.01).

Micrographs of cancerous areas stained with Victoria blue and H&E in patients with a high *ANGPTL2* mRNA level and a high degree of vascular invasion [patient no. 4 (mRNA level 2.52) and patient no. 14 (mRNA level 2.81)] are shown in Fig. 2. The arrows in Fig. 2A and B show the tumor cells in the blood vessels. Fig. 2C shows the micrograph of cancerous areas, stained with H&E, of patients with a low *ANGPTL2* mRNA level [patient no. 5 (mRNA level 0.42)]. The arrow in Fig. 2C indicates no tumor cells in the blood vessels.

Since primary cancer with a high degree of vascular invasion has often already metastasized (14,22,23), accurate pathological diagnoses of vascular invasion could inform assessments of metastatic status. Pathological diagnoses of vascular invasion, however, are elusive. A biomarker of vascular invasion would aid the detection of metastatic cancer. As shown in Table IV, the *ANGPTL2* mRNA level was correlated with vascular invasion.

An ROC analysis was conducted to explore this possibility. Fig. 3 contains the ROC curve (n=15) for the analysis of *ANGPTL2* mRNA levels and the degree of vascular invasion. An area under the curve of 0.92 (95% confidence interval, 0.78-1.00; P=0.01) indicated a high diagnostic potential. The results of ROC analysis also indicated that *ANGPTL2* mRNA may be useful for assessing gastric cancer metastasis.

Table II. Level of *ANGPTL2* mRNA, degree of differentiation, tumor invasion depth, lymph node metastasis, distant metastasis, tumor stage, and degrees of lympho-vascular invasion and of vascular invasion.

Patient no.	Sex	Diagnosis	*ANGPTL2* mRNA[a]	Degree of differentiation	Tumor invasion depth	Lymph node metastasis	Distant metastasis	Stage	Lympho-vascular invasion	Vascular invasion
1	Male	Tubular adenocarcinoma	1.11	High	Mucosal	N0	M0	IA	0	0
2	Male	Adenocarcinoma	0.99	Poor	Mucosal	N0	M0	IA	0	0
3	Female	Signet-ring cell carcinoma	1.81	Poor	Mucosal	N0	M0	IA	0	0
4	Male	Tubular adenocarcinoma	2.52	High	Submucosal	N0	M0	IA	0	1
5	Male	Tubular adenocarcinoma	0.42	Moderate	Submucosal	N0	M0	IA	3+	0
6	Male	Adenocarcinoma	1.27	Poor	Submucosal	N2	M0	IIA	1	0
7	Male	Adenocarcinoma	1.46	High	MP	N1	M1	IV	1	0
8	Male	Tubular adenocarcinoma	2.13	Moderate	MP	N1	M0	IIA	2	2
9	Male	Adenocarcinoma	0.69	Poor	MP	N1	M0	IIA	1	0
10	Female	Adenocarcinoma	1.02	Poor	MP	N0	M0	IB	0	0
11	Female	Adenocarcinoma	1.22	Poor	Subserosal	N0	M0	IIA	0	0
12	Male	Tubular adenocarcinoma	1.64	High	SE	N2	M0	IIIB	2+	2+
13	Male	Adenocarcinoma	1.97	Poor	SE	N1	M0	IIIA	1	0
14	Male	Adenocarcinoma	2.81	Poor	SE	N1	M0	IIIA	1	2
15	Male	Adenocarcinoma	1.53	Poor	SE	N3a	M0	IIIC	3	2

[a]*ANGPTL2* mRNA level in the noncancerous areas taken to be 1.0; ANGPTL2 mRNA levels in the cancerous areas were calculated with the $2^{-\Delta\Delta q}$ method. ANGPTL2, angiopoietin-like protein 2; MP, tunica muscularis propria; SE, serosa-exposed.

Table III. Level of ANGPTL2 mRNA, degree of differentiation, lymph node metastasis, distant metastasis, tumor stage, and degrees of lympho-vascular invasion and of vascular invasion by degree of tumor invasion depth.

Parameters	Mucosal (n=3)	Submucosal (n=3)	MP (n=4)	SE/SS (n=5)	Total (n=15)
ANGPTL2 mRNA level[a]	1.31±0.44	1.41±1.06	1.33±0.62	1.83±0.61	1.51±0.66
Degree of differentiation, n					
Poor	2	1	2	4	9
Moderate	0	1	1	0	2
High	1	1	1	1	4
Lymph node metastasis, n					
N0	3	2	1	1	7
N1	0	0	3	2	5
N2	0	1	0	1	2
N3a	0	0	0	1	1
Distant metastasis, n					
M0	3	3	3	5	14
M1	0	0	1	0	1
Tumor stage, n					
IA	3	2	0	0	5
IB	0	0	1	0	1
IIA	0	1	2	1	4
IIIA	0	0	0	2	2
IIIB	0	0	0	1	1
IIIC	0	0	0	1	1
IV	0	0	1	0	1
Lympho-vascular invasion, n					
0	3	1	1	1	6
1	0	1	2	2	5
2/2+	0	0	1	1	2
3/3+	0	1	0	1	2
Vascular invasion, n					
0	3	2	3	2	10
1	0	1	0	0	1
2/2+	0	0	1	3	4

[a]Data are presented as mean ± standard deviation. ANGPTL2, angiopoietin-like protein 2; MP, tunica muscularis propria; SE/SS, serosa-exposed and subserosal.

Next, the optimal cutoff using by Youden's index method for the *ANGPTL2* mRNA level for assessing the presence of vascular invasion in gastric cancer. The optimal cutoff was determined to be a relative expression level of 1.50. All patients with vascular invasion had a level ≥1.50, while only 20% lacking vascular invasion had a level at or above this cutoff. Thus, the cutoff of 1.50 produces a high true-positive rate and low false-positive rate.

The present study demonstrated that *ANGPTL2* mRNA in FFPE tissues is a potential biomarker for informing the pathological diagnosis of gastric cancer. Since numerous medical institutions retain FFPE tissues, this discovery may lead to a widely usable diagnostic procedure for more accurately assessing gastric cancer compared with the conventional pathological diagnosis. The present study also showed that *ANGPTL2* mRNA may be predictive of vascular invasion, which is an indicator of metastasis in gastric cancer. An

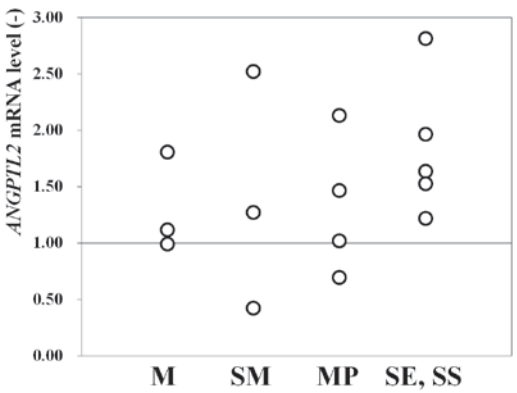

Figure 1. *ANGPTL2* mRNA levels in cancerous areas in formalin-fixed paraffin-embedded tissues of patients with M, SM, MP and SE/SS cancer. An expression level of 1.0 indicates the reference level determined from noncancerous tissue. M, mucosal; SM, submucosal; MP, tunica muscularis propria; SE/SS, serosa-exposed/subserosal; ANGPTL2, angiopoietin-like protein.

Table IV. Correlations between angiopoietin-like protein 2 mRNA concentration and various parameters.

Parameter	Correlation coefficient	P-value
Age, years	-0.31	0.26[a]
Body mass index, kg/m^2	0.09	0.75[a]
CEA level	0.04	0.90[a]
CA19-9 level	0.03	0.91[a]
Degree of differentiation	0.21	0.46[b]
Tumor invasion depth	0.35	0.21[b]
Lymph node metastasis	0.31	0.26[b]
Distant metastasis	0.00	1.00[b]
Stage	0.33	0.23[b]
Lympho-vascular invasion	0.07	0.80[b]
Vascular invasion	0.66	0.01[b]

[a]By Pearson's correlation; [b]by Spearman's rank correlation. CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

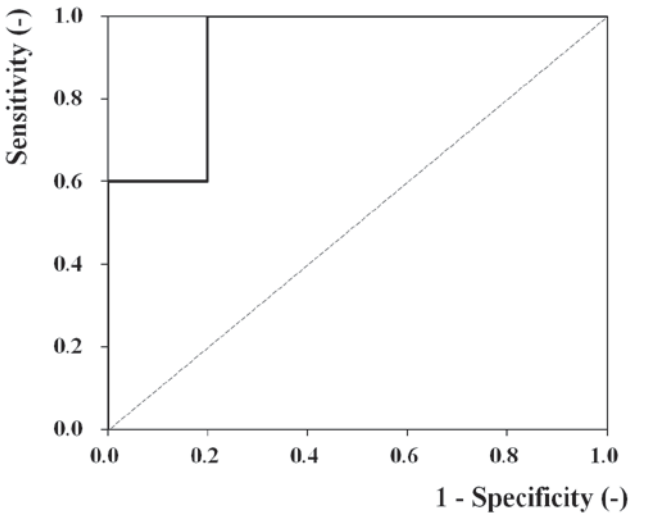

Figure 3. Receiver operating characteristic curve of the angiopoietin-like protein mRNA level and vascular invasion in patients with gastric cancer (n=15).

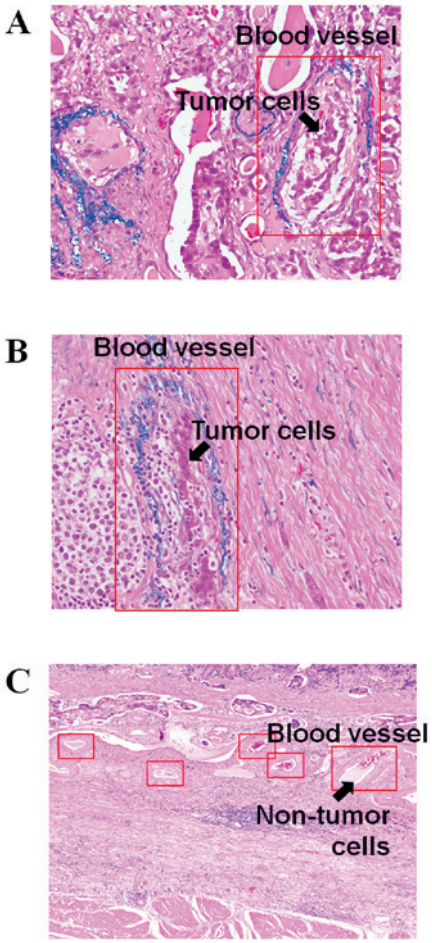

Figure 2. (A and B) Micrographs of cancerous areas, stained with Victoria blue and H&E in patients with a high *ANGPTL2* mRNA level and a high degree of vascular invasion: (A) patient no. 4 (mRNA level, 2.52) and (B) patient no. 14 (mRNA level, 2.81). The arrow indicates tumor cells in the blood vessels. (C) Micrographs of cancerous areas, stained with H&E in a patient with a low *ANGPTL2* mRNA level and no vascular invasion (patient no. 5; mRNA level, 0.42). The arrow indicates no tumor cells in the blood vessels. ANGPTL2, angiopoietin-like protein; H&E, hematoxylin and eosin. The samples were examined at x200 magnification.

increase in the number of samples of cancer metastasis is necessary to clarify whether the *ANGPTL2* mRNA level is actually correlated with metastasis.

The present study focused on *ANGPTL2*, and comparisons with other tumor markers were not performed. Previous studies have reported microRNAs, human epidermal growth factor receptor 2 and proteomic profiling in FFPE as gastric cancer biomarkers (24-26). Our future studies will measure and compare other biomarkers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MY, TT, HN and TY conceived and designed the experiments. Data collection and experiments were performed by ST, EH, AT and ET. TY analyzed the data and all authors contributed to the writing of the manuscript.

Ethics approval and consent to participate

The study design was approved by the Ethics Committee of Nanpuh Hospital (Kagoshima Kyosaikai, Public Interest Inc. Association, Japan). Written informed consent was obtained from all patients.

Patient consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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