

学位論文

**「Comparison of depth-specific gene expression levels  
in primary tumors in advanced gastric cancer」**

(進行胃癌における原発巣内での遺伝子発現の比較)

DM10026 成毛 哲

北里大学大学院医療系研究科医学専攻博士課程

内科学群 消化器内科学

指導教授 小泉 和三郎

## 著者の宣言

本学位論文は、著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

## 要旨

### 背景

多くの悪性腫瘍内で細胞異質性が存在することは広く認められ、腫瘍が進展していく過程で、分化型癌から未分化型癌に組織型が変化する“脱分化”という現象を胃癌の中にも認めることがあるが、脱分化した腫瘍細胞は遺伝子にも広範囲に変化をきたしていることが明らかになってきている。しかしながら、同一腫瘍内の組織型の変化があるにもかかわらず、腫瘍内の部位間での遺伝子発現の変化についてはいまだに検討が少ない。

また、本邦では、Stagell-III (T1 症例や T3 (SS) N0 症例を除く) 進行胃癌症例の標準的治療は胃切除+D2 リンパ節郭清後、術語補助化学療法として、5-Fluorouracil (FU) 系の経口抗癌剤である S-1 (大鵬薬品, 東京, 日本) の内服加療となっている。昨今では、腫瘍内でのフルオロピリミジン代謝関連遺伝子発現レベルが胃癌患者の臨床効果予測因子となる可能性が示唆され、さらに上皮増殖因子受容体 (EGFR), 血管内皮細胞増殖因子 (VEGF), 低酸素誘導因子-1 $\alpha$  (HIF1 $\alpha$ ) の腫瘍内遺伝子発現も胃癌患者の臨床効果予測因子となりうることが示唆されている。そこで、本研究では、フルオロピリミジン代謝関連遺伝子として、チミジレートシンターゼ (TS), チミジンホスホリラーゼ (TP), デヒドロピリミジンデヒドロゲナーゼ (DPD)、血管新生因子として、上皮増殖因子受容体 (EGFR), 血管内皮細胞増殖因子 (VEGF), 低酸素誘導因子-1 $\alpha$  (HIF1 $\alpha$ )、以上の 6 種類を標的遺伝子として選択した。

本研究は、前治療のないステージ II-III の進行胃癌の手術検体を用い、原発巣内における部位毎の遺伝子発現の差異を明らかにすることを目的に行われた。

### 方法

症例は、2001 年 1 月から 2004 年 3 月までに北里大学東病院にて診断加療された連続的な 29 例で、全例が ACTS-GC trial に登録されており、さらに全例から文書による検体組織の実験的使用の同意書は得られている。全例が病理学的に Stagell-III (T1 症例は除く) の進行胃癌症例であり、R0 の胃切除+D2 リンパ節郭清を施行されている。また、臨床評価項目は胃癌取り扱い規約第 13 版 (日本胃癌学会) に基づいて評価された。

ホルマリン固定パラフィン切片の手術検体から、レーザーキャプチャーマイクロダイセクションを用いて以下の 3 箇所より組織を採取した: ①正常粘膜部、②原発巣表層部 (原発巣の粘膜層)、③原発巣先進部 (原発巣筋層以深で腫瘍の最も先進した部分)。採取した組織から mRNA を抽出し、cDNA を作成。gDNA などのコンタミネーションを除去後、標的遺伝子を増幅した試料から、定量的リアルタイム PCR にて遺伝子発現を測定した。

### 結果

症例数は 29 例。男性 18 例、女性 11 例であった。平均年齢は 60.0 歳。原発巣表層部の病理組織型は分化型腺癌が 9 例、未分化型腺癌が 20 例であった。肉眼型では、IIc 類似進行胃癌が 2 例、type1 が 1 例、type2 が 6 例、type3 が 12 例、type4 が 2 例、type5 が 6 例であった。T 因子に関しては、T2 症例が 9 例、T3 症例が 19 例、T4 症例が 1 例であった。N 因子では、N0 症例が 4 例、N1 症例が 16 例、N2 症例が 9 例であった。Stage は、Stagell が 11 例、StageIIA が 12 例、StageIIIB



が6例であった。

同一原発巣内で組織型の変化があったものは4例、全て原発巣表層部は分化型腺癌であり、腫瘍が先進するにつれ未分化型腺癌に変化していた。これらは原発巣表層部が分化型癌であった症例の44.4%であった(4/9例)。リンパ節転移巣で原発巣表層部の組織型が変化していたのは4例で、全て原発巣表層部は分化型腺癌であったものが、リンパ節転移巣では未分化型腺癌に変化していた。これは原発巣表層部が分化型癌であった症例の44.4%であった(4/9例)。

標的遺伝子に関しては、原発巣先進部の TP, EGFR, HIF1 $\alpha$  の発現は、原発巣表層部と比べ、有意に高くなっていた (TP:  $p=0.041$ , EGFR:  $p=0.043$ , HIF1 $\alpha$ :  $p=0.005$ )。また、全ての標的遺伝子において、遺伝子発現は採取部位が深くなるほど高発現し、これらに正の相関を認めた (TS:  $r=0.309$ ;  $p=0.007$ , TP:  $r=0.464$ ;  $p<0.001$ , DPD:  $r=0.313$ ;  $p=0.007$ , EGFR:  $r=0.306$ ;  $p=0.008$ , VEGF:  $r=0.316$ ;  $p=0.006$ , HIF1 $\alpha$ :  $r=0.426$ ;  $p<0.001$ )。また、今回の検討では、脱分化の有無と遺伝子発現の差異との検討も行ったが、統計学的有意差は認められなかった。

## 結論

胃癌において、腫瘍の進展やリンパ節転移に伴って、分化型癌が未分化型癌に変化する脱分化を認めることがある。我々の研究でも、原発巣表層部が分化型癌であったものが腫瘍の先進部では未分化型癌に変化する症例や原発巣表層部が分化型癌であったものがリンパ節転移巣で未分化型癌に変化した症例が認められた。今回、脱分化と遺伝子発現の関係は明らかにできなかったが、遺伝子の変化は脱分化のような組織型の変化に先立つものである。

さらに、本研究では、腫瘍内の TP, EGFR, HIF1 $\alpha$  の遺伝子発現は原発巣表層部よりも原発巣先進部において有意に高くなっており、標的遺伝子全てにおいて、遺伝子発現は採取部位が深くなるほど高発現する、正の相関があることを明らかにできた。

TP は低酸素で誘導されるアポトーシスに対し細胞を耐性にし、腫瘍浸潤や血管新生、転移能獲得にも関与していると考えられており、また、HIF1 $\alpha$  は、低酸素状態になると高発現し、血管新生因子を含む様々な増殖因子の転写を誘導し、血管新生や腫瘍浸潤、転移にも関与しているといわれている。TP と HIF1 $\alpha$  が同時に上昇しているのは、近年明らかになりつつある血管新生のパスウェイに矛盾しない。

TP は腫瘍先進部で高発現し、病理学的検討でも TP や HIF1 $\alpha$  は分化型胃癌と比べ、低分化型胃癌でより高発現するとの報告もある。

今回の実験結果を説明できるようなパスウェイの存在は証明されておらず、遺伝子発現の変化と血管新生などとの関連性は更なる研究が必要であるが、腫瘍細胞内のいくつかの遺伝子はその位置する部位により発現が変化し、その発現は深度が深くなるほど高くなることが示された。これは、腫瘍細胞が先進するにつれて、悪性度などの活動性が増加する可能性を示唆しているのかもしれない。

# 目次

	頁
1. Introduction	1
2. Materials and Methods	
2-1. Clinical methods	3
2-2. Laboratory methods	
2-2-1. LCM of primary tumors	4
2-2-2. RNA extraction and cDNA synthesis	5
2-2-3. Multiplex preamplification of cDNA targets	5
2-2-4. Real-time PCRs	6
2-2-5. Quantification of target gene mRNA levels	6
2-2-6. Statistical analysis	7
3. Results	
3-1. Clinicopathological characteristics	8
3-2. Target gene expression levels	9
4. Discussion	11
5. Reference	14
6. Figure Legends	
6-1. Figure 1 Sites of specimen collection	18
6-2. Figure 2 A typical case with the histologic change among the sites in tumor (H&E staining)	18
6-3. Figure 3 Box and whisker plots of the target genes and correlations among expression levels	18
7. Tables	
7-1. Table 1 Primer sequences and probe sequences	20
7-2. Table 2 Characteristics of the patients	21
7-3. Table 3 Histological heterogeneity	22
7-4. Table 4 Target gene expression	23
8. Figures	
8-1. Figure 1 Sites of specimen collection	24
8-2. Figure 2 A typical case with the histologic change among the sites in tumor (H&E staining)	25
8-3. Figure 3 Box and whisker plots of the target genes and correlations among expression levels	26

## 1. Introduction

Gastric cancer remains one of the most common malignancies worldwide, as well as one of the leading causes of cancer-related death <sup>1</sup>.

Clinically, we often encounter tumors showing different histologic types in intramucosal and submucosal sections of the same primary tumors in patients with gastric cancer. The fact that some differentiated gastric cancers become undifferentiated cancers during the course of development has received considerable attention <sup>2,3</sup>.

It is widely accepted that many malignant tumors contain heterogeneous subpopulations of cells. This heterogeneity is associated with a wide range of genetic, biochemical, and immunologic characteristics. Previous studies have suggested that specific tumor cells within larger heterogeneous tumor specimens are the forerunners of distant metastases <sup>4</sup>. Furthermore, interactions of tumor cells with their environment may accentuate differences among tumor cells <sup>5</sup>. Despite the presence of different histologic types of tumor cells within the same lesion, few studies have examined whether gene expression levels differ among sites in the same tumor.

In Japan, the standard treatment for advanced gastric cancer of Stage II-III (except pT1 and pT3 (SS) pN0) is S-1 (Taiho Pharmaceutical Company, Tokyo, Japan) as adjuvant chemotherapy after the standard gastrectomy with D2 lymph node dissection<sup>6</sup>.



## Depth-specific gene expression levels

5-FU is the 5-fluorouracil (FU) (fluoropyrimidine) oral anticancer drug. It is suggested that the expression levels of several fluoropyrimidine metabolism related genes can become the accurate predict marker of the clinical outcome in patients received 5-FU (fluoropyrimidine) based chemotherapy<sup>7</sup>. Furthermore, in late years it was revealed that EGFR, VEGF and HIF1 $\alpha$  were concerned with tumor progression, tumor related angiogenesis and metastasis, and it is suggested that these can become the predictive marker of the clinical outcome of the gastric cancer<sup>8-10</sup>. Therefore we chose thymidylate synthase (TS), thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and hypoxia-inducible factor 1 $\alpha$ (HIF1 $\alpha$ ) as target genes for this study.

This study was performed for the purpose of clarifying a difference of the gene expression levels in advanced gastric cancer among sites in especially the primary tumor, using formalin-fixed, paraffin-embedded specimens surgically resected from patients with previously untreated stage II-III advanced gastric cancer.

## **2. Materials and Methods**

### **2-1. Clinical methods**

This retrospective study was approved by the Institutional Review Boards of Kitasato University and was performed in accordance with the Declaration of Helsinki as amended in Somerset West.

The study group comprised consecutive 29 patients with stage II or III advanced gastric cancer who were enrolled in the Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer (ACTS-GC)<sup>11</sup>, a randomized controlled study. All 29 patients were enrolled in ACTS-GC trial. Written informed consent for the use of tissue specimens was obtained from all patients in Kitasato University East Hospital. The patients underwent surgical resection of their primary tumors (gastrectomy) in the Department of Surgery, Kitasato University East Hospital from January 2001 through March 2004. All patients had a histopathological diagnosis of stage II (excluding T1 tumors), IIIA, or IIIB gastric cancer; no evidence of residual tumor (R0), including specimens obtained by D2 lymph node dissection; and no evidence of hepatic metastasis or peritoneal dissemination, with negative results of cytologic analysis of peritoneal washings. No patient had received preoperative chemotherapy. All conditions were in accordance with the eligibility criteria for the ACTS-GC trial.



## Depth-specific gene expression levels

The following clinicopathological characteristics were recorded according to the Japanese Classification of Gastric Carcinoma<sup>12</sup>: age, sex, histologic type, depth of tumor invasion (T), extent of lymph node metastasis (N), and disease stage.

### **2-2. Laboratory methods**

#### **2-2-1. LCM of primary tumors**

A representative formalin-fixed, paraffin-embedded specimen of the primary tumor surgically resected before adjuvant chemotherapy were selected by examining slides stained with hematoxylin and eosin. Tissue sections (thickness, 10 µm) were stained with nuclear fast red to enable visualization of histological features for LCM (Arcturus XT microdissection instrument, Sunnyvale, California, USA) and to ensure that only tumor cells were studied.

Specimens were obtained by LCM from the following 3 regions (Figure 1): (1) the normal mucosa (normal mucosa), (2) the surface layer of the primary tumor (surface sections), obtained from the mucosa, and (3) the deepest layer of the primary tumor (deep sections), obtained from the most invasive section of the muscularis propria, the subserosa or the serosa. Specimens were carefully and selectively obtained from the designated sites, avoiding contamination with materials other than tumor cells.

### **2-2-2. RNA extraction and cDNA synthesis**

Total RNA was isolated from tissue specimens obtained by LCM, using a NucleoSpin<sup>®</sup> FFPE RNA/DNA kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Subsequently, a TURBO DNA-free<sup>™</sup> kit (Life Technologies Corporation, Inc., Carlsbad, California, USA) was used according to the instruction manual to completely remove all gDNA contamination. cDNA was synthesized with random primers and reverse transcriptase with the use of a High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Inc.), following the manufacturer's instructions.

### **2-2-3. Multiplex preamplification of cDNA targets**

To increase the sensitivity for quantification of relative gene expression levels, a multiplex PCR preamplification of the 6 target gene cDNAs and  $\beta$ -actin cDNA was performed using a TaqMan PreAmp Master Mix Kit (Life Technologies Corporation, Inc.), following the manufacturer's instructions. The pooled assay mix was 7 primers together in a final concentration of 0.2  $\mu$ M. Subsequently, 12.5  $\mu$ l of the pooled assay mix (0.2  $\mu$ M) was combined with each cDNA sample and 25  $\mu$ l of TaqMan PreAmp

## Depth-specific gene expression levels

Master Mix (2×) in a final volume of 50 µl. Thermal cycling conditions were as follows: initial hold at 95°C for 10 min and 10 preamplification cycles of 15 sec at 95°C and 4 min at 60°C. Primer sequences for the following target gene cDNAs and β-actin cDNA were shown in Table 1.

### **2-2-4. Real-time PCRs**

Real-time PCRs were carried out using LightCycler Software 2.0 (Roche Diagnostics, Indianapolis, Indiana, USA). Probe and primer sequences for the following target gene cDNAs and β-actin cDNA were shown in Table 1. PCR was carried out in a final volume of 20 µl with LightCycler® TaqMan® Master Mix (Roche Diagnostics), using 5.0 µl of preamplified cDNA, 10 µmol/l of each primer, and 10 µmol/l of probe for each target gene cDNA. Cycling conditions were 95°C for 10 min followed by 45 cycles at 95°C for 10 sec, 60°C for 20 sec, and 72°C for 1 sec.

### **2-2-5. Quantification of target gene mRNA levels**

Relative gene expression levels were determined by the standard curve method. Standard curves for target gene cDNAs and β-actin cDNA were generated using a five-fold serially diluted solution of preamplified cDNA from Stratagene QPCR Human

## Depth-specific gene expression levels

Reference Total RNA (#750500, Stratagene Co., Orange County, California, USA).

The target gene expression level was calculated from the standard curve, and quantitative normalization of cDNA in each sample was performed using the expression of the  $\beta$ -actin gene as an internal control. Finally, target gene cDNA levels were expressed as ratios relative to the  $\beta$ -actin cDNA level.

### **2-2-6. Statistical analysis**

All statistical analyses were carried out using SPSS, version 17.0 (SPSS Japan Inc., Tokyo, Japan). The Mann-Whitney test was used to evaluate the expression level of each target gene in each section studied. Spearman's rank correlation coefficient was used to evaluate correlations between target gene and site. P values of less than 0.05 were considered to indicate statistical significance.



### **3. Results**

#### **3-1. Clinicopathological characteristics**

The study group comprised 29 patients (18 male and 11 female) with a mean age of 60.0 years. The histologic type of surface sections was differentiated adenocarcinoma in 9 patients and undifferentiated adenocarcinoma in 20. The macroscopic type of the primary tumors was type IIc-like advanced type in 2 patients, type 1 in 1, type 2 in 6, type 3 in 12, type 4 in 2, and type 5 in 6. The depth of wall invasion by the primary tumor (T) was T2 in 9 patients, T3 in 19, and T4 in 1. Lymph node metastasis (N) was N0 in 4 patients, N1 in 16, and N2 in 9. Disease stage was stage II in 11 patients, stage IIIA in 12, and stage IIIB in 6. The patients' characteristics are summarized in Table 2.

In 4 patients, the histologic type within the primary tumor changed depending on depth. In all 4 patients, the surface layer of the primary tumor was differentiated adenocarcinoma, which changed to undifferentiated adenocarcinoma as the depth of the tumor progression (Table 3). The histologic type of the surface layer of the primary tumor differed from that of synchronous lymph node metastases in 4 patients. In all 4 patients, the histologic type of the surface layer of the primary tumor was differentiated adenocarcinoma, whereas that of the lymph node metastases had become undifferentiated adenocarcinoma (Table 3). We showed a typical case with the

## Depth-specific gene expression levels

histologic change among the sites in tumor in Figure 2A-C.

### 3-2. Target gene expression levels

Expression levels (relative cDNA levels) of the target genes were presented as ratios of the gene of interest to the internal reference gene ( $\beta$ -actin), which provided a normalization factor for the amount of cDNA. Table 4 showed the expression levels of each target gene. When compare tumor tissues with normal mucosa, gene expression levels of TS, TP and VEGF in surface sections were significantly higher than those in normal mucosa, and all target gene expression levels in deep section were significantly higher than those in normal mucosa.

Each target gene expression levels among different sites of primary tumors were shown in Figure 3A-F.

TS, DPD and VEGF gene expression levels had no significantly difference among dissected sites in tumor (Figure 3A, C, E).

In the primary tumor, TP, EGFR, and HIF1 $\alpha$  gene expression levels in deep sections were significantly higher than those in surface sections ( $p=0.041$ ,  $p=0.043$ ,  $p=0.005$ ) (Figure 3B, D, F).

All six target genes showed positive correlations between gene expression levels and

## Depth-specific gene expression levels

dissected sections (TS:  $r=0.309$ ;  $p=0.007$ , TP:  $r=0.464$ ;  $p<0.001$ , DPD:  $r=0.313$ ;  $p=0.007$ , EGFR:  $r=0.306$ ;  $p=0.008$ , VEGF:  $r=0.316$ ;  $p=0.006$ , HIF1 $\alpha$ :  $r=0.426$ ;  $p<0.001$ ) (Figure 3A-F).

#### 4. Discussion

In gastric cancer, tumor progression and lymph node metastasis are sometimes associated with the dedifferentiation of differentiated adenocarcinoma to undifferentiated adenocarcinoma<sup>2, 3, 13</sup>. In our study, in 4 patients, the surface layer of the primary tumor was differentiated adenocarcinoma, which changed to undifferentiated adenocarcinoma as the depth of the tumor progression. This phenomenon was found in 44.4% of patients in whom the histologic type of the surface layer was differentiated adenocarcinoma. In another 4 patients, the histologic type of the surface layer of the primary tumor was differentiated adenocarcinoma, whereas that of the lymph node metastases had become undifferentiated adenocarcinoma. This phenomenon was found in 44.4% of patients in which the histologic type of the surface sections was differentiated adenocarcinoma.

Submucosal transformation to poorly differentiated adenocarcinoma is also thought to increase malignant potential and promote lymphatic vessel invasion and lymph node metastasis<sup>14</sup>. Genetic changes most likely precede histopathological changes.

Our study showed that expression levels of the TP, EGFR, and HIF1 $\alpha$  genes were higher in the deep section than in the surface section of the primary tumor, and gene expression levels and dissected sections had positive correlations in all target genes,



## Depth-specific gene expression levels

gene expression levels became higher as tumor progression. Concentration ratio of EGFR and HIF1 $\alpha$  were low, but these gene expression levels in deep section became higher to approximately threefold of those in surface section.

The finding that the TP and HIF1 $\alpha$  genes were simultaneously expressed at high levels is consistent with angiogenesis pathways proposed by recent studies<sup>15</sup>. However, many aspects of the relations among different genes remain unclear, further elucidation must also await the results of future studies.

TP has been shown to convey resistance to hypoxia-induced apoptosis<sup>16</sup> and may also participate in tumor invasion and metastasis in gastric cancer<sup>17-19</sup>.

The HIF1 $\alpha$  gene is expressed at high levels under hypoxic conditions and finally binds to hypoxia responsive elements (HREs), its target gene, thereby inducing transcription of various growth factors, such as VEGF and Glucose transporter 1 (GLUT1), which is involved in active transport of glucose<sup>20-22</sup>. In gastric cancer, HIF1 $\alpha$  also participates in angiogenesis, tumor invasion, and metastasis<sup>23</sup>. However, recently, The HIF1 $\alpha$  gene expression may receive the control at the transcription level by some kind of other factors as well as hypoxia<sup>24</sup>.

TP expression is high at the invasive edge of tumors<sup>25</sup>. Histopathologically, TP expression is higher in undifferentiated cancer than in differentiated cancer<sup>26</sup>. HIF1 $\alpha$

## Depth-specific gene expression levels

expression is also increased in undifferentiated cancer<sup>27</sup>.

These findings together with our results suggest that tumor cells undergo genetic changes to adapt to their environment either before or simultaneously with dedifferentiation-induced structural changes in response to various conditions including hypoxia.

The existence of pathway which can explain these laboratory finding is not proved, and it is thought that a further study was necessary to clarify the association between angiogenesis and the change of gene expression level at present, however, our study demonstrated that some genes in tumor cells could cause changes in their expression level even within the same tumor in response to environmental factors. As tumor invades, this may suggest the possibility that malignant activity increases. This is a pilot study, we should increase cases and collection parts. We think that it is necessary to examine further study in future.

## 5. References

1. Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. *CA Cancer J Clin.* 2005;55(2):74-108.
2. Endoh Y, Tamura G, Watanabe H, et al. The common 18-base pair deletion at codons 418-423 of the E-cadherin gene in differentiated-type adenocarcinomas and intramucosal precancerous lesions of the stomach with the features of gastric foveolar epithelium. *J Pathol.* 1999;189(2):201-6.
3. Saito A, Shimoda T, Nakanishi Y, et al. Histologic heterogeneity and mucin phenotypic expression in early gastric cancer. *Pathol Int.* 2001;51(3):165-71.
4. Portera CA, Jr., Berman RS and Ellis LM. Molecular determinants of colon cancer metastasis. *Surg Oncol.* 1998;7(3-4):183-95.
5. Fidler IJ. Critical determinants of metastasis. *Semin Cancer Biol.* 2002;12(2):89-96.
6. Japanese gastric cancer treatment guidelines 2010 (ver. 3). *Gastric Cancer.* 2011;14(2):113-23.
7. Ichikawa W. Prediction of clinical outcome of fluoropyrimidine-based chemotherapy for gastric cancer patients, in terms of the 5-fluorouracil metabolic pathway. *Gastric Cancer.* 2006;9(3):145-55.
8. Lieto E, Ferraraccio F, Orditura M, et al. Expression of vascular endothelial growth

factor (VEGF) and epidermal growth factor receptor (EGFR) is an independent prognostic indicator of worse outcome in gastric cancer patients. *Ann Surg Oncol.* 2008;15(1):69-79.

9. Wang X, Cao W, Mo M, et al. VEGF and cortactin expression are independent predictors of tumor recurrence following curative resection of gastric cancer. *J Surg Oncol.* 2010;102(4):325-30.
10. Nakamura J, Kitajima Y, Kai K, et al. Hypoxia-inducible factor-1alpha expression predicts the response to 5-fluorouracil-based adjuvant chemotherapy in advanced gastric cancer. *Oncol Rep.* 2009;22(4):693-9.
11. Sakuramoto S, Sasako M, Yamaguchi T, et al. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med.* 2007;357(18):1810-20.
12. Japanese Gastric Cancer A. Japanese Classification of Gastric Carcinoma - 2nd English Edition. *Gastric Cancer.* 1998;1(1):10-24.
13. Nakamura T, Yao T, Kabashima A, et al. Loss of phenotypic expression is related to tumour progression in early gastric differentiated adenocarcinoma. *Histopathology.* 2005;47(4):357-67.
14. Mita T and Shimoda T. Risk factors for lymph node metastasis of submucosal invasive differentiated type gastric carcinoma: clinical significance of histological



heterogeneity. *J Gastroenterol.* 2001;36(10):661-8.

15. Fox SB, Gasparini G and Harris AL. Angiogenesis: pathological, prognostic, and growth-factor pathways and their link to trial design and anticancer drugs. *Lancet Oncol.* 2001;2(5):278-89.
16. Kitazono M, Takebayashi Y, Ishitsuka K, et al. Prevention of hypoxia-induced apoptosis by the angiogenic factor thymidine phosphorylase. *Biochem Biophys Res Commun.* 1998;253(3):797-803.
17. Yu EJ, Lee Y, Rha SY, et al. Angiogenic factor thymidine phosphorylase increases cancer cell invasion activity in patients with gastric adenocarcinoma. *Mol Cancer Res.* 2008;6(10):1554-66.
18. Maeda K, Kang SM, Ogawa M, et al. Combined analysis of vascular endothelial growth factor and platelet-derived endothelial cell growth factor expression in gastric carcinoma. *Int J Cancer.* 1997;74(5):545-50.
19. Takebayashi Y, Miyadera K, Akiyama S, et al. Expression of thymidine phosphorylase in human gastric carcinoma. *Jpn J Cancer Res.* 1996;87(3):288-95.
20. Semenza GL. Regulation of physiological responses to continuous and intermittent hypoxia by hypoxia-inducible factor 1. *Exp Physiol.* 2006;91(5):803-6.
21. Semenza GL. Regulation of tissue perfusion in mammals by hypoxia-inducible

- factor 1. *Exp Physiol*. 2007;92(6):988-91.
22. Ikeda E. Cellular response to tissue hypoxia and its involvement in disease progression. *Pathol Int*. 2005;55(10):603-10.
23. Isobe T, Aoyagi K, Koufuji K, et al. Clinicopathological significance of hypoxia-inducible factor-1 alpha (HIF-1alpha) expression in gastric cancer. *Int J Clin Oncol*. 2012.
24. Yoshida T, Hashimura M, Mastumoto T, et al. Transcriptional upregulation of HIF-1alpha by NF-kappaB/p65 and its associations with beta-catenin/p300 complexes in endometrial carcinoma cells. *Lab Invest*. 2013.
25. Shimaoka S, Matsushita S, Nitanda T, et al. The role of thymidine phosphorylase expression in the invasiveness of gastric carcinoma. *Cancer*. 2000;88(10):2220-7.
26. Kimura H, Konishi K, Kaji M, et al. Correlation between expression levels of thymidine phosphorylase (dThdPase) and clinical features in human gastric carcinoma. *Hepatogastroenterology*. 2002;49(45):882-6.
27. Qiu MZ, Han B, Luo HY, et al. Expressions of hypoxia-inducible factor-1alpha and hexokinase-II in gastric adenocarcinoma: the impact on prognosis and correlation to clinicopathologic features. *Tumour Biol*. 2011;32(1):159-66.

## **6. Figure Legends**

### **6-1. Figure 1 Sites of specimen collection**

Formalin-fixed, paraffin-embedded tumor tissues were dissected from (1) the normal mucosa (normal mucosa), (2) the surface layer of the primary tumor (surface sections), obtained from the mucosa, and (3) the deepest layer of the primary tumor (deep sections), obtained from the most invasive section of the muscularis propria, the subserosa or the serosa by the laser captured microdissection technique.

### **6-2. Figure 2 A typical case with the histologic change among the sites in tumor (H&E staining)**

A) Microscopic perspective of the primary tumor, B) The histologic type of the surface layer of the primary tumor was differentiated adenocarcinoma, C) The histologic type of the deepest layer of the primary tumor was undifferentiated adenocarcinoma

A)  $\times 1$ ; B), C)  $\times 200$

### **6-3. Figure 3 Box and whisker plots of the target genes and correlations among expression levels**

A) TS, thymidylate synthase; There was no significantly difference between the surface

## Depth-specific gene expression levels

section and the deep section in primary tumor. There was positive correlation between TS gene expression levels and dissected sections ( $r = 0.309$ ,  $p = 0.007$ ). B) TP, thymidine phosphorylase; TP gene expression level in deep section was significantly higher than in surface section (\*1:  $p = 0.041$ ). There was positive correlation between TP gene expression levels and dissected sections ( $r = 0.464$ ,  $p < 0.001$ ). C) DPD, dihydropyrimidine dehydrogenase; There was no significantly difference between the surface section and the deep section in primary tumor. There was positive correlation between DPD gene expression levels and dissected sections ( $r = 0.313$ ,  $p = 0.007$ ). D) EGFR, epidermal growth factor receptor; EGFR gene expression level in deep section was significantly higher than in surface section (\*2:  $p = 0.043$ ). There was positive correlation between EGFR gene expression levels and dissected sections ( $r = 0.306$ ,  $p = 0.008$ ). E) VEGF, vascular endothelial growth factor; There was no significantly difference between the surface section and the deep section in primary tumor. There was positive correlation between VEGF gene expression levels and dissected sections ( $r = 0.316$ ,  $p = 0.006$ ). F) HIF1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; HIF1 $\alpha$  gene expression level in deep section was significantly higher than in surface section (\*3:  $p = 0.005$ ). There was positive correlation between HIF1 $\alpha$  gene expression levels and dissected sections ( $r = 0.426$ ,  $p < 0.001$ ).



## 7. Tables

### 7-1. Table1 Primer sequences and probe sequences

**Table 1** Primer sequences and probe sequences

Gene	Primer and probe	Sequence
$\beta$ -actin	Forward primer	5'-GAGCGCGGCTACAGCTT-3'
	Reverse primer	5'-TCCTTAATGTCACGCACGATTT-3'
	Probe	5'-(FAM)ACCACCACGGCCGAGCGG(TAM)-3'
TS	Forward primer	5'-GCCTCGGTGTGCCTTTCA-3'
	Reverse primer	5'-CCCGTGATGTGCGCAAT-3'
	Probe	5'-(FAM)TCGCCAGCTACGCCCTGCTCA(TAM)-3'
TP	Forward primer	5'-CCTGCGGACGGAATCCT-3'
	Reverse primer	5'-GCTGTGATGAGTGGCAGGCT-3'
	Probe	5'-(FAM)CAGCCAGAGATGTGACAGCCACCGT(TAM)-3'
DPD	Forward primer	5'-AGGACGCAAGGAGGGTTTG-3'
	Reverse primer	5'-GTCCGCCGAGTCCTTACTGA-3'
	Probe	5'-(FAM)CAGTGCCTACAGTCTCGAGTCTGCCAGTG(TAM)-3'
EGFR	Forward primer	5'-TGCGTCTCTTGCCGGAAT-3'
	Reverse primer	5'-GGCTCACCCTCCAGAAGCTT-3'
	Probe	5'-(FAM)ACGCATTCCCTGCCTCGGCTG(TAM)-3'
VEGF	Forward primer	5'-AGTGGTCCCAGGCTGCAC-3'
	Reverse primer	5'-TCCATGAACTTCACCACTTCGT-3'
	Probe	5'-(FAM)ATGGCAGAAGGAGGAGGGCAGAATCA(TAM)-3'
HIF1 $\alpha$	Forward primer	5'-CGCTGGAGACACAATCATATC-3'
	Reverse primer	5'-TCCTCAAGTTGCTGGTCATC-3'
	Probe	5'-(FAM)TTTGGCAGCAACGACACAGAACT(TAM)-3'

TS, thymidylate synthase; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; HIF1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ .

7-2. Table 2 Characteristics of the patients

**Table 2** Characteristics of the patients

Characteristic	(n = 29)
Age (years)	
mean $\pm$ SD (range)	60.0 $\pm$ 8.8 (41-75)
Sex	
Male	18
Female	11
Surface histologic type	
Differentiated	9
Undifferentiated	20
Macroscopic type	
Ilc-like advanced	2
Type 1	1
Type 2	6
Type 3	12
Type 4	2
Type 5	6
T	
2	9
3	19
4	1
N	
0	4
1	16
2	9
Stage	
II	11
IIIA	12
IIIB	6

T, depth of tumor invasion;  
N, extent of lymph node metastasis.

### 7-3. Table 3 Histological heterogeneity

**Table 3** Histologic heterogeneity

Surface histologic type: Differentiated adenocarcinoma	n=9
Histologic heterogeneity of primary tumor (%)	4 (44.4)
Histologic heterogeneity between surface section and lymph node metastasis (%)	4 (44.4)

Patient	Surface section	Middle section	Deep section	Lymph node metastasis
No.1	Diff.	Diff.	Undiff.	None
No.2	Diff.	Undiff.	Undiff.	None
No.3	Diff.	Diff.	Undiff.	Undiff.
No.4	Diff.	Diff.	Undiff.	Undiff.
No.5	Diff.	Diff.	Diff.	Undiff.
No.6	Diff.	Diff.	Diff.	Undiff.

Diff., Differentiated adenocarcinoma;

Undiff., Undifferentiated adenocarcinoma

## 7-4. Table 4 Target gene expression

**Table 4** Target gene expression

(mean  $\pm$  SD, (range))

	Normal mucosa	Surface section	Deep section
TS	0.20 $\pm$ 0.45 (0.00-1.54)	0.32 $\pm$ 0.35 (0.00-1.22)	0.45 $\pm$ 0.54 (0.00-1.71)
TP	0.73 $\pm$ 1.59 (0.00-4.72)	1.94 $\pm$ 2.11 (0.00-7.42)	5.37 $\pm$ 5.67 (0.00-23.34)
DPD	1.45 $\pm$ 2.56 (0.00-7.63)	2.17 $\pm$ 3.45 (0.00-13.44)	3.21 $\pm$ 3.41 (0.00-10.36)
EGFR	0.02 $\pm$ 0.05 (0.00-0.15)	0.02 $\pm$ 0.03 (0.00-0.11)	0.06 $\pm$ 0.14 (0.00-0.69)
VEGF	0.21 $\pm$ 0.38 (0.00-1.13)	0.49 $\pm$ 0.47 (0.00-1.66)	0.58 $\pm$ 0.59 (0.00-2.76)
HIF1 $\alpha$	0.04 $\pm$ 0.11 (0.00-0.38)	0.05 $\pm$ 0.13 (0.00-0.60)	0.14 $\pm$ 0.15 (0.00-0.59)

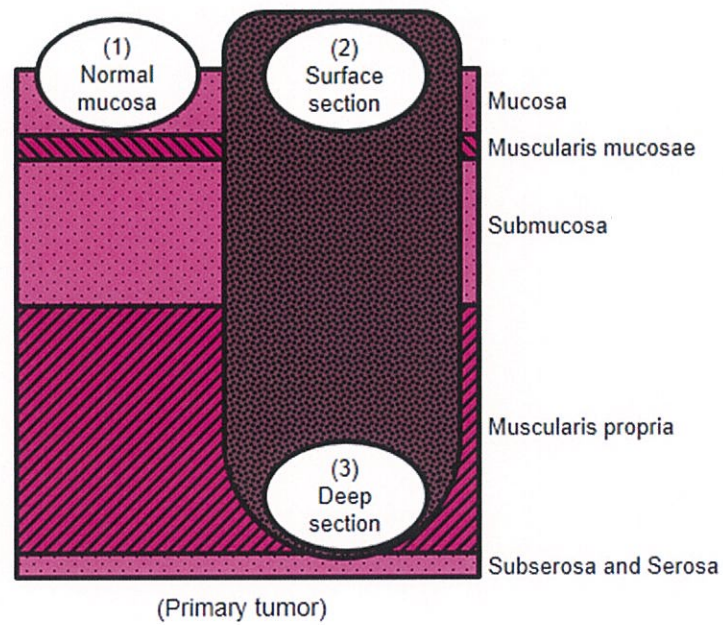
TS, thymidylate synthase; TP, thymidine phosphorylase;  
 DPD, dihydropyrimidine dehydrogenase;  
 EGFR, epidermal growth factor receptor;  
 VEGF, vascular endothelial growth factor;  
 HIF1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ .



## 8. Figures

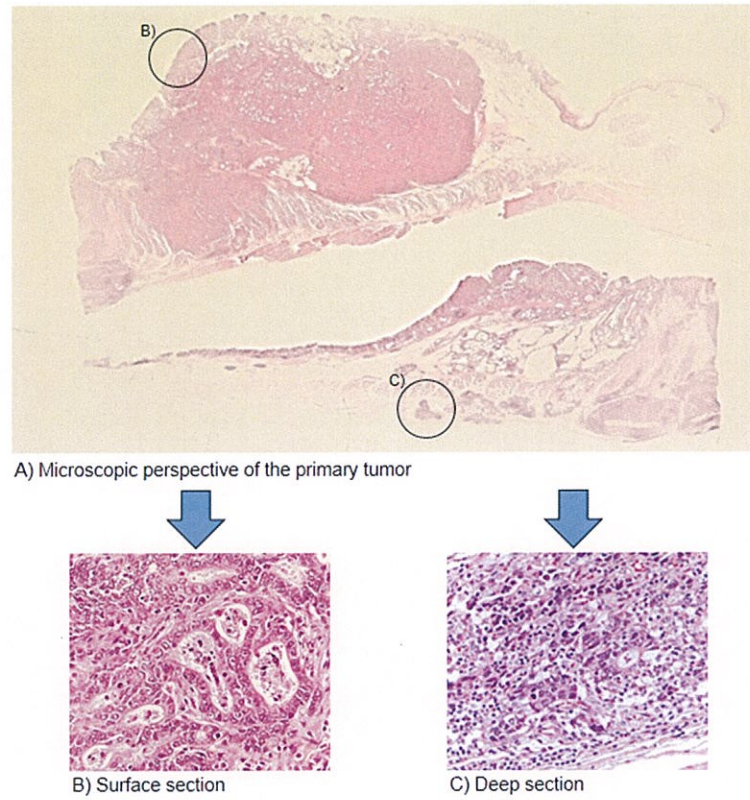
### 8-1. Fig. 1 Sites of specimen collection

Figure 1



**8-2. Fig. 2 A typical case with the histologic change among the sites in tumor**

**Figure 2**



8-3. Fig. 3 Box and whisker plots of the target genes and correlations among expression levels

