

Comparison of site-specific gene expression levels in primary tumors and synchronous lymph node metastases in advanced gastric cancer

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Received: 7 November 2013 / Accepted: 8 February 2014 / Published online: 21 March 2014
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Abstract

Background Many malignant tumors consist of heterogeneous subpopulations of cells. This heterogeneity is associated with genetic characteristics. However, it remains unclear whether gene expression levels differ among specific sites of tumors in gastric cancer.

Methods We studied differences in gene expression levels among specific sites of primary tumors and synchronous lymph node metastases, using formalin-fixed, paraffin-embedded specimens resected surgically from 48 patients with previously untreated advanced gastric cancer. Specimens were obtained by laser-captured microdissection from five regions: (1) nonneoplastic mucosa, (2) surface layer (mucosa) of the primary tumor (surface sections), (3) middle layer (submucosa) of the primary tumor (middle sections), (4) the deepest layer of the primary tumor (muscularis propria or deeper) at the site of deepest invasion (deep sections), and (5) level 1 synchronous lymph node metastasis (lymph node metastases). Expression levels of the following target genes were determined by quantitative real-time polymerase chain reaction: thymidylate synthase (TS), thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD), epidermal growth factor receptor (EGFR), vascular endothelial

growth factor (VEGF), and hypoxia-inducible factor-1 α (HIF1 α).

Results TP, DPD, EGFR, and HIF1 α gene expression levels were significantly higher in deep sections than in surface sections. TP, EGFR, VEGF, and HIF1 α gene expression levels were significantly higher in lymph node metastases than in surface sections. TP, DPD, EGFR, VEGF, and HIF1 α gene expression levels were positively correlated with the specific samples harvested from the tumors.

Conclusions Our results show that the expression levels of some genes in tumor cells can change in specific sites of tumors and can become higher in association with tumor progression.

Keywords Advanced gastric cancer · Site-specific gene expression level · Laser-captured microdissection

Introduction

Gastric cancer remains one of the most common malignancies worldwide, as well as one of the leading causes of cancer-related death [1].

Clinically, the histological types of intramucosal and submucosal sections of the same primary tumor often differ in patients with gastric cancer. The fact that some differentiated gastric cancers become undifferentiated cancers during the course of development has received considerable attention [2, 3].

It is widely accepted that many malignant tumors consist of heterogeneous subpopulations of cells. This heterogeneity is associated with a wide range of genetic, biochemical, and immunological characteristics. Previous studies have suggested that specific tumor cells within large heterogeneous tumor specimens are the forerunners of distant metastases [4].

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Many biological differences thus exist between tumor cells in primary lesions and those in metastatic lesions. Furthermore, interactions of tumor cells with their environment may accentuate differences among tumor cells [5]. Despite the presence of different histological types of tumor cells within the same lesion, few studies have examined whether gene expression levels differ among specific sites in the same tumor or between primary tumors and metastatic lesions.

This study was designed to clarify differences in gene expression levels among specific sites in the same tumor or between primary tumors and synchronous lymph node metastases in advanced gastric cancer. Formalin-fixed, paraffin-embedded specimens surgically resected from patients with previously untreated stage II or III advanced gastric cancer were studied.

Laser-captured microdissection (LCM) was used to harvest cells from specific sites. LCM is an innovative technique that offers researchers a simple, reliable, rapid, and accurate tool for precise, contamination-free procurement of cells from tissue sections under direct visualization [6, 7]. We quantified gene expression levels in tissue specimens obtained by LCM by real-time polymerase chain reaction (PCR), performed after preamplification.

In Japan, the standard treatment for stage II or III advanced gastric cancer [excluding pT1 and pT3 (penetrates serosa) pN0 disease] is S-1 (Taiho Pharmaceutical, Tokyo, Japan) as adjuvant chemotherapy after standard gastrectomy with D2 lymph node dissection [8]. S-1 is an oral formulation of 5-fluorouracil (5-FU, a fluoropyrimidine). It has been suggested that the expression levels of several genes related to fluoropyrimidine metabolism can be used to predict clinical outcomes in patients who receive 5-FU (fluoropyrimidine)-based chemotherapy [9]. Furthermore, recent studies have revealed that epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and hypoxia-inducible factor 1 α (HIF1 α) participate in tumor progression, tumor-related angiogenesis, and metastasis. Gene expression levels of these factors might thus be useful predictors of clinical outcomes in gastric cancer [10–12]. We therefore selected thymidylate synthase (TS), thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD), EGFR, VEGF, and HIF1 α as target genes in this study.

Materials and methods

Clinical methods

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

The study group comprised 48 consecutive patients with stage II or III advanced gastric cancer. Written informed consent for the use of tissue specimens was obtained from all subjects 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 December 2004. All patients had a histopathological diagnosis of stage IIA or IIB (excluding T1 tumors), IIIA, IIIB, or IIIC 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 cytological analysis of peritoneal washings. No patient had received preoperative chemotherapy.

The following clinicopathological characteristics were recorded according to the Japanese Classification of Gastric Carcinoma, issued by the Japanese Gastric Cancer Association [13]: age, sex, histological type, depth of tumor invasion (T), extent of lymph node metastasis (N), and disease stage.

Laboratory methods

LCM of primary tumors and lymph node metastases

Representative formalin-fixed, paraffin-embedded specimens of the primary tumor and synchronous lymph node metastasis surgically resected before adjuvant chemotherapy were selected by examining slides stained with hematoxylin and eosin. Tissue sections (thickness, 10 μ m) were stained with nuclear fast red to enable visualization of histological features for LCM (Arcturus XT microdissection instrument; Arcturus, Sunnyvale, CA, USA) and to ensure that only tumor cells were studied.

Specimens were obtained by LCM from the following five sites: (1) the nonneoplastic mucosa (adjacent normal sections); (2) the surface layer of the primary tumor (surface sections), obtained from the mucosa; (3) the middle layer of the primary tumor (middle sections), obtained from the middle of the submucosa; (4) the deepest layer of the primary tumor (deep sections), obtained from sections at the site of deepest invasion of the muscularis propria, subserosa, or serosa; and (5) synchronous lymph node metastasis (lymph node metastases), obtained from the level 1 lymph node metastases. Specimens were carefully and selectively obtained from the designated sites, avoiding contamination with materials other than tumor cells.

RNA extraction and cDNA synthesis

Total RNA was isolated from tissue specimens obtained by LCM, using a NucleoSpin FFPE RNA/DNA kit (Takara

Table 1 Primer sequences and probe sequences

Gene	Primer and probe	Sequence
β -actin	Forward primer	5'-GAGCGCGGCTACAGCTT-3'
	Reverse primer	5'-TCCTTAATGTACGCACGATTT-3'
	Probe	5'-(FAM)ACCACCACGCCGAGCGG(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'-AGGACGCAAGGAGGGTTG-3'
	Reverse primer	5'-GTCCGCCGAGTCCTTACTGA-3'
	Probe	5'-(FAM)CAGTGCCTACAGTCTCGAGTCTGCCAGTG(TAM)-3'
EGFR	Forward primer	5'-TGCGTCTCTTGCCGGAAT-3'
	Reverse primer	5'-GGCTCACCTCCAGAAGCTT-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 α	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 α hypoxia-inducible factor-1 α

Bio, Otsu, Japan) according to the manufacturer's instructions. Subsequently, a TURBO DNA-free kit (Life Technologies, Carlsbad, CA, 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), following the manufacturer's instructions.

Multiplex preamplification of cDNA targets

To increase the sensitivity for quantification of relative gene expression levels, a multiplex PCR preamplification of the six target gene cDNAs and of β -actin cDNA was performed using a TaqMan PreAmp Master Mix Kit (Life Technologies), following the manufacturer's instructions. The pooled assay mix contained seven primers together in a final concentration of 0.2 μ M. Subsequently, 12.5 μ l of the pooled assay mix (0.2 μ M) was combined with each cDNA sample and 25 μ l of TaqMan PreAmp Master Mix (2 \times) in a final volume of 50 μ l. Thermal cycling conditions were as follows: initial hold at 95 $^{\circ}$ C for 10 min and ten preamplification cycles of 15 s at 95 $^{\circ}$ C and 4 min at 60 $^{\circ}$ C. Primer sequences for the target gene cDNAs and β -actin cDNA are shown in Table 1.

Real-time PCR

Real-time PCR was carried out using LightCycler Software 2.0 (Roche Diagnostics, Indianapolis, IN, USA). Probe and primer sequences for the target gene cDNAs and β -actin cDNA are shown in Table 1. PCR was carried out in a final volume of 20 μ l with LightCycler TaqMan Master Mix (Roche Diagnostics), using 5.0 μ l preamplified cDNA, 10 μ mol/l each primer, and 10 μ mol/l probe for each target gene cDNA. Cycling conditions were 95 $^{\circ}$ C for 10 min followed by 45 cycles at 95 $^{\circ}$ C for 10 s, 60 $^{\circ}$ C for 20 s, and 72 $^{\circ}$ C for 1 s.

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 fivefold serially diluted solution of preamplified cDNA from Stratagene QPCR Human Reference Total RNA (#750500; Stratagene, Orange County, CA, 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 β -actin gene as an internal control. Finally, target gene cDNA

Table 2 Characteristics of the patients

Characteristics	<i>n</i> = 48
Age (years)	
Mean \pm SD (range)	60.9 \pm 8.9 (41–78)
Sex	
Male	33
Female	15
Histological type of surface section of primary tumor	
pap	0
tub1	7
tub2	9
por1	18
por2	7
sig	4
muc	3
Macroscopic type	
Type 1	2
Type 2	9
Type 3	19
Type 4	4
Type 5	14
T	
2	3
3	16
4a	28
4b	1
N	
0	8
1	14
2	12
3a	9
3b	5
Stage	
IIA	4
IIB	13
IIIA	12
IIIB	6
IIIC	13

pap papillary adenocarcinoma, *tub1* well-differentiated tubular adenocarcinoma, *tub2* moderately differentiated tubular adenocarcinoma, *por1* poorly differentiated adenocarcinoma solid type, *por2* poorly differentiated adenocarcinoma nonsolid type, *sig* signet-ring cell carcinoma; *muc*, mucinous adenocarcinoma, *T* depth of tumor invasion, *N* extent of lymph node metastasis

levels were expressed as ratios relative to the β -actin cDNA level.

Statistical analysis

All statistical analyses were carried out using SPSS, version 17.0 (SPSS Japan, Tokyo, Japan). The Mann–Whitney

Table 3 Histological heterogeneity

Histological type of surface section: tub1 or tub2 adenocarcinoma				<i>n</i> = 16
Histological heterogeneity of primary tumor (%)				5 (31.3)
Histological heterogeneity between surface section and lymph node metastasis (%)				7 (43.8)
Patient	Surface section	Middle section	Deep section	Lymph node metastasis
No. 1	tub2	tub2	por1	
No. 2	tub2	por2	por2	
No. 3	tub1	tub1	por1	por1
No. 4	tub2	tub2	por1	por1
No. 5	tub2	tub2	por1	por1
No. 6	tub1	tub1	tub1	por1
No. 7	tub1	tub1	tub2	por1
No. 8	tub2	tub2	tub2	por1
No. 9	tub2	tub2	tub2	por1

tub1 well-differentiated tubular adenocarcinoma, *tub2* moderately differentiated tubular adenocarcinoma, *por1* poorly differentiated adenocarcinoma solid type, *por2* poorly differentiated adenocarcinoma nonsolid type

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 <0.05 were considered to indicate statistical significance.

Results

Clinicopathological characteristics

The study group consisted of 48 patients (33 men and 15 women) with a mean age of 60.9 years. The histological type of the surface sections of the primary tumors was papillary adenocarcinoma (pap) in no patient, well-differentiated tubular adenocarcinoma (tub1) in 7, moderately differentiated tubular adenocarcinoma (tub2) in 9, poorly differentiated adenocarcinoma solid type (por1) in 18, poorly differentiated adenocarcinoma nonsolid type (por2) in 7, signet-ring cell carcinoma (sig) in 4, and mucinous adenocarcinoma (muc) in 3. The macroscopic type of the primary tumors was type 1 in 2, type 2 in 9, type 3 in 19, type 4 in 4, and type 5 in 14. The depth of wall invasion by the primary tumor (T) was T2 in 3 patients, T3 in 16, T4a in 28, and T4b in 1. Lymph node metastasis (N) was N0 in 8 patients, N1 in 14, N2 in 12, N3a in 9, and N3b in 5. Disease stage was stage IIA in 4 patients, stage IIB in 13, stage IIIA in 12, stage IIIB in 6, and stage IIIC in 13. Patient characteristics are summarized in Table 2.

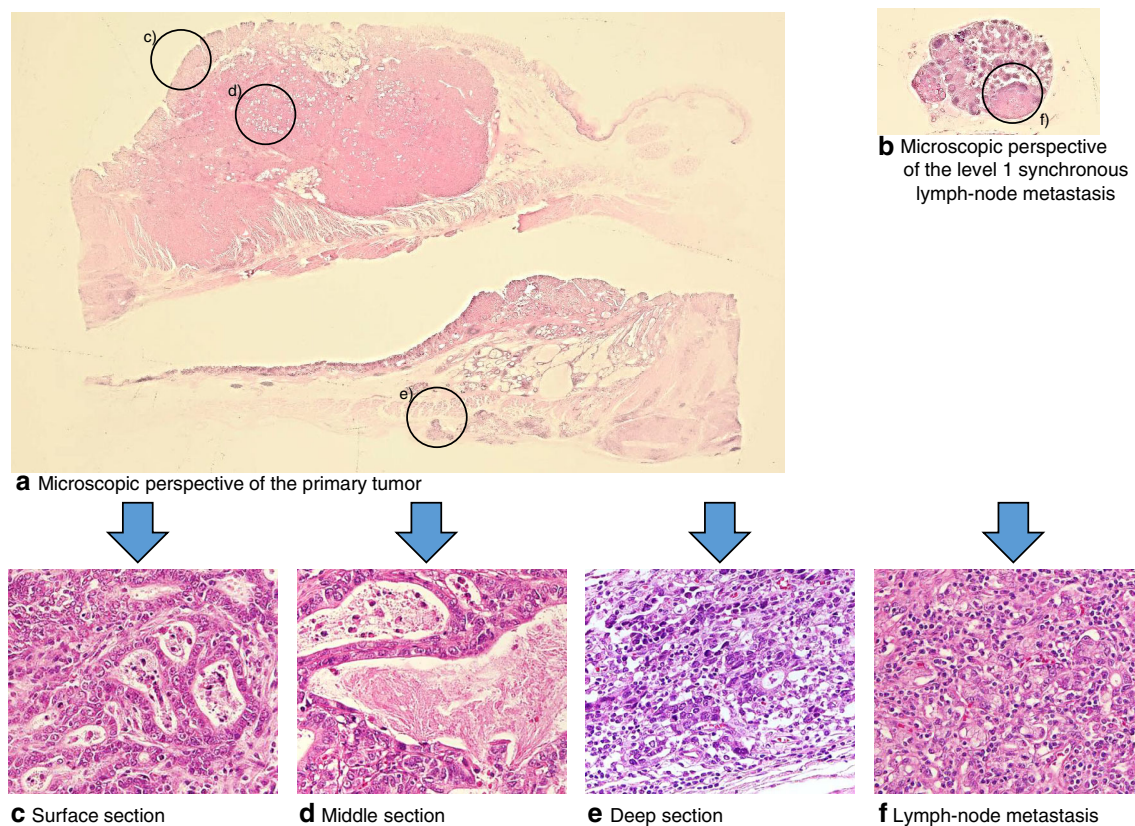


Fig. 1 A typical case showing the histological heterogeneity among different sites of tumor [hematoxylin and eosin (H&E) staining]. **a** Microscopic perspective of the primary tumor. **b** Microscopic perspective of the level 1 synchronous lymph node metastases. **c** Histological type of the surface section of the primary tumor was well-differentiated tubular adenocarcinoma (tub1). **d** Histological

type of the middle section of the primary tumor was well-differentiated tubular adenocarcinoma (tub1). **e** Histological type of the deep section of the primary tumor was poorly differentiated adenocarcinoma, solid type (por1). **f** Histological type of the level 1 synchronous lymph node metastasis was poorly differentiated adenocarcinoma, solid type (por1). **a, b** $\times 1$; **c–f** $\times 200$

In the five patients with histological heterogeneity in their primary tumors, the histological type of the surface sections of their primary tumors was tub1 or tub2 adenocarcinoma, which changed to por1 or por2 adenocarcinoma as the depth of the tumor invasion increased (Table 3). In the seven patients showing histological heterogeneity between the surface sections of their primary tumors and lymph node metastases, the histological type of the surface sections of the primary tumors was also tub1 or tub2 adenocarcinoma (Table 3). A typical case showing histological heterogeneity among specific tumor sites is shown in Fig. 1a–f.

Target gene expression levels

Expression levels (relative cDNA levels) of the target genes are presented as ratios of the gene of interest to the internal reference gene (β -actin), which provided a normalization factor for the amount of cDNA. Table 4 shows the expression levels of each target gene. Nearly all target gene expression levels were higher in tumor tissue than in

nonneoplastic mucosa. The expression levels of the TS, EGFR, and HIF1 α genes did not differ significantly between the surface sections and the nonneoplastic mucosa (Table 4).

In each of the dissected sites, there were no significant differences in target gene expression levels among the histological types (pap, tub1, tub2 or por1, por2, muc). There was no correlation between the gene expression levels and clinical outcomes.

Target gene expression levels in specific sites of primary tumors and synchronous lymph node metastases are shown in Fig. 2a–f.

TS gene expression levels did not differ significantly among the dissected sites of the tumors (Fig. 2a).

TP, DPD, EGFR, and HIF1 α gene expression levels in deep sections were significantly higher than those in surface sections ($p = 0.002$, $p = 0.035$, $p = 0.016$, $p < 0.001$, respectively) (Fig. 2b–d, f). The HIF1 α gene expression level was significantly higher in deep sections than in middle sections and was significantly higher in middle sections than in surface sections ($p = 0.017$, $p = 0.021$, respectively) (Fig. 2f).

Table 4 Target gene expression [mean \pm SD, (range)]

	Non-neoplastic mucosa	Surface section	Middle section	Deep section	Lymph-node metastasis
TS	0.17 \pm 0.36 (0.00-1.54)	0.24 \pm 0.32 (0.00-1.22)	0.35 \pm 0.51 (0.00-2.01)	0.32 \pm 0.46 (0.00-1.71)	0.23 \pm 0.28 (0.00-1.43)
		$p=0.101$	$*p=0.025$	$*p=0.014$	$*p=0.015$
TP	0.65 \pm 1.48 (0.00-5.01)	1.96 \pm 3.78 (0.00-22.74)	2.94 \pm 3.69 (0.00-15.20)	4.18 \pm 5.03 (0.00-23.34)	5.47 \pm 6.38 (0.00-31.17)
		$*p=0.009$	$*p<0.001$	$*p<0.001$	$*p<0.001$
DPD	1.03 \pm 2.16 (0.00-7.63)	1.58 \pm 2.92 (0.00-13.44)	1.85 \pm 2.75 (0.00-11.39)	2.10 \pm 2.93 (0.00-10.36)	0.94 \pm 0.85 (0.00-3.90)
		$*p=0.039$	$*p=0.002$	$*p=0.001$	$*p=0.002$
EGFR	0.01 \pm 0.04 (0.00-0.15)	0.02 \pm 0.03 (0.00-0.11)	0.04 \pm 0.08 (0.00-0.41)	0.13 \pm 0.56 (0.00-3.52)	0.04 \pm 0.07 (0.00-0.34)
		$p=0.238$	$*p=0.011$	$*p=0.001$	$*p<0.001$
VEGF	0.20 \pm 0.33 (0.00-1.13)	0.42 \pm 0.52 (0.00-2.35)	0.55 \pm 0.60 (0.00-2.31)	0.63 \pm 0.77 (0.00-4.03)	0.96 \pm 1.47 (0.00-8.60)
		$*p=0.037$	$*p=0.005$	$*p<0.001$	$*p<0.001$
HIF1 α	0.02 \pm 0.08 (0.00-0.38)	0.05 \pm 0.11 (0.00-0.60)	0.07 \pm 0.12 (0.00-0.52)	0.13 \pm 0.15 (0.00-0.69)	0.14 \pm 0.14 (0.00-0.51)
		$p=0.127$	$*p=0.001$	$*p<0.001$	$*p<0.001$

TS thymidylate synthase, TP thymidine phosphorylase, DPD dihydropyrimidine dehydrogenase, EGFR epidermal growth factor receptor, VEGF vascular endothelial growth factor, HIF1 α hypoxia-inducible factor-1 α

TP, EGFR, VEGF, and HIF1 α gene expression levels were significantly higher in lymph node metastases than in surface sections of primary tumors ($p = 0.002$, $p = 0.002$, $p = 0.005$, $p < 0.001$) (Fig. 2b, d–f). The HIF1 α gene expression level was significantly higher in lymph node metastases than in the middle sections of primary tumors ($p = 0.025$) (Fig. 2f).

TP, DPD, EGFR, VEGF, and HIF1 α gene expression levels positively correlated with the dissected sections of the tumors in a site-specific manner (TP: $r = 0.399$, $p < 0.001$; DPD: $r = 0.250$, $p = 0.001$; EGFR: $r = 0.346$, $p < 0.001$; VEGF: $r = 0.323$, $p < 0.001$; HIF1 α : $r = 0.449$, $p < 0.001$) (Fig. 2b–f).

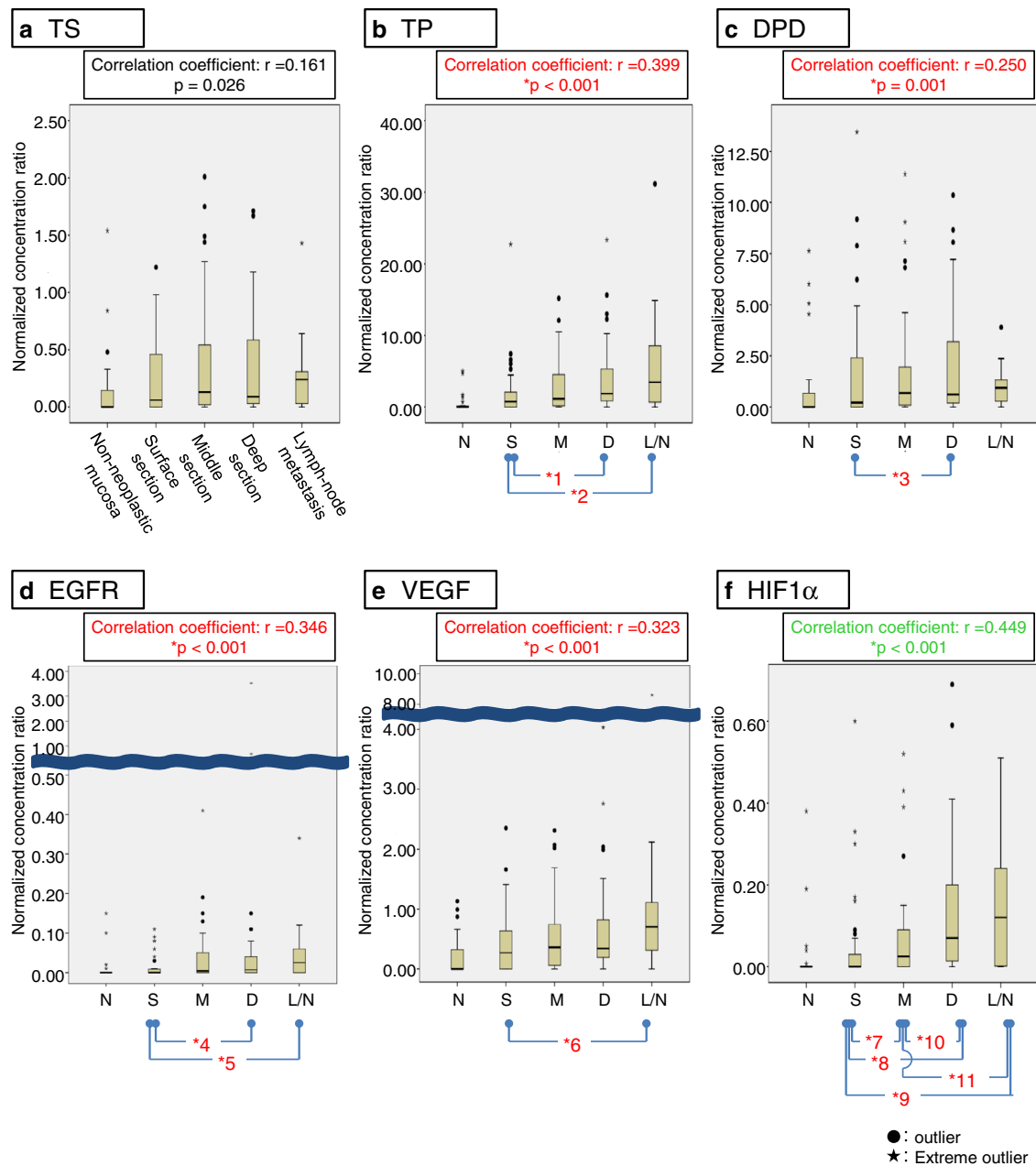
Discussion

Our study showed that expression levels of the TP, DPD, EGFR, and HIF1 α genes were greater in deep sections than in surface sections of primary tumors. In addition, expression levels of the TP, EGFR, VEGF, and HIF1 α genes were higher in lymph node metastases than in surface sections of primary tumors. All target genes except TS showed site-specific positive correlations between gene expression level and the dissection sites of the tumors (including lymph node metastases) in our study. Several studies have also reported high expression levels of genes related to growth factors, cell cycles, and cell motility in lymph node metastases [14], which is in part consistent with our results.

In gastric cancer, tumor progression and lymph node metastases are sometimes associated with the dedifferentiation of differentiated adenocarcinoma to undifferentiated adenocarcinoma [2, 3, 15]. Among the 16 patients in whom the histological type of the surface layer was tub1 or tub2 adenocarcinoma in our study, 5 patients (31.3 %) showed histological heterogeneity within their primary tumors and 7 (43.8 %) showed histological heterogeneity between their primary tumors and lymph node metastases. Submucosal transformation to poorly differentiated adenocarcinoma is thought to increase malignant potential and to promote lymphatic vessel invasion and lymph node metastasis [16].

Few studies have compared the histological type of primary tumors with that of synchronous lymph node metastases. Genetic changes most likely precede histopathological changes.

The finding that the TP, VEGF, and HIF1 α genes were simultaneously expressed at high levels is consistent with angiogenesis pathways proposed by recent studies [17]. However, many aspects of the relationships among different genes remain uncertain. Further elucidation must await the results of future studies. Whether there is a direct causal relationship between TP and VEGF gene expression remains unclear, but TP is known to participate in angiogenesis [18]. TP expression in tumor cells is closely linked to microvascular density, and microvascular density in tumors strongly correlates with VEGF expression [19]. In addition, TP has been shown to convey resistance to hypoxia-induced apoptosis [20] and may also participate in tumor invasion and metastasis in gastric cancer [21–23]. However, vascular endothelial growth factor receptor (VEGFR) was not examined in this study. Therefore, we could not clarify the relationship between angiogenesis and expression of the target genes.



*1: $p = 0.002$, *2: $p = 0.002$, *3: $p = 0.035$, *4: $p = 0.016$, *5: $p = 0.002$, *6: $p = 0.005$,
*7: $p = 0.021$, *8: $p < 0.001$, *9: $p < 0.001$, *10: $p = 0.017$, *11: $p = 0.025$

Fig. 2 Box and whisker plots of the target genes and correlations among expression levels: target gene expression levels in specific sites of primary tumors and synchronous lymph node metastases. Five of the six genes (excluding TS) showed site-specific positive correlations between the expression level and dissected site of the

tumors (including lymph node metastases): thymidylate synthase (TS) (a); thymidine phosphorylase (TP) (b); dihydropyrimidine dehydrogenase (DPD) (c); epidermal growth factor receptor (EGFR) (d); vascular endothelial growth factor (VEGF) (e); hypoxia-inducible factor-1 α (HIF1 α) (f)

The HIF1 α 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 [24–26]. In gastric cancer, HIF1 α also participates in angiogenesis, tumor invasion, and metastasis [27]. However, HIF1 α gene expression may be

controlled at the transcription level by some other factor(s) besides hypoxia [28].

As tumors grow in diameter, the tissue becomes hypoxic [29]. We did not measure tissue oxygen tension in our study and therefore could not clarify the relationship between hypoxia and expression levels of the target genes.

TP expression is high at the invasive edge of tumors [30]. Histopathologically, TP expression is higher in undifferentiated cancer than in differentiated cancer [31]. HIF1 α expression is also increased in undifferentiated cancer [32]. In fact, the expression levels of some genes in our study were positively correlated with tumor progression, although there were no significant differences among the histological types (pap, tub1, tub2 or por1, por2, muc) in any dissected sites studied.

Our results together with these findings suggest that tumor cell genes change as a response to environmental factors such as hypoxia, either before or simultaneously with dedifferentiation-induced histological changes or the acquisition of metastatic activity. It is still unclear whether environmental factors or malignant potential initially influences changes in the expression of some genes; however, we suppose that malignant potential increases as a result of changes in gene expression in a similar manner as genetic changes precede histological changes.

Because we consider malignant potential a cell-specific factor, even if tumor cells originally have it, stimulation by environmental factors is the trigger of gene expression changes. We are planning to investigate the effects of Ki-67 gene and protein expression on cell proliferation potential in a similar site-specific situation. The results of these studies may shed light on which factors predominantly affect gene expression.

The existence of a pathway that can explain these laboratory findings is not yet proven, and further studies are needed to clarify the relationship between angiogenesis and changes in gene expression levels. In summary, our study demonstrated that the expression levels of some genes in tumor cells can change in specific sites of tumors and can become higher in association with tumor progression.

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

Clarification of site-specific differences in gene expression levels in gastric cancer is likely to contribute to our understanding of the properties of tumors and to provide useful information for the design of therapeutic strategies for gastric cancer.

Conflict of interest The authors disclose no potential conflicts of interest.

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