学位論文

「Gene expression profile of genes related to canceration, invasion or conversion for metastasis in patients with poorly differentiated gastric adenocarcinoma treated by gastric endoscopic submucosal dissection.」

(内視鏡治療された胃低分化腺癌における、
癌化・浸潤・形質転換に関連する遺伝子プロファイル)

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1. Introduction

Although the incidence of gastric cancer is decreasing worldwide, it remains the fifth-ranked malignancy in incidence and the third in the mortality rate.(1) While efforts to eradicate Helicobacter pylori continue, in Japan, gastric cancer remains the second most common malignancy in incidence and the third in the rate of mortality.(2,3) Therefore, the diagnosis and treatment of this disease remain important issues.

Endoscopic examinations have improved the diagnoses of gastric cancer in the early stages, and the widespread use of magnifying endoscopy and image-enhanced endoscopy has led to even further improvements in recent years. Due to the increase in the rate of endoscopic screening in the general population,(4) approximately half of all new gastric cancers are identified in the early stages of the disease. Endoscopic treatment of early-stage gastric cancer, which is at present commonly performed at most medical facilities in Japan, has led to an increase in the numbers of patients receiving treatment and those who are completely cured, and a decrease in unexpected symptoms and complications.(5)

In addition to conventional endoscopy, the use of novel techniques, such as chromoendoscopy (e.g., endoscopic dye spraying), image-enhanced endoscopy, and endoscopic ultrasound are considered useful for the determination of tumor invasion depth. However, based on the postoperative histopathologic findings, several patients are still

diagnosed with lesions that are outside the guidelines or were subjected to non-curative resections. The Japanese Gastric Cancer Treatment Guidelines(6) has established both absolute and relative indications based on lymph node metastasis rates, and it recommends that all patients who do not qualify for either of those indications should undergo lymph node dissection as part of the surgical treatment. However, as the actual postoperative lymph node metastasis rate is approximately 15%,(7) there are many patients who do not require an additional resection.(8) In addition, as the population in Japan is aging steadily, surgical treatments are often avoided due to issues, such as low patient tolerance for surgery and problems related to the patient's quality of life (QOL); therefore, the number of cases wherein the disease course is simply routinely observed in follow-up examinations is increasing. Because it is necessary to balance the need for surgery against the risks related to metastasis and prognosis a new diagnostic examination must be developed to assess the risk of lymph node metastasis.

Currently, the postoperative histopathologic findings are the only basis to determine whether a resection was curative or non-curative. Moreover, to our knowledge, there are no diagnostic criteria for this determination based on the features of the tumor itself. However, even for cases with the same diagnosis, some include lymph node metastasis and others do not, which indicates the possibility that the features of the tumors themselves

may be different.

Naruke et al.(9) reported on changes in the degree of expression of different tissue types and the genes involved in the same tumors in advanced cancer cases according to factors such as the layer of the tumor and sites of lymph node metastasis. The deeper the depth of tumor invasion, the more the genes were expressed, which in turn suggests the possibility that higher gene expression is indicative of a higher degree of malignancy and activity. There has been scant molecular biological examination of specimens that were endoscopically resected from early-stage cancer cases because the size of each specimen is exceedingly small.

Thus, in the present study, we investigated the changes in the degree of gene expression in early-stage gastric cancer, especially poorly differentiated adenocarcinoma with poor prognosis, as well as changes in the degree of expression according to the sites within the same tumor. We also analyzed the genes related to tumor proliferation or transformation and metastasis. Therefore, we investigated the trends in the expression of genes related to early-stage cancer.

2. Materials and methods

2-1. Patients selection

The specimens of 14 patients with poorly differentiated adenocarcinoma of a primary tissue type based on postoperative histopathologic diagnosis were assessed in this study. They were selected from among patients diagnosed with early-stage gastric cancer and who underwent endoscopic submucosal dissection at the Kitasato University Hospital. The following clinicopathological characteristics were defined and listed in accordance with the 15th Edition of the Japanese Classification of Gastric Carcinoma(10): age, sex, macroscopic classification, histological classification, depth of tumor invasion, vascular invasion, and degree of complete recovery (the "eCura" system).11

2-2. Laboratory methods

2-2-1. Specimen collection

The endoscopic submucosal dissection specimens collected at this hospital were divided into approximately 3-mm-wide sections, fixed in formalin, and embedded in paraffin. The tissue sections were then prepared as 10-μm-thick slices and subjected to nuclear staining with Kernechtrot to improve tissue visibility during laser microdissection (LMD) using the Arcturus XT Microdissection System (Thermo Fishier Scientific, Waltham, MA, USA) LMD7000 and LMD7 (Leica Microsystems, Wetzlar, Germany).

The specimens were classified according to the following regions: noncancerous mucosa

(N), mucosa (M), and submucosa (SM) of the malignant tumor (Figure 1). The structures in the vicinity (e.g., red blood cells, microvasculature, and lymphocyte masses) were carefully separated and removed to selectively isolate the target sites.

2-2-2. RNA extraction and cDNA synthesis

Following the manufacturer's instructions and using a NucleoSpin totalRNA FFPE (Macherey-Nagel, Düren, Germany), we extracted total RNA from each specimen obtained using LMD. We then performed cDNA synthesis using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany).

2-2-3. Preamplification

To increase the sensitivity to the level of gene expression, the target and housekeeping genes were preamplified. We mixed the primer utilized when using commercially available plates (RT2 PreAmp pathway primer mix: PBH-090Z Human Epithelial Mesenchymal Transition and PBH-176Z Human Cancer Stem Cells, QIAGEN) and the primer mix used with the custom plates (Target gene primer: RT2 qPCR Primer Assay, QIAGEN) according to the manufacture's instructions. In both cases, we used preAmp Master Mix (RT2 Pre AMP cDNA Synthesis Kit, QIAGEN). The instructions were followed in all cases except

when increasing the number of amplification cycles. The cycle parameters were: initial hold at 95℃ for 10 minutes, and preamplification at 95℃ for 15 seconds and then at 60℃ for 4 minutes for a total of 12 cycles.

2-2-4. Real-time PCR

We mixed the cDNA synthesis reaction mixture of the specimens subjected to preamplification with RT2 SYBR Green/Fluorescein qPCR Master mix, placed it on a commercially available plate, and performed real-time PCR using LightCyler 96 (Roche Diagnostics, Indiana, USA) according to the manufacturer's instructions.

2-3. Target genes

To screen for the increased expression of cancer-related, metastasized, and invasion-related genes, commercially available plates were used. We selected two plates: 1. Human Cancer Stem Cells (RT2 Profiler PCR Array; PAHS-176Z, QIAGEN), 2. Human Epithelial Mesenchymal Transition (EMT) (RT2 Profiler PCR Array; PAHS-090Z, QIAGEN). We simultaneously measured a total of 158 genes on each of the 2 plates previously set with 84 types of target gene primers, excluding some overlapping genes. All the genes analyzed are shown in Figure 2. We then selected 12 genes that showed an increasing expression

and a tendency of increasing expression with increasing depth (CALD1, CD44, COL1A2, COL3A1, DKK1, FN1, FZD7, TGFB3, THY1, TIMP1, ZEB1, and ZEB2) to create custom plates (Table 1). The expression of the 5 types of housekeeping genes (ACTB, B2M, GAPDH, HPRT1, RPLP0) on all the plates were simultaneously measured.

2-4. Statistical analysis

The PCR cycle values for all specimens were standardized using the mean values for the 5 housekeeping genes. The relative ratios for the degree of expression of the target genes in the tumor (M or SM) were calculated using the $2^{-\Delta\Delta}$ method and were calibrated to the target genes in the proximal noncancerous mucosa (N).

Statistical analyses were performed using Microsoft Excel. Intracase comparisons were performed using the two-sided t-test and comparisons of each site were performed using the Mann-Whitney U-test. The correlations between the target genes and the specimen sites were assessed using Spearman's rank correlation coefficient. Values of $P < 0.05$ were considered to indicate statistical significance.

2-5. Institutional Review Board

This was a retrospective study approved by the Institutional Review Board of Kitasato University (No. B17-028) and was conducted in accordance with the precepts of the Helsinki Declaration. Written consent was obtained from all the patients to use their treatment data and specimens while undergoing this treatment, and they were informed of their right to opt out of the study at any time.

3. Results

3-1. Clinicopathological characteristics

A total of 1020 patients underwent endoscopic submucosal dissection at Kitasato University Hospital between 2015 and 2018. Twenty-five patients (2.45%) were diagnosed with poorly differentiated adenocarcinoma as the primary tissue type. Of those 25 patients, 14 were selected for investigation based on tumor volume and invasion depth. The clinicopathological characteristics of the patients and specimens are shown in Table 2. Six patients had intramucosal cancer, and 8 patients had submucosal invasion. Of those 8 patients, 5 had superficial submucosal invasion (SM1, $<500\ \mu$ m), and 3 had deep submucosal invasion (SM2, $\geqq 500\ \mu$ m). The primary tissue type in all cases was poorly differentiated adenocarcinoma (por). Of those, 5 patients were diagnosed with por, 4 patients with por mixed with signet-ring cell carcinoma (sig), and 5 patients with por

mixed with tubular adenocarcinoma (tub). Investigation of the sites of the specimens obtained using LMD indicated that of the por cases, 14 were in the mucosa (M), and 8 were in the submucosa (SM). Surrounding noncancerous mucosa (N) was collected from all 14 patients.

3-2. Target gene expression levels

Comparison of M to N indicated that 9 (CALD1, CD44, COL1A2, COL3A1, FN1, THY1, TIMP1, ZEB1, ZEB2) of the 12 target genes had significantly higher levels of expression in M. Also, comparison of SM to N indicated that 11 genes (all but DKK1) had significantly higher levels of expression in SM. Comparison of SM to M indicated that 8 genes (COL1A2, COL3A1, FN1, FZD7, TGFB3, THY1, TIMP1, ZEB2) had significantly higher levels of expression in SM.

Stratification analysis of the levels of gene expression resulted in division into 3 patterns.

a) N < M < SM: Depth dependent. Gene expression increased in order of depth: noncancerous tissue, mucosa, submucosa.

b) N < M = SM: Cancerous sites had higher expression levels than normal tissue. No significant increases even at deeper depths.

c) N = M < SM: Although there were no clear increases in the mucosa, however there were

in the submucosa.

The fold changes of the gene expression levels are shown in Figure 3.

4. Discussion

Even after endoscopic treatment, gastric cancer can cause lymph node metastasis; and if it recurs, the prognosis is generally poor. Although the lymph node metastasis rate for early-stage gastric cancer is not significantly high, there are cases in which it does occur.(11) Because it is difficult to identify cases in which metastasis is more likely, it is recommended that all cases that do not meet the indication guidelines for endoscopic treatment undergo additional surgical treatment. However, one of the most common clinical problems is that there are many patients for whom it is difficult to perform additional surgery due to the risk of complications, the negative effect on the patient's QOL, and poor tolerability of surgery due to advanced age. Therefore, there have been multiple attempts at identifying risk factors and high-risk groups.(8,11-13) Hatta et al.(11) analyzed the survival of patients after undergoing treatment of lesions not indicated for endoscopic treatment and who did not undergo additional surgery due to the score of the risk of lymph node metastasis by calculating the tumor size, venous invasion, and depth. Sekiguchi et al.(13) advocated the use of a scoring model in which the risk of lymph node

invasion is rated on an 11-point scale. However, these points were determined only by using the histopathologic diagnosis. Although there have been reports on factors related to lymph node metastasis and prognostic predictors that take into consideration biological issues,(14,15) a consensus has yet to be reached. As a result, there have been few studies of any type that investigated cases indicated for endoscopic treatment.

In the present study, we used commercially available plates for cancer stem cells and EMT and investigated 158 genes to identify their stem cells and EMT characteristics. Determination of whether these genes showed increased expression in tumors and exclusion of those that did not show a certain level of increased expression allowing us to select 12 genes (CALD1, CD44, COL1A2, COL3A1, DKK1, FN1, FZD7, TGFB3, THY1, TIMP1, ZEB1, and ZEB2) that exhibited a tendency toward increased expression. Of the genes we selected, DKK1 showed only a small amount of expression; and although it was ultimately excluded from our analysis, because its PCR cycle values indicated many specimens that were below detection sensitivity, all 11 of the remaining genes showed significantly increased expression in cancerous sites in comparison to the surrounding noncancerous sites.

Nine genes showed increased gene expression in the mucosa compared to that in the surrounding noncancerous mucosa (CALD1, CD44, COL1A2, COL3A1, FN1, THY1,

TIMP1, ZEB1, ZEB2). Of these, COL1A2, COL3A1, FN1, THY1, TIMP1, and ZEB2 gene expression was higher in the mucosa than that in the noncancerous mucosa and increased as the tumor developed (Figure 3a). COL1A2 and COL3A1 both from the collagen family, have been reported to show increased expression in gastric and other cancers.(16-19) The large glycoprotein FN1 governs cell adhesion and is related to migration and metastasis.(20) COL1A2 and FN1 have been reported to be correlated to the gene expression levels and prognosis in gastric cancer cases,(17,21) which is consistent in that the expression levels increase as the invasion depth and malignancy increase. THY1 (CD90) is a glycoprotein expressed in many cells, including T cells, nerve cells, and fibroblasts.(22) It regulates cell adhesion, neurite outgrowth, tumor proliferation, migration, and apoptosis. TIMP1 has been reported to induce transdifferentiation from normal fibroblasts to activated fibroblasts.(23) It contributes to cell adhesion and metastasis by regulating the ability of the MMPs (matrix metalloproteinases) to degrade the extracellular matrix. It has been reported to show particularly increased expression in scirrhous gastric cancer.(24) These 6 genes can be expected to show increased expression in cases with increasing depth as well as in metastatic tumors. Therefore, those genes may be useful as tumor development markers because they reflect tumor activity and malignancy.

Although CALD1, CD44, and ZEB1 show higher expression in M than in N, they did not show higher expression in M than in SM (Figure 3b). CD44 is a typical cancer stem cell marker expressed in the stomach.(25) In the present study, we confirmed that it shows increased expression not only in advanced cancer but also in the early stages of cancer. That its expression level increases in the mucosa to the level already attained in the submucosa and because it shows nearly the same expression level in M cancers with no SM invasion suggest that it may attain high levels in the mucosa even at relatively early stages, and it may maintain those levels as the invasion depth increases. The same tendencies are displayed by ZEB1, which is important for EMT. Because ZEB1 is essential for CD44 stem cell activation,(26) the two are related. CALD1 codes the caldesmon protein, which is a calmodulin binding protein and a cytoskeleton related protein.(27) It is involved in the differentiation of smooth muscle and regulates EMT during metastasis.(28) These genes already show significantly increased expression when they are in the mucosa but undergo little change as depth increases; and, as a result, it is possible that they indicate the character of the tumor itself and are not related to invasion depth. However, in some cases they show gradual increases in expression; therefore, further study is warranted to determine whether they remain at the same level of expression in the mucosa or increase with increased invasion depth.

Although significant changes in the expression of TGFB3 and FZD7 were not observed in the mucosa, a significant increase in their expression was observed in the submucosa (Figure 3c). TGFß3 is a TGFß subtype that is a factor that promotes the transformation of fibroblasts. TGFß has a suppressive action in early-stage cancer but promotes metastasis in advanced-stage cancer.(29,30) Therefore, it has a dual function with respect to the stage of cancer development. In the present study, we observed almost no cases of increased expression in early disease stages that were restricted to the mucosa; however, there were several cases in which expression decreased. The expression did increase when the submucosa was invaded, which supports the aforementioned account of its characteristics. FZD7 does not have a particularly high positive rate in gastric cancers overall. And according to Schmuck et al.,(31) it appears in 25% of T1 tumors and in approximately 40% of T2 and deeper tumors; however, the incidence does not increase as the depth increases. The expression increases in SM, although the degree of increase with increasing depth is only slight; however, the disparities in the gastric cancers themselves may account for why we were unable to identify any clear trends in FZD7 expression. This must be examined in studies with larger sample sizes to more accurately determine the FZD7-expression positivity rate.

There have been few studies examining gene expression trends in other cancers that make

a distinction between surrounding noncancerous mucosa and early stage cancer. In their investigation of squamous cell lung cancer, Koper et al.(32) divided the samples into noncancerous mucosa, noninvasive cancer, and invasive cancer. However, although they found a tendency toward increased expression as depth increased, many genes showed marked changes in invasive lesions as compared to noninvasive lesions, and the amount of change did not increase with increased invasion depth thereafter. This suggests that the character of the tumor undergoes some major change when invasion occurs. There have been some comparisons to adenoma, which is a precancerous lesion, in cases of colorectal cancer. Pesson et al.(33) reported differential expression of all genes between colorectal adenoma and adenocarcinoma, and they and other investigators found that most cases were between colorectal adenoma and normal epithelia.(33-35)

The wide variety of studies on the molecular biomarkers for lymph node metastasis in gastric cancer cases include studies with comprehensive investigations using microarrays(14) and large-scale studies in which the TCGA (The Cancer Genome Atlas) data sets were used.(15) However, since all these studies include patients in all disease stages and mixed tissue types, a consensus has not been reached to date, and no definitive marker has been identified. Gastric cancer tumors present with uniform tissue types, and it is possible that their molecular pathological characteristics may differ. Therefore, it is

difficult to identify a universal prognostic factor. Further studies of risk factors linked to tissue types and disease stages are warranted.

In the present study, we focused our investigation on poorly differentiated adenocarcinomas of pT1 cancers that are indicated for endoscopic treatment because there have been few studies of such cases. We found that there are already changes in gene expression in the early disease stages. However, as there are very few cases of lymph node metastasis among patients indicated for endoscopic treatment, and because even in patients indicated for surgery the lymph node metastasis rate is only between 8% and 20% in cases of pT1 tumor,(7) we did not investigate such cases in the present study. The present study was a retrospective single-center study with a small number of subjects. In addition, as we selected 14 from 25 patients because of area or depth of tumors due to PCR technical problem, there must be considered selection bias in this study. The genetic trends identified in this study require further study to determine what invasion depth and metastasis trends can be identified based on expression levels in the early stages; and, therefore, they should include cases of metastasis. We identified multiple cancer-related genes that undergo changes in expression level during the early stages of poorly differentiated gastric adenocarcinoma, when endoscopic treatment may be indicated. We also identified those in which the expression level increases in stages as the tumor

develops and invasion depth increases, which indicates that this may be related to tumor malignancy, proliferation, and/or transformation. We expect this data will lead to the clarification and stratification of metastasis risks through combining conventional histologic diagnosis with other techniques and modalities.

5. Conflict of Interest

There is no conflict of interest (COI) to be declared.

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7. Figure Legends

7-1. Figure 1: Sites of specimen collection

7-2. Figure 2: Target genes in commercial plates

Cancer stem cells and epithelial-to-mesenchymal transition play important roles in proliferation and differentiation as well as invasion and metastasis.

7-3. Figure 3: Fold changes of the expression of target genes at tumor mucosal sites (M) and tumor submucosal sites (SM) in comparison to the surrounding non-cancerous mucosa (N) in each case.

(a) N<M<SM: Depth-dependent. Gene expression increased in order of depth: Non-cancerous tissue, mucosa, submucosa

(b) N<M=SM: Cancerous sites had higher expression levels than normal tissue. No significant increases even at deeper depths.

(c) N=M<SM: Although no clear increases were observed in the mucosa, there were significant increases only in the submucosal section.

8. Tables

8-1. Table 1: Targeted genes selected for custom plates

CALD1	*caldesmon 1*
CD44	*CD44 molecule*
COL1A2	*collagen, type I, alpha 2*
COL3A1	*collagen, type III, alpha 1*
DKK1	*Dikkopf homolog 1*
FN1	*Fibronectin 1*
FZD7	*Fizzled family receptor 7*
TGFB3	*Transforming growth factor, beta 3*
THY1	*Thy-1 cell surface antigen*
TIMP1	*TIMP metallopeptidase inhibitor 1*
ZEB1	*Zinc finger E-box binding homeobox 1*
ZEB2	*Zinc finger E-box binding homeobox 2*

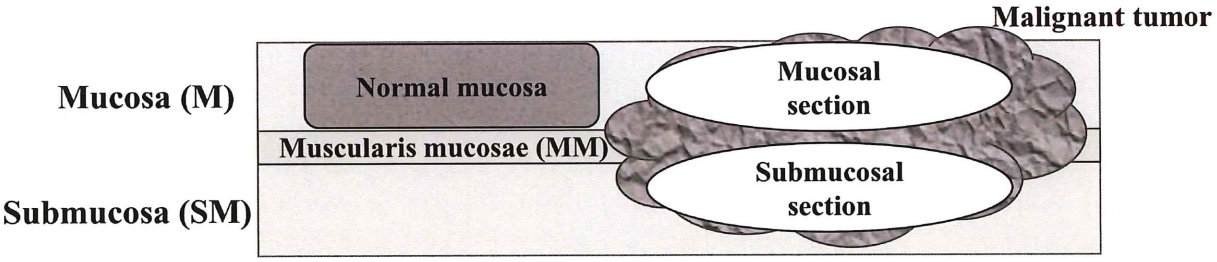
8-2. Table 2: Patients characteristics

Age (years)	
mean±SD (range)	73.4±11.0 (50-86)
Sex	
Male	10
Female	4
Size(mm)	
Median (range)	15 (8-42)
Location	
U	2
M	1
L	9
Others	2
Macroscopic type	
0-IIa+IIc	1
0-IIc	12
0-IIc+IIa	1
Depth of tumor	
Mucosa (M)	6
Submucosa (SM)	8
SM1/SM2	5/3
Histological type	
por	5
por>sig	4
por>tub	5
Lymphovascular invasion	
Ly present/absent	5/9
V present/absent	3/11
Curativity	
eCura B	4
eCura C2	10
Additional surgical resection (%)	5(35.7)

por: Poorly differentiated adenocarcinoma, sig: Signet-ring cell carcinoma, tub: Tubular adenocarcinoma

9. Figures

9-1. Figure 1: Sites of specimen collection



9-2: Figure 2: Target genes in commercial plates

Human Cancer Stem Cells (PAHS-176Z)

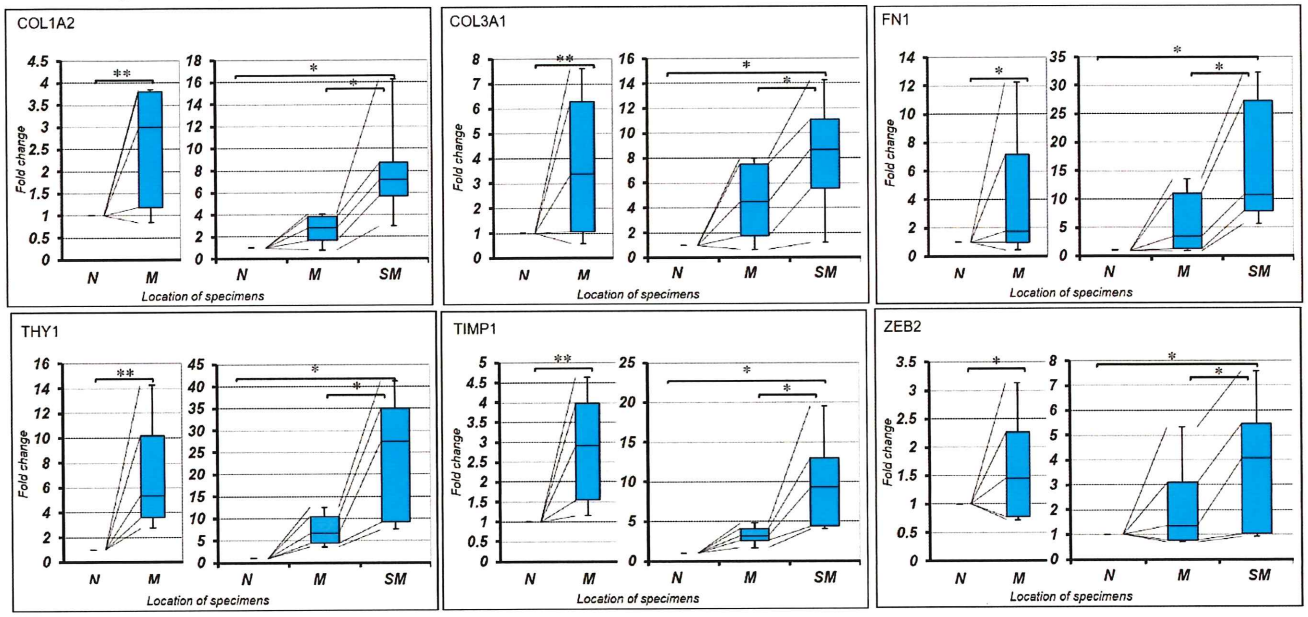
ABCB5	ABCG2	ALCAM	ALDH1A1	ATM	ATXN1	AXL	BMI1	BMP7	CD24	CD34	CD38
CD44	CHEK1	DACH1	DDR1	DKK1	DLL1	DLL4	DNMT1	EGF	ENG	EPCAM	ERBB2
ETFA	FGFR2	FLOT2	FOXA2	FOXP1	FZD7	GATA3	GSK3B	HDAC1	ID1	IKBKB	CXCL8
ITGA2	ITGA4	ITGA6	ITGB1	JAG1	JAK2	KIT	KITLG	KLF17	KLF4	LATS1	LIN28A
LIN28B	MAML1	MERTK	MS4A1	MUC1	MYC	MYCN	NANOG	NFKB1	NOS2	NOTCH1	NOTCH2
PECAM1	PLAT	PLAUR	POU5F1	PROM1	PTCH1	PTPRC	SAV1	SIRT1	SMO	SNAI1	SOX2
STAT3	TAZ	TGFBR1	THY1	TWIST1	TWIST2	WEE1	WNT1	WWC1	YAP1	ZEB1	ZEB2

Human Epithelial-Mesenchymal Transition (PAHS-090Z)

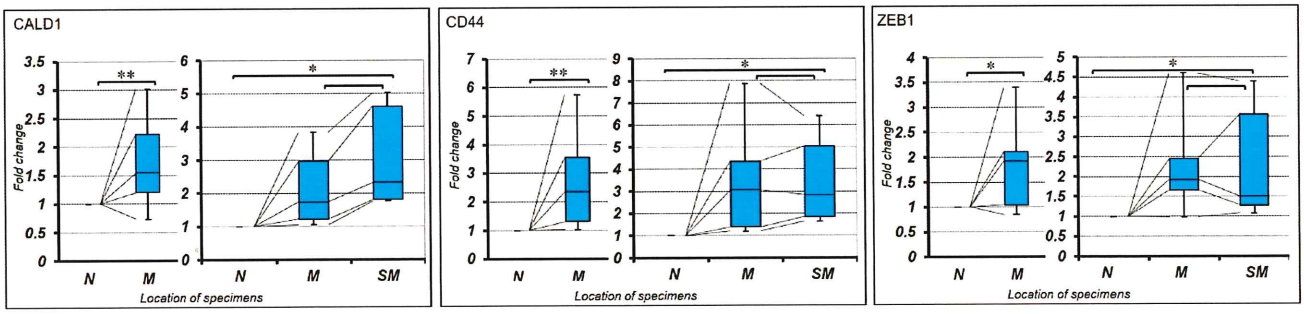
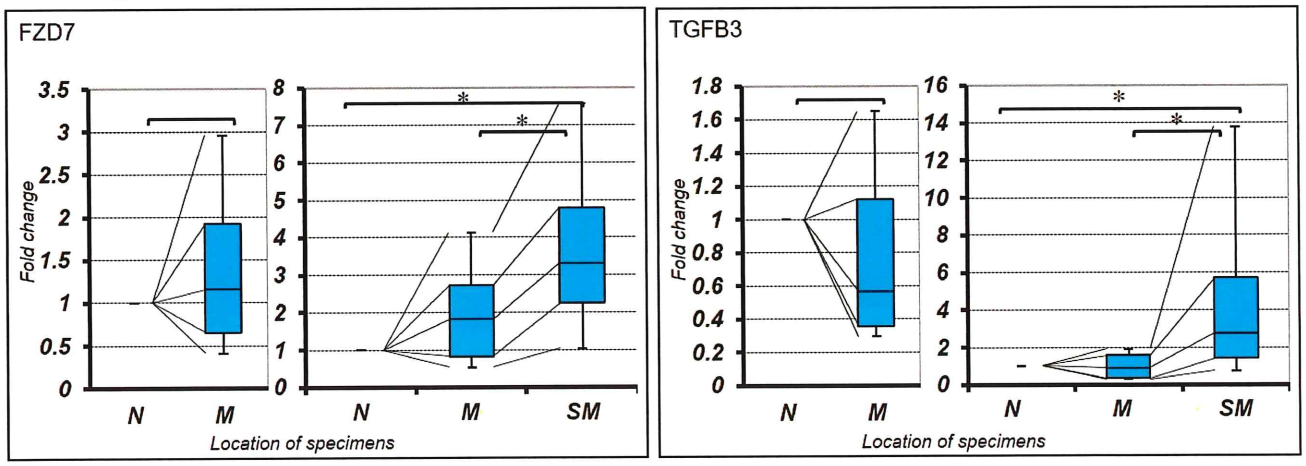
AHNAK	AKT1	BMP1	BMP2	BMP7	CALD1	CAMK2N1	CAV2	CDH1	CDH2	COL1A2	COL3A1
COL5A2	CTNNB1	DSC2	DSP	EGFR	ERBB3	ESR1	F11R	FGFBP1	FN1	FOXC2	FZD7
GNG11	GSC	GSK3B	IGFBP4	IL1RN	ILK	ITGA5	ITGAV	ITGB1	JAG1	KRT14	KRT19
KRT7	MAP1B	MMP2	MMP3	MMP9	MSN	MST1R	NODAL	NOTCH1	NUDT13	OCLN	PDGFRB
PLEK2	DESI1	PTK2	PTP4A1	RAC1	RGS2	SERPINE1	GEMIN2	SMAD2	SNAI1	SNAI2	SNAI3
SOX10	SPARC	SPP1	STAT3	STEAP1	TCF3	TCF4	TFPI2	TGFB1	TGFB2	TGFB3	TIMP1
TMEFF1	TMEM132A	TSPAN13	TWIST1	VCAN	VIM	VPS13A	WNT11	WNT5A	WNT5B	ZEB1	ZEB2

9-3. Figure 3: Fold changes of the expression of target genes at tumor mucosal sites (M) and tumor submucosal sites (SM) in comparison to the surrounding non-cancerous mucosa (N) in each case.

9-3-1. Figure 3-(a)



9-3-2. Figure 3-(b)



9-3-3. Figure 3-(c)