

# Gene expression profiles of tumor regression grade in locally advanced rectal cancer after neoadjuvant chemoradiotherapy

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**Abstract.** Tumor regression grading (TRG) reportedly has prognostic value in rectal cancer patients after pre-operative chemoradiotherapy (CRT). The aim of this retrospective study was to differentiate gene expression profiles based on TRG in residual cancer cells after CRT. We evaluated pathological response using the criteria of four TRG systems: the Japanese Society for the Cancer of Colon and Rectum (JSCCR), Mandard, Dworak and Rödel. Total RNA was obtained using microdissection from 52 locally advanced rectal cancer specimens from patients who underwent pre-operative CRT to examine the expression levels of 20 genes [*PCNA*, *MKI67*, *CDKN1A* (*p21<sup>Cip1</sup>*), *CDK2*, *CHEK1*, *PDRG1*, *LGR5*, *PROM1* (*CD133*), *CD44*, *SOX2*, *POU5F1* (*OCT4*), *LKB1*, *VEGF*, *EGFR*, *HGF*, *MET*, *HIF1*, *GLUT1*, *BAX* and *BCL2*] using real-time quantitative RT-PCR. Gene expression was compared across the four TRG systems. *LGR5* gene expression levels in CRT non-responders were significantly higher than in responders in all four grading systems. Patients with elevated *PDRG1* and *GLUT1* gene expression had poor pathological response in three TRG systems (JSCCR, Dworak and Rödel). *MKI67* gene expression in non-responders was significantly higher than in responders in two grading systems (JSCCR and Rödel). While, *BAX* gene expression in responders was significantly higher than in non-responders in the Mandard TRG system. The results of this study suggest that TRG may reflect characteristics, such as proliferative activity, stemness potency and resistance to hypoxia, of residual cancer cells following pre-operative CRT.

## Introduction

Pre-operative CRT followed by surgery is the standard treatment for patients with locally advanced rectal cancer. However, disease recurrence remains the major cause of mortality in these patients (1,2). Identifying predictors for disease recurrence or poor prognosis would aid in the successful treatment of these patients. Tumor regression grade (TRG) following pre-operative CRT is determined by quantifying the proportion of residual cancer cells to the stroma of the entire tumor bed. In rectal cancer, several studies have found that TRG or pathologic response is a predictor of clinical outcome, including disease recurrence and survival (3-8). Hence, it is possible that gene expression correlated with TRG might reflect the characteristics, including resistance to CRT, of residual cancer cells and might be associated with prognosis in patients with rectal cancer after pre-operative CRT. DNA repair pathways (9), cell cycle pathways (10), hypoxia (11,12), anti-apoptosis (13) and cancer stem cells (14,15) have been implicated in the mechanisms of CRT resistance. Twenty genes were selected for a comparison in expression levels between CRT responders and non-responders: *PCNA* and *MKI67* (*Ki67*) as proliferative markers; *CDKN1A* (*p21<sup>Cip1</sup>*) and *CDK2* as cell cycle associated markers after irradiation; *CHEK1* and *PDRG1* (*p53* and DNA downregulated gene) as DNA damage associated makers; *LGR5*, *PROM1* known as *CD133*, *CD44*, *SOX2*, *POU5F1* known as *OCT4* and *LKB1* known as *SKT11*, as stem cell associated markers; *VEGFA*, *HGF* and *MET* as growth factors; *HIF1* and *GLUT1* as hypoxia associated markers; *BAX* and *BCL2* as apoptosis associated markers in this transcriptional analysis. The aim of this retrospective study was to examine the expression of certain genes in TRGs and determine if the two are associated with clinical outcome in patients with locally advanced rectal cancer after pre-operative CRT.

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**Key words:** gene expression profiles, tumor regression grade, neoadjuvant chemoradiotherapy, rectal cancer

## Materials and methods

**Patients and specimens.** From 2001 to 2008, 64 patients with rectal cancer received pre-operative CRT followed by surgery at Mie University Hospital. The following criteria were used for induction of pre-operative CRT. Patients must i) be no more

Table I. Primer sequences of target genes.

Gene symbol	Forward	Reverse
<i>PCNA</i>	GAAGCACCAAACCAGGAGAA	TATCGGCATATACGTGCAA
<i>MKI67</i>	TGAGCCTGTACGGCTAAAACA	TTGACTTCCTTCCATTCTGAAG
<i>CDKN1A</i>	GACTCTCAGGGTCGAAAACG	GGATTAGGGCTTCTCTTGG
<i>CDK2</i>	CATTCCTCTTCCCTCATCA	TTTAAGGTCTCGGTGGAGGA
<i>CHEK1</i>	GACTGGGACTTGGTGCAAAC	CACTGCGACTGATTCAG
<i>PDRG1</i>	CCTCACCTGAGACAAAGGA	GGCGGTTGACCTTCACTTTA
<i>LGR5</i>	GATGTTGCTCAGGGTGGACT	GGGAGCAGCTGACTGATGTT
<i>PROM1</i>	GCTTTGCAATCTCCCTGTTG	TGATCCGGTTCTTACCTG
<i>CD44</i>	CGGACACCATGGACAAGTTT	CACGTGGAATACACCTGCAA
<i>SOX2</i>	CAAGATGCACAACTCGGAGA	GCTTAGCCTCGTCGATGAAC
<i>POU5F1</i>	CTGGAGAAGGAGAAGCTGGA	CAAATTGCTCGAGTTCTTTCTG
<i>LKB1</i>	CTCTTACGGCAAGGTGAAGG	TTGTGCCGTAACCTCCTCAG
<i>VEGFA</i>	CAGAAGGAGGAGGGCAGAA	CTCGATTGGATGGCAGTAGC
<i>EGFR</i>	CCTATGTGCAGAGGAATTATGATCTTT	CCACTGTGTTGAGGGCAATG
<i>HGF</i>	ATTTGGCCATGTTTTGACC	AGCTGCGTCCTTTACCAATG
<i>MET</i>	AGGTGTGGGAAAAACCTGA	ATTCAGCTGTTGCAGGGAAG
<i>HIF1</i>	CCGCTGGAGACACAATCATA	CTTCCTCAAGTTGCTGGTCA
<i>GLUT1</i>	CCTGCAGTTTGGCTACAACA	GTGGACCCATGTCTGGTTG
<i>BAX</i>	CTTTGCCAGCAAACCTGGTG	CAGCCCATGATGGTTCTGA
<i>BCL2</i>	TCGCCCTGTGGATGACTGA	CAGAGACAGCCAGGAGAAATCAA
<i>GAPDH</i>	GGAAGGTGAAGGTCCGGAGTC	AATGAAGGGGTCATTCATGG
<i>ACTB</i>	ACAGAGCCTCGCCTTTG	GCGGCGATATCATCATCC

*PCNA*, proliferation cell nuclear antigen; *MKI67*, *Ki67*; *CDKN1A*, *p21<sup>Cip1</sup>*; *CDK2*, cyclin-dependent kinase 2; *PDRG1*, p53 and DNA-damage regulated 1; *LGR5*, leucine-rich repeat-containing G protein-coupled receptor 5 known as GPR49; *CHEK1*, checkpoint kinase 1; *PROM1*, CD133; *POU5F1*, OCT4; *LKB1*, serine/threonine kinase 11 known as STK11; *VEGFA*, vascular endothelial growth factor; *EGFR*, epidermal growth factor receptor; *HGF*, hepatocyte growth factor; *MET*, HGF receptor; *HIF1*, hypoxia inducible factor 1 $\alpha$ ; *GLUT1*, glucose transporter 1; *ACTB*,  $\beta$ -actine.

than 80 years of age; ii) be in clinical stage II/III based on the International Union Against Cancer TNM classification, with no evidence of distant metastases; iii) exhibit no invasion of the external sphincter muscle or elevator muscle of the anus; and iv) show no evidence of deep venous thrombosis. A total of 52 cases that received a curative operation and excluded pathological complete response were available for this study. The study design was approved by the hospital ethics review board. All patients signed informed consent forms allowing use of their tissues in this study.

**5-Fluorouracil-based chemoradiotherapy regimen.** Patients with rectal cancer were treated with short-course (a dose of 20 Gy in four fractions) or long-course (a dose of 45 Gy in 25 fractions) radiotherapy using a four-field box technique with concurrent chemotherapy to take advantage of 5-fluorouracil (5-FU) radio-sensitization. Patients underwent concurrent pharmacokinetic modulation chemotherapy (5-FU given by intravenous infusion, 600 mg/m<sup>2</sup> for 24 h and tegafururacil (UFT) given orally, 400 mg/m<sup>2</sup> orally for 5 days (16). Forty-one patients received short-course radiotherapy with chemotherapy over 1 week. The remaining eleven patients received long-course radiotherapy with chemotherapy for

4 weeks. The time interval between pre-operative CRT and surgery was 2-3 weeks for short-course irradiation patients, and 4-6 weeks for long-course irradiation patients. All patients underwent standard surgery, including total mesorectal excision, and received 5-FU-based adjuvant chemotherapy after surgery for 6 months to 1 year.

**Clinical and pathological response to CRT.** The clinical response after pre-operative CRT was evaluated by barium enema, endoscopy, computed tomography and magnetic resonance imaging, and was then graded as a complete response, a partial response, no change or progressive disease. Histological sections were sliced at a thickness of 5  $\mu$ m and stained with hematoxylin and eosin. The TRG method for sampling and examining the tumor site from colorectal excision specimens removed following neoadjuvant therapy was found in Bateman *et al* (17). The median number of sections per case examined was 4.5 (range, 2-7). TRG was evaluated using criteria from four sources: Japanese Society for Cancer of the Colon and Rectum (JSCCR) (18), Mandard *et al* (19), Dworak *et al* (20) and Rödel *et al* (3). Each TRG was evaluated by two investigators (K. Tanaka and Y. Okugawa) in a blinded fashion without knowledge of the clinical and pathological information.

**Microdissection and RNA extraction from formalin-fixed paraffin-embedded specimens.** Microdissection of formalin-fixed paraffin-embedded (FFPE) was performed as previously described (21). Microdissected specimens were digested with proteinase K in lysis buffer containing Tris-HCl, ethylenediaminetetraacetic acid and sodium dodecyl sulfate, as previously published with minor modifications (22). RNA was purified by phenol/chloroform extraction and precipitated using ethanol. The concentration and quality of RNA was measured with UV absorbance at 260 and 280 nm (A260/280 ratio).

**cDNA synthesis.** The fragmented mRNA from FFPE tissue materials was reverse transcribed using random hexamer priming instead of oligo(dt)-based priming. cDNA was synthesized with random hexamers and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

**Quantitative real-time polymerase chain reaction.** Real-time quantitative RT-PCR analysis was performed using a fluorescence-based real-time detection method (TaqMan) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). Although SYBR-Green-based detection is less specific than TaqMan-based detection, we used SYBR-Green due to time and cost constraints. Primers were strictly selected or designed to span introns to avoid amplification from contaminating genomic DNA. Target sequences were kept as small as possible (~100 bp) to ensure the detection of fragmented and partially degraded RNA. To confirm primer specificity, a single band of expected amplicon size for each target gene was verified using gel electrophoresis on a 2% agarose gel and visualized with ethidium bromide. Primers for *PCNA*, *MKI67*, *CDKN1A* (*p21<sup>Cip1</sup>*), *CDK2*, *CHEK1*, *PDRG1*, *LGR5*, *PROM1* (*CD133*), *CD44*, *SOX2*, *POU5F1* (*OCT4*), *LKB1*, *VEGF*, *EGFR*, *HGF*, *MET*, *HIF1*, *GLUT1*, *BAX*, *BCL2*, *GAPDH* and *ACTB* ( $\beta$ -actin) were designed with Primer3 software (Biology Workbench Version 3.2, San Diego Supercomputer Center, University of California, San Diego). Primer sequences are shown in Table I. PCR was performed in a final volume of 25  $\mu$ l with a SYBR-Green PCR Master Mix using 1  $\mu$ l cDNA and 400 nM of each primer for the respective genes. Cycling conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

**Relative mRNA levels of target genes.** The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The Ct is inversely proportional to the amount of cDNA, i.e., a higher Ct value means that more PCR cycles are required to reach a certain level of detection. Relative mRNA levels were determined using a standard curve. Standard curves and line equations were generated using 5-fold serially diluted solutions of cDNA from the LoVo colon cancer cell line or from qPCR Human Reference Total RNA (Clontech, Mountain View, CA). All standard curves were linear in the analyzed range with an acceptable correlation coefficient ( $R^2$ ). Target gene expression was calculated using the standard curve. Quantitative normalization of cDNA in each sample was performed using expression of the *ACTB* and *GAPDH* gene as internal controls. Finally, mRNA

levels of the target gene were presented as ratios between the genes of interest and the internal reference gene. Real-time PCR assays were performed twice for each sample and mean values were used for calculations of mRNA levels.

**Statistical analyses.** All statistical analyses were performed using Stat View 5.0 for Windows (SAS Institute Inc., Cary, NC). Values of each target gene are expressed as median values (inter-quartile range) in tables. Associations between continuous variables and categorical variables were evaluated using the Mann-Whitney U test for two groups. Recurrence-free survival (RFS) and overall survival (OS) time were calculated from the date of surgery to the date of disease recurrence and death, respectively. RFS and OS probability were calculated using the Kaplan-Meier product limit method; intergroup differences were determined using a log-rank test. Two-sided P-values of <0.05 were considered statistically significant.

## Results

**Patient and tumor characteristics.** The median age of the patients was 64.5 years (range, 38-77 years) and the median follow-up period was 52 months (range, 3-129 months). The male to female ratio was 2.9:1. The median age was 64.5 years (range, 37-77 years) and the male to female ratio was 3.7:1. The post-CRT pathological T stages were pT1 (n=5), pT2 (n=12), pT3 (n=33) and pT4 (n=2). Seventeen patients (33%) had lymph node metastases. Forty-four tumors (84.6%) showed well or moderately differentiated adenocarcinoma histology. Three patients (5.7%) had local recurrence alone. A total of 12 patients (23.1%) had distant recurrence. Patterns of distant recurrence were seen as liver and lung metastases in 2 patients, lung metastasis alone in 6 patients and peritoneal metastasis in 2 patients (Table II).

**Evaluation of pathological response by TRG.** Table III shows the pathological response after CRT using each TRG. Responders were categorized as patients with JSCCR TRG 2, Mandard TRG 2, Dworak and Rödel TRG 3. The others were non-responders. We excluded the cases with pathological complete response and no regression in this study (JSCCR TRG 0 and 3, Mandard TRG 1 and 5, Dworak TRG 0 and 4, Rödel TRG 0 and 4).

**Gene expression profiles of residual cancer according to TRG.** Total RNA was isolated from 52 specimens and transcriptional analysis was performed for 20 genes. Table IV shows the gene expression levels of residual cancer according to TRG. *LGR5* gene expression in non-responders was significantly higher than in responders among all TRG systems. Patients with elevated *PDRG1* and *GLUT1* gene expression had poor pathological response using the JSCCR, Dworak and Rödel TRG criteria. *MKI67* gene expression in non-responders was significantly higher than in responders in the JSCCR and Rödel TRG systems. While, *BAX* gene expression in responders was significantly higher than in non-responders in Mandard TRG system. Patients with poor pathological response based on the Rödel criteria had significantly higher gene expression of *MKI67*, *PDRG1*, *LGR5*, *LKB1*, *EGFR*, *MET* and *GLUT1*.

Table II. Patient and tumor characteristics.

Variables	n=52 (%)
Age, median 64.5 years (range, 38-77)	
Gender	
Male	41 (79.0)
Female	11 (21.0)
Pre-T classification	
1/2	10 (19.2)
3/4	42 (80.8)
Pre-N classification	
Negative	17 (32.7)
Positive	35 (67.3)
Pre-stage	
I/II	17 (32.7)
III	35 (67.3)
Down staging	
No	29 (55.8)
Yes	23 (44.2)
Post-T classification	
1/2	17 (32.7)
3/4	35 (67.3)
Post-N classification	
Negative	35 (67.3)
Positive	17 (32.7)
Lymphatic invasion	
Negative	13 (25.0)
Positive	39 (75.0)
Vascular invasion	
Negative	21 (40.4)
Positive	31 (59.6)
Histology	
Well/moderately	44 (84.6)
Poorly/signet/mucinous	8 (15.4)
Post-operative stage	
I/II	33 (63.5)
III	19 (36.5)
Radiation	
Short, 20 Gy/4 fractions	41 (78.8)
Long, 45 Gy/25 fractions	11 (21.2)
Recurrence	
None	37 (71.2)
Local alone	3 (5.7)
Distant with/without local failure	12 (23.1)

Recurrence-free and overall survival based on expression levels of *PDRG1*, *LGR5* and *GLUT1*. We investigated whether the gene expression levels correlated with TRG were also associated with patient prognosis after pre-operative CRT followed by curative surgery because it has been reported as a predictor of clinical outcome. Four genes were selected (*MKI67*, *PDRG1*, *LGR5* and *GLUT1*) that were significantly correlated with more than two TRG systems. The median value of *MKI67*,

Table III. Evaluation of pathological response by TRGs.

Criteria	n=52 (%)
JSCCR	
TRG 0	-
TRG 1a	13 (25.0)
TRG 1b	30 (57.6)
TRG 2	9 (17.4)
TRG 3	-
Mandard	
TRG 1	-
TRG 2	15 (28.8)
TRG 3	23 (44.2)
TRG 4	14 (27.0)
TRG 5	-
Dworak	
TRG 0	-
TRG 1	14 (27.0)
TRG 2	32 (61.5)
TRG 3	6 (11.5)
TRG 4	-
Rödel	
TRG 0	-
TRG 1	10 (19.2)
TRG 2	22 (42.3)
TRG 3	20 (38.5)
TRG 4	-

TRG, tumor regression grade. Cases with pathological complete response and no regression (JSCCR TRG 0 and 3, Mandard TRG 1 and 5, Dworak TRG 0 and 4, Rödel TRG 0 and 4) were excluded in this study.

*PDRG1*, *LGR5* and *GLUT1* gene expression was 0.00000895, 0.0308, 6.295 and 0.566, respectively. We categorized the case with more than the median value as the high gene expression group, and the remaining as the low group. Fig. 1 shows the survival curve for RFS according to each gene expression level using Kaplan-Meier analysis. Patients with *LGR* expression levels above the cut-off values showed a significantly poorer RFS than did patients with expression levels below the cut-off values ( $P=0.0262$ ). Patients in high *MKI67* expression group had poorer RFS than that in low group without significant difference ( $P=0.0549$ ). Patients with high *GLUT1* gene expression had significantly poorer OS than those with low one (*GLUT1*  $P=0.0093$ ). *MKI67*, *PDRG1* and *LGR5* gene expression were not associated with OS (*MKI67*,  $P=0.6018$ ; *PDRG1*,  $P=0.5493$ ; *LGR5*,  $P=0.6487$ ) (data not shown).

## Discussion

Rectal cancer is one of the most common cancers in Japan and the Western world. The introduction of pre-operative CRT and total mesorectal excision for the management of locally advanced rectal cancer significantly decreased local recur-

Table IV. Gene expression profiles of residual cancer according to TRG systems.

Gene symbol (range)	JSCCR			Mandard			Dworak			Rödel		
	Non-responder (n=43)	Responder (n=9)	P-value	Non-responder (n=37)	Responder (n=15)	P-value	Non-responder (n=46)	Responder (n=6)	P-value	Non-responder (n=32)	Responder (n=20)	P-value
<i>PCNA</i> (0-0.433)	0.0248	0.0057	0.2122	0.0248	0.0104	0.2975	0.0221	0.0063	0.1396	0.0414	0.0091	0.0585
<i>MKI67</i> (0-46.647)	2.1635	0.0002	0.0282 <sup>a</sup>	2.4304	0.2072	0.0554	2.0224	0.0003	0.2521	2.7135	0.3099	0.0207 <sup>a</sup>
<i>CDKN1A</i> (0-11.562)	1.4291	1.9966	0.3393	1.4604	1.4038	0.7542	1.4448	1.5462	0.9999	1.6539	1.2498	0.3766
<i>CDK2</i> (0-32.813)	0.4628	0.2893	0.7333	0.5166	0.2893	0.4644	0.4405	0.0733	0.4115	0.5279	0.2484	0.2332
<i>CHEK</i> (0-0.318)	0.0205	0.0037	0.6646	0.0238	0.0022	0.1475	0.0192	0.0055	0.7974	0.0258	0.0044	0.5879
<i>PDRG1</i> (0-0.308)	0.0485	0.0091	0.0077 <sup>a</sup>	0.0438	0.0096	0.0841	0.0409	0.0048	0.0296 <sup>a</sup>	0.1068	0.0118	0.0135 <sup>a</sup>
<i>LGR5</i> (0-54.643)	7.7440	0.1810	0.0018 <sup>a</sup>	10.6660	2.0120	0.0037 <sup>a</sup>	7.2345	0.0905	0.0039 <sup>a</sup>	9.7080	7.4985	0.0105 <sup>a</sup>
<i>PROM1</i> (0-0.523)	0.1070	0.0280	0.4533	0.1070	0.0660	0.6937	0.1110	0.0195	0.2519	0.1040	0.0820	0.5662
<i>CD44</i> (0-1.274)	0.1500	0.1080	0.9130	0.1500	0.1080	0.9354	0.1510	0.0525	0.4549	0.1740	0.1050	0.1025
<i>SOX2</i> (0-71.914)	2.2590	3.2150	0.3273	2.2590	0.2541	0.1404	2.4000	1.6905	0.2290	2.8365	1.7595	0.0923
<i>POU5F1</i> (0-600.343)	6.7220	5.7540	0.2506	6.7220	5.7540	0.2623	9.6385	3.2130	0.0758	6.0060	10.9950	0.5473
<i>LKB1</i> (0-0.442)	0.0233	0.0027	0.0712	0.0233	0.0163	0.2793	0.0226	0.0095	0.2623	0.0319	0.0057	0.0110 <sup>a</sup>
<i>VEGFA</i> (0-0.092)	0.0289	0.0331	0.7555	0.0278	0.0316	0.9378	0.0309	0.0316	0.6733	0.0278	0.0316	0.7618
<i>EGFR</i> (0-0.256)	0.0128	0.0183	0.4835	0.0142	0.0104	0.2886	0.0129	0.0189	0.7518	0.0148	0.0093	0.0377 <sup>a</sup>
<i>HGF</i> (0-1.2323)	0.1334	0.0316	0.7226	0.1792	0.0078	0.2741	0.14405	0.0158	0.4005	0.2008	0.03055	0.0621
<i>MET</i> (0-3.477)	0.26	0.066	0.1239	0.26	0.066	0.1851	0.257	0.059	0.0801	0.526	0.063	0.0310 <sup>a</sup>
<i>HIF1</i> (0-0.390)	0.0775	0.1553	0.5836	0.0775	0.1388	0.3581	0.0776	0.1130	0.9088	0.0776	0.0722	0.8361
<i>GLUT1</i> (0-8.189)	0.7059	0.3017	0.0151 <sup>a</sup>	0.6598	0.5140	0.2295	0.6408	0.1904	0.0274 <sup>a</sup>	0.7259	0.3981	0.0352 <sup>a</sup>
<i>BAX</i> (0-15.062)	0.7128	1.0788	0.0696	0.6855	1.0548	0.0339 <sup>a</sup>	0.7171	1.0668	0.1118	0.7751	0.7063	0.8655
<i>BCL2</i> (0-0.617)	0.0336	0.1139	0.6801	0.0366	0.0745	0.9204	0.0316	0.1696	0.2986	0.0393	0.0607	0.9835

Values of each target gene are expressed as the median value. JSCCR, Japanese Society for Cancer of the Colon and Rectum. Mann-Whitney U test. <sup>a</sup>P<0.05.

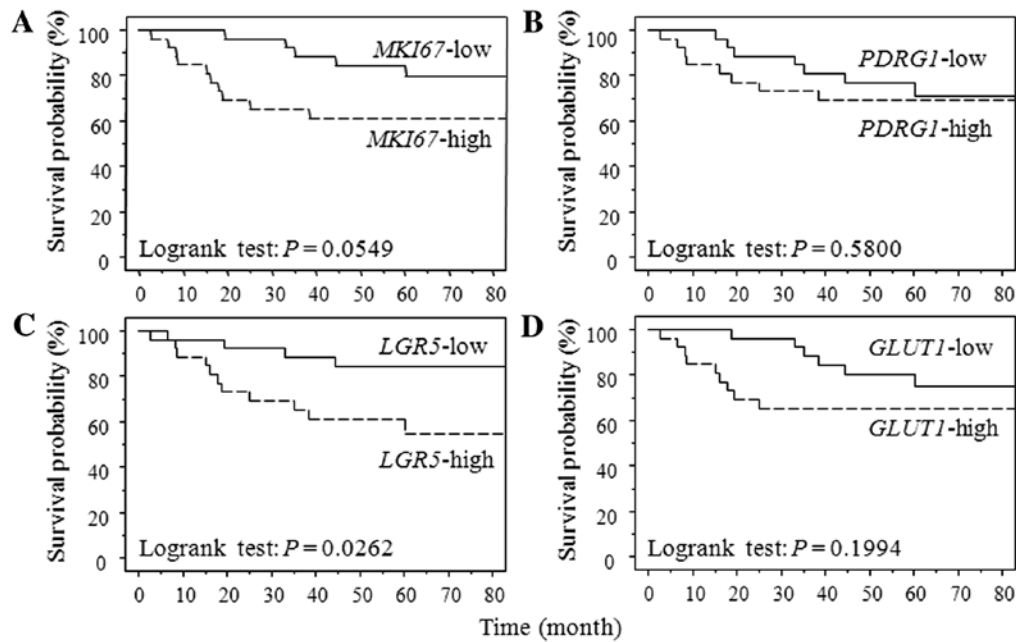


Figure 1. Kaplan-Meier curve for recurrence-free survival based on the expression of (A) *MKI67*, (B) *PDRG1*, (C) *LGR5* and (D) *GLUT1*.

rence rates and improved sphincter preservation and patient survival (23-26). However, tumor recurrence remains the major cause of mortality in these patients, and the mechanism of tumor recurrence after pre-operative CRT remains unclear. In this study, the expression levels of 20 genes were correlated with TRG to determine if gene expression levels can be associated with tumor recurrence in locally advanced rectal cancer after pre-operative CRT.

Tumor down-staging, post-operative stage, N, T classification and TRG have been identified as important prognostic factors in rectal cancer patients following pre-operative CRT (3-8,27-29). We observed that advanced post-operative stage, the presence of lymph node metastasis and poor pathological response based on JSCCR and Rödel TRGs were significantly associated with recurrence-free survival in this study of 52 patients (log-rank test; post-operative stage,  $P=0.0137$ ; presence of lymph node metastasis,  $P=0.0244$ ; JSCCR,  $P=0.0464$ ; Rödel,  $P=0.0338$ ) (data not shown). Our data were similar to previous studies although down-staging and T classification were not associated with clinical outcome. Transcriptional analyses of gene expression according to TRG were then performed. To the best of our knowledge, there are few reports on the correlation between TRG and gene expression of residual cancer cells after pre-operative CRT. The expression levels of eight genes were correlated with TRGs. *LGR5* gene expression levels in non-responders was significantly higher than those in responders in all four TRG systems. *LGR5* is a potential marker for stem cells in the small intestine and colon (30). Overexpression of *LGR5* has also been implicated in colorectal carcinogenesis (31). Elevated *PDRG1* and *GLUT1* gene expression level was significantly correlated with poor pathological response in three of the four TRG systems (JSCCR, Dworak and Rödel). *PDRG1* is regulated by DNA damage due to ultraviolet radiation and is implicated in tumor cell growth (32,33). *GLUT1* serves as a hypoxic indicator and is a hypoxic marker in colorectal cancer

(34). Immunohistochemistry of *GLUT1* expression in pre-treatment rectal cancer biopsy samples suggests that *GLUT1* might be a useful predictive marker of response to CRT in rectal cancer (35). *MKI67*, *LKBI*, *EGFR*, *MET* and *BAX* expression levels were significantly correlated with one to two TRG systems. *MKI67* is a proliferative marker (36) and *LKBI* has been implicated in the maintenance of hematopoietic stem cells and tumorigenesis through the suppression of apoptosis (37-39). *BCL2/BAX* act as anti- or pro-apoptotic regulator. We observed that high *BAX*, but not *BCL2*, expression was correlated with better pathological response based on Mandard TRG system. Reduction of *BAX* function in human colorectal cancer cells has been associated with resistance to chemotherapy (40) and *BAX* expression was correlated with outcome to neoadjuvant CRT (41) using immunohistochemistry in pre-CRT samples. Our result suggested that post-CRT *BAX* expression also might reveal the correlation of resistance to CRT. We examined whether gene expression of TRGs were associated with prognosis in rectal cancer after pre-operative CRT followed by surgery. We observed that patients with high *LGR5* expression had significant correlation to RFS. Elevated *GLUT1* expression was significantly associated with poor OS.

Biomarkers for tumor recurrence and prognosis after pre-operative CRT followed by curative surgery remain to be established. Our results may help identify prognostic predictors and clarify the strategy of adjuvant therapy for tumor recurrence after pre-operative CRT. However, it is not clear if the gene expressions correlated with TRGs are inherent or acquired after CRT due to the inability to compare expression levels between pre- and post-CRT samples. Therefore, these results did not directly show the predictive value of response to pre-operative CRT. We plan to examine the gene expression levels between pre-CRT biopsy and post-CRT samples.

In conclusion, TRG may reflect certain characteristics, such as proliferative activity, stemness potency and resistance

to hypoxia, of residual cancer cells after pre-operative CRT. Moreover, the gene expression levels correlated with TRG were associated with poor recurrence-free survival in patients with locally advanced rectal cancer after neoadjuvant CRT. However, data in this study should be interpreted with some caution. The major limitation was the small number of patients, especially for patients with recurrence, and the retrospective nature of the study. A larger study population will allow us to validate these conclusions.

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