The role of KLF5 and its mechanism for treatment resistance in preoperative chemoradiation therapy for rectal cancer

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Directed by Professor Nam Kyu Kim

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Jeong Yeon Kim

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ABSTRACT

The role of KLF5 and its mechanism for treatment resistance in preoperative chemoradiation therapy for rectal cancer

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Background

The identification of predictive molecular markers of tumor response to preoperative radiotherapy would provide an additional tool for selecting patients most likely to benefit from treatment.

The aim of this study was to determine whether Krüppel-like factor 5 (KLF5) expression in pre-irradiation tumor biopsies is a useful predictive marker of tumor response in patients with rectal cancer. Additionally, we verified the up- and downstream effectors of KLF5 in chemoradiation therapy resistance.

Method

Immunohistochemistry for KLF5, Ki-67, vascular endothelial growth factor (VEGF), p53, and C-ern2 was performed on 60 pre-irradiation biopsies from patients with completely responsive (ypT0) or nonresponsive tumors after preoperative radiotherapy. We multiplied intensity and percentage of KLF5 staining to produce a weighted score for each case ranging from 0 to 12. Each

factor was evaluated using several scoring methods and the association between KLF5 expression and tumor response was compared.

Result

The median patient age was 59 years. The clinical cancer stage was T3 and T4 in 50 (83.3%) and 10 (16.7%) patients, respectfully. Clinically positive lymph nodes were observed 29 patients (43.8%). Complete remission was achieved by 9 patients. VEGF, p53, and KLF5 were significantly associated with complete remission (p=0.04, p=0.05, p=0.02, respectfully). Additionally, KLF5 score was significantly associated with post-chemoradiotherapy pathologic T stage (p=0.032) and the presence of KRAS mutations (p=0.028). In vitro study, we western blot of KLF5 and DNA sequencing to check KRAS, BRAF mutation in colon cell line, HCT116, SW48, CaCO2, DLD-1, HT29. KRAS mutations were detected in DLD-1, HCT 116, and SW48 colorectal adenocarcinoma cell lines, and *BRAF* mutations were detected in HT-29 cell line. Stress, such as chemotherapy, radiation therapy, and UV therapy, stabilized KLF5 protein levels in a time- and dose-depended manner in HCT 116 and CaCO2 cells. We made KLF5 overexpression stable cell line with HCT 116(HCT 116 KLF5 OE). HCT 116 KLF5 OE exhibited significantly better cell viability compared to control cells, suggesting that KLF5 mediates cell survival.

Conclusion

Overexpression of KLF5 might be predictive of poor tumor regression after

preoperative CRT. Although KLF5 was significantly associated with the presence of KRAS mutations, its increased expression following chemo or radiation therapy in cell lines was independent of KRAS mutation status. Our study suggests KLF5 as a possible biomarker to predict chemoradiation response.

Key words : rectal cancer, preoperative chemoradiation therapy, KLF5, prognostic factor

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I. INTRODUCTION

Preoperative chemoradiation therapy (CRT) has become the standard treatment for patients with locally advanced rectal cancer.[1-2] This treatment has been shown to improve survival and may reduce local recurrence rates. In addition, tumor down staging may be achieved with preoperative radiotherapy, leading to greater sphincter preservation. Many studies have suggested that a patient's tumor regression grade is significantly associated with prognosis; specifically, patients with complete regression have a good prognosis.[3-4] However, 15~30% of patients still progress while being treated with CRT and go on to develop distant metastasis.[5-8] Therefore, predicting tumor response before treatment can significantly influence the selection of patients for preoperative CRT as well as potentially modify postoperative treatment plans. However, the currently available imaging modalities, including endorectal ultrasound, computed

tomography (CT), magnetic resonance imaging, or positron emission tomography used to restage patients after preoperative CRT lack the accuracy needed for this prediction.[9-10] For this reason, oncologists have a great interest in identifying molecular predictors of rectal cancer response to CRT.[11-12] Many molecular markers have been investigated, but as yet, the availability of markers able to predict CRT response is lacking. Typically, current markers are only able to identify secondary indicators of tumor response, typically clinical parameters including complete remission or tumor regression grade.

Recently Hur et al.[13] reported that incorporating multiple significant prognostic factors could increase the accuracy of predicting tumor response to CRT. In other words, one factor alone cannot explain or predict tumor response to CRT, suggesting that determining the detailed mechanism of tumor response to CRT is necessary.

Krüppel-like factor 5 (KLF5) is a zinc finger-containing transcription factor that is involved in diverse physiological processes including proliferation and differentiation of intestinal epithelial cells.[14] KLF5 has a pro-proliferative effect in cultured cells through activation of cell cycle regulatory proteins such as cyclin D1, cyclin B1, and Cdc2.[15,16] KLF5 is known to be increased by oncogenic $KRAS^{V12}$ and the BRAF-ERK-MEK cascade.[17] The aim of this study was to validate the role of KLF5, which is known to be downstream of the KRAS-BRAF cascade, in predicting tumor response to CRT.

II. MATERIALS AND METHODS

1. Patient selection

In four multicenters, 60 consecutive patients who received neoadjuvant chemoradiation for locally advanced (radiological T3–T4 or N+ and/or clinically bulky) colorectal cancer were enrolled in this study. Patients with distant metastases, recurrent disease, previous chemotherapy, pelvic radiotherapy, abnormal liver, kidney, or bone marrow function, or those aged less than 18 years or more than 75 years, were excluded. The study was approved by the scientific review and ethics committee at our institution. Written, informed consent was obtained from all the patients before the study. The initial clinical stage was based on a digital rectal examination, rigid proctoscopy, abdominopelvic CT, pelvic magnetic resonance imaging, chest CT, whole-body positron emission tomography/CT, complete blood cell count, liver function tests, and the serum carcinoembryonic antigen (CEA) level. The location of the tumor was defined as the distance between the caudal margin of the tumor and the anal verge, and this was measured by a digital examination and rigid proctoscopy. A biopsy was performed before starting chemoradiation therapy.

2. Preoperative chemoradiation therapy protocol

All patients received a total dose of 50.4 Gy with daily fractions of 180 cGy/d

over 5 weeks.

Chemotherapy was administered intravenously, consisting of 5-fluorouracil (5-FU; 425 mg/m^2/d) and leucovorin (20 mg/m^2/d) during the first and fifth weeks of radiotherapy. Experienced surgeons performed the radical surgery, which included total mesorectal excision, high vascular ligation (the inferior mesenteric artery and vein), and *en bloc* resection of the adjacent involved organs, 6 to 8 weeks following the completion of preoperative chemoradiation.

3. Tumor regression grading

The surgery included low anterior resection with colorectal or coloanal anastomosis and abdominoperineal resection. Use of a diverting stoma was subject to the surgeon's decision. The disease staging was based on the final pathological features of the tumor according to the seventh UICC tumor-node metastasis (TNM) staging system. Assessment of the tumor response to chemoradiation was performed by 2 experienced gastrointestinal pathologists according to the pathologic TNM staging and the tumor regression grading (TRG) systems based on the ratio of fibrosis to residual cancer. TRG scores were defined as follows: TRG1 (fibrosis <25%), TRG2 (25%≤ fibrosis <50%), TRG3 (50% ≤ fibrosis <75%), and TRG4 (fibrosis ≥75%).

Figure 1. Scoring of immunohistochemistry with KLF5

Intensitiy/%	**1 [0~25%]**	**2[25~50%]**	**3[50~75%]**	**4[75~100%]**
Weak 1+	**1**	**2**	**4**	**7**
2+	**3**	**5**	**8**	**10**
Strong 3+	**6**	**9**	**11**	**12**

4. Immunohistochemical staining

Immunohistochemical staining was performed using anti-Trp53, vascular endothelial growth factor (VEGF), C-ern, Ki67 . Before labeling, deparaffinized tissue sections were overlaid with 10 mmol/L citrate buffer (pH 6.0) and heated for 20 minutes in a pressure cooker. Next, slides were blocked with 5% bovine serum albumin in PBS and primary antibodies (Abcam, Ltd., Cambridge, United Kingdom) were applied at a 1:50 dilution, followed by incubation with biotinylated anti-rabbit secondary antibody (Dianova, Hamburg, Germany, at a dilution of 1:50 for 1 hour room temperature) and streptavidin/biotinylated alkaline phosphatase for 30 minutes. Finally, a nuclear Fast-Red solution or hematoxylin (37%) was used for counterstaining. Negative control slides, which lacked primary antibody, were included for each staining. The mean percentage of positive tumor cells was determined using an imaging system (Optimas 6.2, Stemmer PC Systeme, Puchheim, Germany) and used to assign each sample to one of the following categories: 0 (<5%), 1 (5-25%), 2 (25-50%), 3 (50-75%),

and 4 (>75%). The intensity of KLF5 immunostaining was scored as: 1+ (weak), 2+ (moderate), and 3+ (intense). The percentage of positive tumor cells and staining intensity were then multiplied to produce a weighted score for each case ranging from 0 to 12 (Figure 2B). Because of the limited number of patients to facilitate further statistical analysis, the weighted KLF5 score was arbitrarily dichotomized: "low-KLF5 expression" was classified as a KLF5 score of 5 or below and "high-KLF5 expression" as a score of 6 or above.

5. KLF5 immunohistochemistry

We especially add KLF5 immunohistochemistry (IHC) in this study. We previously compared the mRNA expression profiles of TRG grade 4 to TRG grade 1 pre-CRT tumor biopsies using a microarray. We found that the mRNA of KLF5 was four times higher in TRG1 specimens. Furthermore, in previous reports showed that KLF5 is a transcriptional factor acting down-stream of RAS-MAPK cascade and easy to work with IHC.[15-17] We made a hypothesis that each mutation of RAS, RAF, MER, ERK also influence the results of chemoradiation response, so, molecule of downstream such RAS-MAPK cascade would be more specific to predict CRT response.

6. Cell culture

The human colorectal adenocarcinoma cell lines, SW48, HCT-116 p53+/+, HCT 116 p53-/-, CaCo2, DLD-1, and HT-29 were obtained from the American Type

Culture Collection (LGC-Promochem, Wiesbaden, Germany). SNU-C4 5-FU-sensitive cell line and SNU-C4 5-FU-resistant cell line (which was generated by exposing cells to 5-FU for more than 6 months to create stable cell lines resistant to 5-FU), were obtained from Korean cell line bank (Seoul, Korea). The cells were maintained in DMEM (Biochrom, Berlin, Germany) and supplemented with 10% heat-inactivated FCS, 1% sodium pyruvate, and 2 mmol/L glutamine (all supplements from Biochrom) at 37°C, 5% CO_2, and 95% humidity.

7. Western immunoblotting

For immunoblotting, cells were washed and lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% deoxycholate] supplemented with protease inhibitors (1 mmol/L phenylmethylsulfonylfluoride, 10 ag/mL pepstatin, 10 ag/mL aprotinin, and 5 ag/mL leupeptin; all from Sigma, Deisenhofen, Germany). Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein (10 ag) were separated on a 12.5% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond C, Amersham, Freiburg, Germany). Membranes were blocked in 5% nonfat dry milk in PBS for 30 minutes at room temperature and probed with rabbit anti-KLF5, KRAS, cyclin D1, b-catenin, a-tubulin, Trp53, P21, Bax, or PUMA antibodies (dilution, 1:1,000, R&D Systems, Wiesbaden, Germany)

overnight at 4°C. Next, membranes were incubated with horseradish peroxidase-linked secondary antibodies (1:200, Dako, Hamburg, Germany) and developed by an enhanced chemoluminescence detection system (ECL, Amersham) and autoradiography (Biomax film, Kodak, Rochester, MN). To confirm equal protein loading, membranes were subsequently reprobed with a 1:2000 dilution of an anti-h-tubulin antibody (Biozol, Eching, Germany) or GAPDH (Biozol, Eching, Germany). For densitometric analysis, scanned autoradiographs were quantified using the AIDA software package (Raytest, Straubenstadt, Germany).

8.KLF5 overexpression stable cell line

For stable overexpression of the KLF5 gene, the fragment encoding the full length cDNA of KLF5 from the pSG5-KLF5 construct was cloned into the SmaI-XhoI sites of pLL-CMV-puro lentiviral vector. Plasmid DNAs were transfected into HCT 116 cells along with lentiviral packaging mix consisting of an envelope and packaging vector to produce lentivirus packed with KLF5 cDNA.

9. MTT assay

NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4, 5-dimethylthiazol-2-yl)-2,

5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. Other closely related tetrazolium dyes including XTT, MTS and the WSTs, are used in conjunction with the intermediate electron acceptor, 1-methoxy phenazine methosulfate (PMS). With WST-1, which is cell-impermeable, reduction occurs outside the cell via plasma membrane electron transport. Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials. MTT assays are usually done in the dark since the MTT reagent is sensitive to light.

9. Statistical Analysis

Statistical evaluation was carried out using the SPSS statistical package for Windows (Version 11.0; SPSS Inc., Chicago, IL). Differences between the two groups were tested with the chi-squared test and student's t-test. A value of $p<0.05$ was considered statistically significant.

III. RESULTS

1. Characteristics of patients

A total of 60 patients were included in this study, 49 males and 11 females. The median age of the patients was 59±18.3 years (range: 35–79 years), and the median distance of the tumor from the anal verge was 7.3±3.1 cm. Thirty-four (56.7%) patients exhibited a poor response to CRT (TRG 1 or 2), whereas 26

(43.3%) patients exhibited a good response (TRG 3 or 4). A pathologic complete response (pCR) or complete remission was observed in 9 patients (15%). Additional patient characteristics are provided in Table 1.

Table 1. Patients characteristics

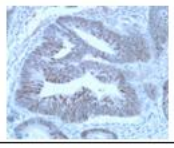
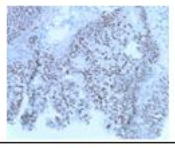
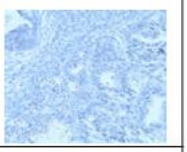
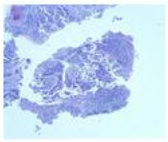
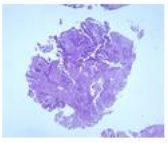
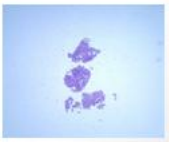
Age	No	(%)
≤60	34	56.7
>60	26	43.3
Sex		
Male	49	81.7
Femal	11	18.3
CEA		
≤5ng	36	60
>5ng	24	40
cT stage		
cT3	50	83.3
cT4	10	16.7
cN stage		
cN0	31	51.7
cN+	29	48.3
Anal verge	7.3±3.1	
Complete response		
pCR	9	15
Non-pCR	51	85
Tumor regression grade		
TRG 1-2	34	56.7
TRG 3-4	26	43.3
N Down grading		

Yes	19	31.7
No	41	68.3

2. TRG and N downstaging according to biomarkers.

Tumors of 6 of the patients (10%) harbored a *KRAS* mutation. High KLF5 immunohistochemical scores were observed 23 patients (38%). We immunohistochemically evaluated the expression of KLF5, as well as several tumor markers including epidermal growth factor receptor, Ki67, C-ern, and EGFR, p53. Additionally, we sequenced each tumor to determine *KRAS* mutation status. Relative expression levels and the correlation of those levels with either TRG or nodal (N) downstaging can be found in Table 2. High KLF5 scores and p53 were significantly associated with TRG (Figure 2). Additionally, a trend towards an association between *KRAS* mutation status and tumor regression was observed, but was not statistically significant. No examined factors related significantly to TRG or N downstaging.

Figure2. Correlation between KLF5 scoring system with TRG grading.

	TRG 1	TRG 2-3	TRG 4
Preoperative colon cancer Biopsy - KLF5 IHC			
KLF scoring	12	6	2
Postoperative Permanent biopsy - H & E			

2.The biomarker expression in tumor tissue for assessment of Tumor regression grade and Down grading

		N	Tumor regression grade			N Down grading		
			TRG 1-2	TRG 3-4	P	Positive	Negative	p
EGFR	N/1+	44	22	22	0.140	13	31	0.550
	2+/3+	16	12	4		6	10	
Ki67	High	44	26	18	0.568	14	30	1.0
	Low	16	8	8		5	11	
C-ern	N/1+	49	30	19	0.18	14	35	0.301
	2+/3+	11	4	7		5	6	
P53	Positive	31	13	18	0.021	9	20	0.573
	Negative	29	21	8		10	21	
KLF5	High score	36	26	10	0.004	14	22	0.167
	Low score	24	8	16		5	19	
Kras	None	52	27	25	0.062	16	35	0.593
	Mutation	8	7	1		3	6	

3. Correlation of clinical factors with pCR

Clinical factors, as well as the protein expression of several tumor-related markers, were evaluated to determine association with pCR (Table 3). By univariate analysis, VEGF and KLF5 expression were found to significantly correlate with pCR; however, after multivariate analysis, only KLF5 expression were still significant prognostic factors.

Table 3.Univariate and multivariate analysis of clinical variation and biomarker associated pCR

		Univariate analysis			Multivariate analysis		
		Yes(n=9)	No(N=51)	p	Yes(n=9)	No(N=51)	p
Age	≤60	8	26	0.064			
	>60	1	25				
Sex	Male	8	41	1.0			
	Female	1	10				
Pre CRT CEA	≤5	7	29	0.29			
	>5	2	22				
Clinical T stage	cT2/cT3	7	43	0.63			
	cT4	2	8				
Clinical N stage	cN(-)	5	26	1.00			
	cN(+)	4	25				
EGFR	N/1+	9	35	0.048	9	35	0.40
	2+/3+	0	16		0	9	
Ki67	High	6	38	0.44			
	Low	3	13				
p53	Posivie	2	24	0.089			
	Negative	7	27				
C-ern	N/1+	7	42	0.66			
	2+/3+	2	9				
KLF5	High	2	34	0.023	2	34	0.012
	Low	7	17		7	17	

4. Correlation of clinical factors with KLF5 scoring

Because KLF5 was determined to correlate with tumor response to treatment, we compared KLF5 expression to several clinical characteristics known to effect tumor response, including ypT and ypN stages, pCR, *KRAS* mutation status, pre CEA, and TNM stage (Table 4). The immunohistochemical KLF5 score significantly correlated with increasing ypT staging (p=0.005), *KRAS* mutation status (p=0.006), and pCR (p=0.005).

Table 4. correlation KLF5 with clinical factors

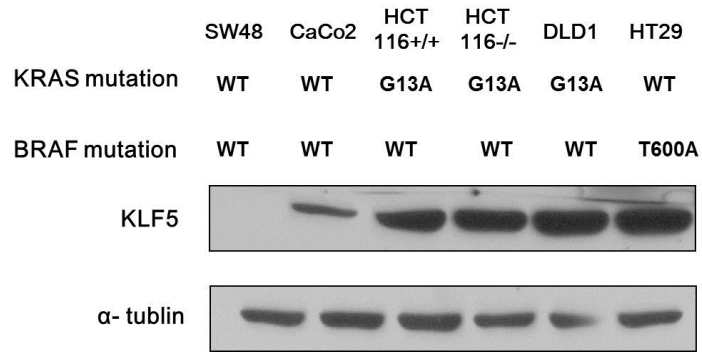
		N	KLF5 score	p-value
ypT stage	ypT0	23	5.13±2.56	0.005
	ypT1-3	37	7.02±2.39	
ypN stage	ypN0	44	6.18±2.76	0.843
	ypN+	16	6.34±2.58	
pCR	Complete	9	4.11±2.61	0.005
	Partial	51	6.68±2.43	
KRAS	Wild	51	5.92±2.54	0.006
	Mutated	9	8.44±1.94	
Pre CEA	≤5	36	6.13±2.78	0.563
	>5	24	6.54±2.35	
TNM stage	Stage 0-I	30	5.59±2.59	0.239
	Stage II-III	30	6.70±2.61	

5. In vitro study

KLF5 levels were elevated in human colorectal cancer cell lines with mutated KRAS

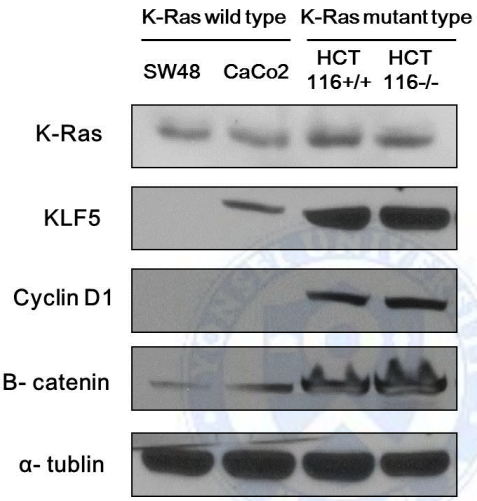
We examined 6 different human colorectal cancer cell lines for mutated KRAS and mutated BRAF. Through DNA sequencing, we determined that DLD-1, SW-48, HCT 116 p53+/+, and HCT 116 p53-/- contained KRAS activating mutations, while HT-29 and Caco-2 contained wild-type KRAS (Figure 3A). Only HT-29 contained a *BRAF* mutation. The level of KLF5 expression in the 6 cell lines correlated with *KRAS* genotype, with those containing mutated *KRAS* having higher levels. It is of interest to note that HT-29, which contains a mutated *BRAF* gene, exhibited higher levels of KLF5, similar to those cell lines harboring *KRAS* mutations (Figure 3A).

Figure 3. KLF5 and up/down-stream on colon cancer cell line



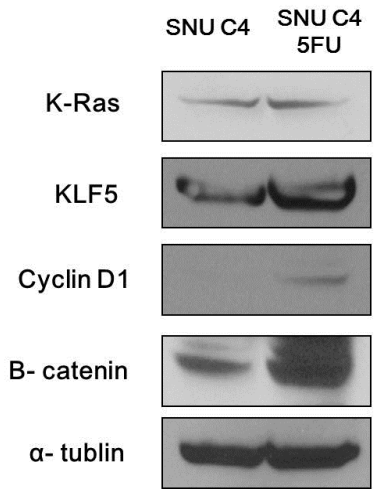
3A. Western of KLF5 depends on KRAS and BRAF mutation in colon cancer cell line

KRAS wild-type cell lines, SW48 and CaC02, had low levels of cyclin D1 and b-catenin, as well as low levels of KLF5. Conversely, HCT 116 p53+/+ and HCT 116 p53-/-, cell lines with activating *KRAS*^{G13D} mutations, exhibited higher levels of cyclin D1, b-catenin, and KLF5 (Figure 3B).



3B. Down-stream of KLF5 with or without KRAS mutation

Lastly, we evaluated the effect of chemo-resistance on KLF5 levels. SNU-C4 5-FU is a stable cell line that has been subjected to long-term exposure of 5-FU and as a result, has developed 5-FU resistance (Figure 3C). The level of KLF5 was increased in SNU-C4 5-FU compared to SNU-C4. Similarly, cyclin D1 and b-catenin were also increased.



3C, The changes in protein of KLF5 getting Chemoresistance in SNU C4

These results suggest that KLF5 has a significant relationship with activated KRAS or BRAF, such that the expression of KLF5 and its downstream effectors is significantly increased in cells with mutated KRAS or BRAF in colon cancer. Furthermore, KLF5 has an important role in cell survival in response to chemotherapy through increases in cyclin D1, leading to increases in the cell cycle.

The increase in KLF5 expression depends on chemo-radiation or UV therapy.

To determine the effect of chemotherapy and ultraviolet therapy on KLF, we compared KLF protein expression in HCT 116 ($KRAS^{G13D}$) cells treated with ultraviolet irradiation (UVR). Additionally, we compared KLF expression in

CaCO2 cells (wild-type *KRAS*) treated with or without 5-FU (Figure 4A). We found that KLF5 protein levels increased soon after DNA damage: levels were maximal after 10 GY of radiation in HCT 116 p53+/+. When similar experiments were carried out on p53-deficient derivatives of HCT 116 (HCT 116 p53 -/-), KLF5 levels increased in an identical fashion (data not shown). Similarly, in CaCo2 cells, KLF5 protein levels were maximal 24 h after 10 nM 5-FU treatment (Figure 4A).

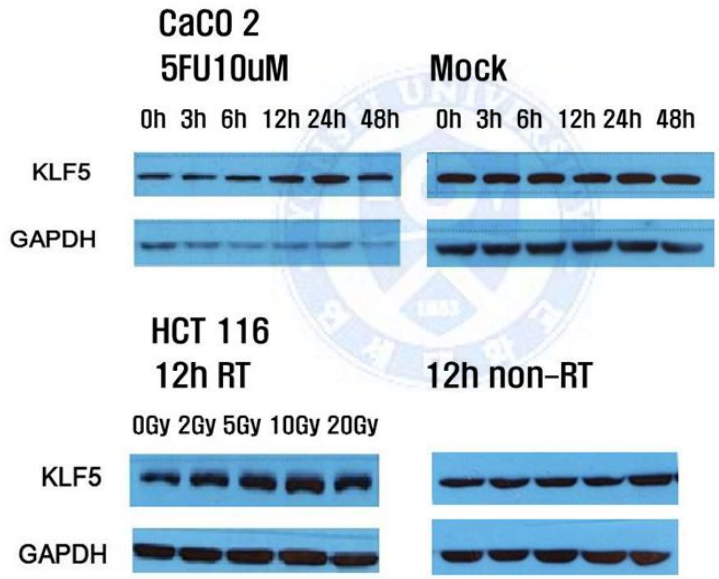


Figure 4A. Radiation therapy , chemotherapy increased KLF5 depends on dose or time regardless KRAS mutation

We performed a similar experiment in HCT 116 p53+/+ cells expressing endogenous KFL and those overexpressing KLF5 (HCT 116 KLF5 OE). Cells

were subjected to UVR for 24h, after which protein levels (Figure 4 B) and cell viability (Figure 4C) were evaluated. In both cell lines, KLF protein levels increased with UVR. As expected, both cyclin D1 and b-catenin were increased in the KLF-overexpressing cells, and this increase was amplified after UVR treatment. After exposure to UVR, HCT 116 KLF5 OE cells exhibited a significantly higher survival rate compared to control HCT 116 cells ($p<0.05$), further suggesting that KLF5 mediates cell survival.

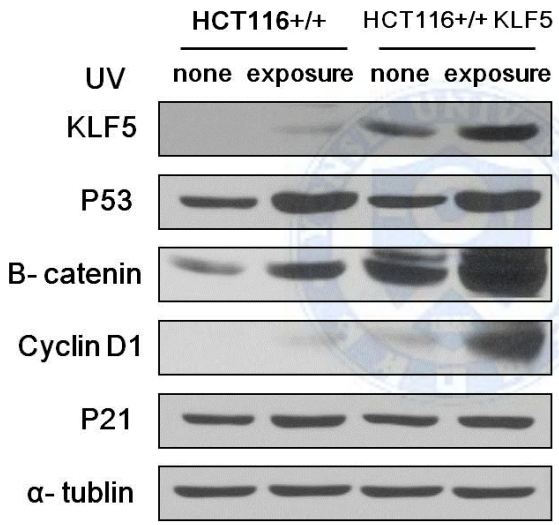


Figure 4B. KLF5 OE increased cell cycle in HCT 116

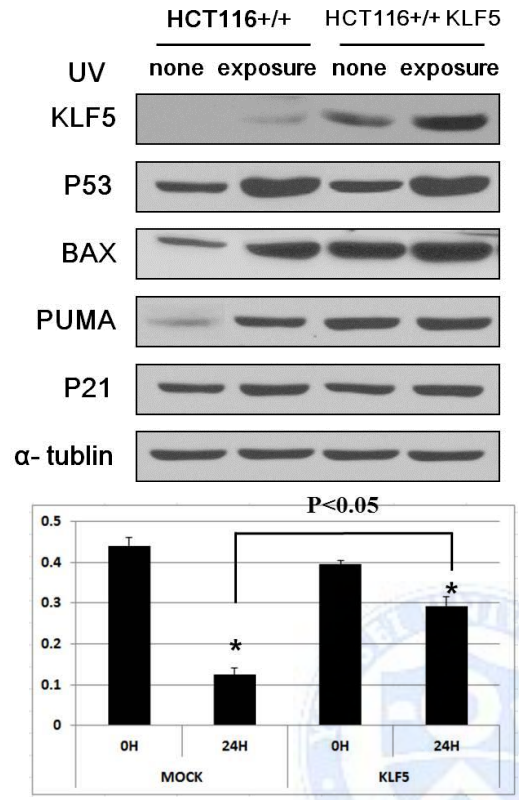


Figure 4C. KLF5 OE shows survival benefits in Cell death signals in MTT assay

IV. DISCUSSION

To the best of our knowledge, our study is the first article to explain the mechanism how tumor can survive in threatened stress like CRT. We found that protein of KLF5 which related with KRAS, BRAF mutation is a crucial mediator to resist CRT in rectal cancer. And that reason, KLF5 expression was could be a specific biomarker to predict TRG response after CRT in rectal cancer patients.

In recent years, several promising candidate markers have been reported potential roles in the prediction of radiation response, including angiogenesis [thymidine phosphorylase (TP), thymidylate synthase(TS), and vascular endothelial growth factor (VEGF)], apoptosis[bax, p53, nuclear factor-kappa B (NfkB), and survivin], proliferation[cyclooxygenase (COX)-2 and proliferating cell nuclear antigen(PCNA)], and cell adhesion or collagenease [CD44, CD133, matrixmetalloproteinase (MMP) 2, and MMP9] with regard to preoperative CRT in rectal cancer.[18-27] Also, few studies have evaluated KRAS as a biomarker for tumor response in rectal cancer patients treated with CRT and TME. Garcia-Aguilar et al[28] described a series of rectal cancer patients treated with preoperative CRT and reported that tumors with wild-type KRAS were more likely to respond to CRT than tumors with mutant KRAS. But such biomarkers are still not specific, so, the various results of significant factor are different.

Our study also showed the tendency of KRAS mutation as a prognostic marker but not having a statistical significance. KRAS mutation was observed only just 15% in our study while as reported 35~40% in previous studies.[29,30] And so in small scale study has limitation that KRAS mutation is being a prognostic factors. But, KRAS mutation is most important factor in treatment in colorectal cancer. [31] So, some other biomarker should be needed to represent the phenomenon of KRAS mutation. So, we select KLF5 IHC method to predict chemoradiation therapy response which could contain the KRAS mutation

effect. Furthermore, IHC of KLF5 is very well stained in normal colonic mucosa, so, its base control is very good. And the study time of IHC stain is short, better to decide patient's treatment plan.

We find out HCT 116 p53+/+, HCT 116 p53_/_, DLD1 has KRAS mutation and HT 29 do not have RAS mutation but RAF mutation. Although HT-29 do not have RAS mutation, protein of KLF5 is significantly high (Fig 3A). Nandan MO et al[17], previous reported that KLF5 is linked to MAPK pathway in colon cancer. In that report, protein level of KLF5 was modulated by RAS mutation or ERK, MEK phosphorylation. On this mechanism, the molecule of downstream such like KLF5 would be more specific biomarker compared to upstream molecule such like RAS. So, we should validate the mechanism to predict CRT response exactly.

Especially, KLF5 stained scoring system showed positive relationship with TRG grade and T down stage. KLF5 is known to be a crucial role in the maintenance of cellular proliferation, cyto-differentiation, and morphology of the crypt-villus axis.[37] And it is amplified in colorectal cancers suggesting a contributory role in tumorigenesis to regulate cell cycle components such as Cyclin D1, Cyclin B1, and Cdc2.[16,36] This result suggested that the response rate of CRT is influenced by the protein level of KLF5 in patients because of KLF5 role in tumor proliferation via cell cycling molecule.

In HCT 116 with KRAS mutation, protein level of KLF5 is increased as dose dependent with radiation therapy or UV therapy was inducted. We also studied

with CaC02, it also had KLF5 protein, although CaCo2 is KRAS wild type. Interestingly, it showed same tendency of increasing protein of KLF5 as time or dose dependent with treated 5-FU chemotherapy or radiation therapy same as previous study already demonstrated.[35] Independent with KRAS mutation, KLF5 is directly influenced by stress such like UV, radiation and chemotherapy. But another important data was that the baseline protein level of KLF5 has decisive effect when stress (radiation, chemotherapy, UVR) would be added. Previous reports already demonstrated, protein level of KLF5 is increased as amount dependent with KRAS mutation.[22] So, wild type colon cancer has low level of KLF5 and less tend to resistance tumor regression by CRT. And our patient's data with KRAS mutation results high KLF5 score in pretreatment biopsy and had trended to relate with poor tumor regression. So, we thought KLF5 protein has been amplified following CRT and disturb Tumor down grading to make active cell cycle via cyclin D1, cyclin B1 and Cdc2.

Last, we also confirmed that KLF5 is important factor for tumor to survive from CRT. We made stable cell line with HCT116 which gets overexpress of KLF5. We made a stress with UV in both HCT116 and HCT116 KLF5 OE. After 24h, we assayed MTT. KLF OE HCT116 showed apoptosis markers such as bax, P53, PUMA. But remained odds of survival cell was significantly higher than MOCK. As long exposure of 5 FU in SNU C4, protein level of KLF5 is signifancly increased (Fig 3C). And our patient's data exactly showed that high KLF5 score patients got poor tumor regression. These results suggested, KLF5

is a crucial medicator to survival when stress inducted. We thought that KLF5 expression would trigger cell cycle activation via cyclin D1 or cyclin B1/CdC[15], and maintain bonding each other or adhesion another site via b-catenin.

Our study has some limitation. But it was the first report to validate the mechanism based on the cascade.

V. CONCLUSION

Overexpression of KLF5 in pretreatment biopsies might be predictive factor of poor tumor regression after preoperative CRT. KLF5 was significantly related with KRAS mutation and KLF5 has crucial role to get resistance from preoperative CRT. Our study suggested one possible mechanism of biomarker to predict chermoradiation response

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ABSTRACT(IN KOREAN)

제목직장암에서 항암방사선 치료 저항성을 갖게 되는 KLF5의 역할과 그 기전 연구

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김 정 연

배경 직장암에서 수술전 항암방사선 치료의 치료 반응에 대한 예후인자를 찾는 노력은 계속되어 왔다. 이 논문에서는 항암방사선 치료 전 암조직을 이용해 Krüppel-like factor 5 (KLF5)라는 전사인자의 발현이 직장암 항암방사선 치료의 예후를 예측하는 예측 인자로써의 가능성을 알아보고자 한다.

방법 60명의 항암방사선 치료를 시행받은 직장암환자에서 항암방사선 치료전 조직을 이용해 KLF5, Ki-67, VEGF, p53과 C-ern2의 조직 면역 화학 염색를 시행하였다. 그리고 KLF5의 검사결과를 염색 정도와 양을 바탕으로 0~12점의 점수를 매기고 그 결과와 항암방사선치료 반응결과 및 임상정보를 비교 분석하였다.

결과 평균연령은 59세였으며, 검사 병기로는 T3가 50명, T4가 10명으로 각각 83.3%, 16,7% 의 비율이었다. 검사 병기에서 림프절 전이가 의심되는 환자가 29명(43.8%)였다. 항암방사선

치료를 통해 완전 관해가 된 환자가 9명이었으며, 이는 VEGF, p53, KLF5와 통계학적으로 연관성이 있는 것으로 확인되었다.
특히 KLF5 점수는 T병기 반응과의 연관성을 강하게 보여주었으며 (p=0.032) , KRAS의 변이와도 연관성이 있었다 (p=0.028).
그리고 in-vitro연구에서 대장암 세포주인 DLD-1, HCT116, SW48, HT29, CaCO2에서 KLF5의 western검사와 KRAS mutation을 DNA sequencing 검사를 통해 알아보았다. 그 중 DLD-1과 HCT116, SW48에서는 KRAS mutation을 관찰할 수 있었으며, HT29에서는 BRAF mutation을 관찰할 수 있었다. 또한, KRAS와 BRAF mutation이 관찰된 세포주에서는 KLF5의 단백질 양이 증가되어 있는 것을 알 수 있었고 그 하위로 알려진 cyclin D1의 단백질 양도 같이 증가되어 있는 것을 알수 있었다. 또한 CaCO2와 HCT 116에서 5-flurouracil (5FU)을 처리했을때 KLF5의 단백질 양이 5FU의 용량에 비례하여 같이 증가하는 것을 알 수 있었다.
HCT116 세포주에 KLF5를 과발현 시켰으며 과발현 시킨 세포주에서 정상세포주에 비하여 cell death가 적게 일어나는 것으로 MTT assay를 통해 확인하였다.

결론 KLF5의 과발현은 직장암에서 수술 전 방사선치료의 반응을 낮추는 예후인자임을 알 수 있다. KLF5는 KRAS 의 변이와 연관성이 있으나 변이와 상관없이 항암 방사선 치료에서 그 단백질의 발현이 증가되는 경향이 있다. KLF5는 직장암

환자에서 항암방사선치료의 좋은 예측인자가 될 것이라고 생각된다.

핵심되는 말 : 직장암, 항암방사선치료, 예측인자, KLF5