Prediction of pathologic complete remission after preoperative chemoradiation in rectal cancer with multidimensional approach including 3D cell culture

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Directed by Professor Nam Kyu Kim

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ABSTRACT

Prediction of pathologic complete remission after preoperative chemoradiation in rectal cancer with multidimensional approach including 3D cell culture

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Background: Preoperative chemoradiotherapy (preop-CRT) followed by total mesorectal excision for locally advanced rectal cancers has been adopted widely. Although pretreatment factors that can predict preop-CRT responses are continually investigated for their applicability to risk-adaptive therapy, little has been validated simultaneously using the same patient cohort. The aim of this study is to develop a system for predicting good tumor response after preop-CRT using multidimensional parameters including clinicopathological factors, endoscopy, MRI and 3D cell culture.
Materials and Methods: The patients for this study were divided into the study/validation set and the experimental set. Between 2012 - 2014, 60 patients with locally advanced rectal cancer treated with preop-CRT were retrospectively selected. All patients had undergone pre- and post-CRT MRI and endoscopy after completion of CRT, and these patients constituted the study set. Between August 2015 and March 2017, 49 patients with locally advanced rectal cancer that underwent preop-CRT were prospectively collected and assigned to the validation set. Among these 49 patients, 3D cell culture results were available for 26, and these patients comprised the experimental set. Univariate and multivariate logistic regression analysis of clinical, endoscopy, and MRI findings were used to predict good tumor response in the study

set. A nomogram was developed for the study set (n=60) and validated in the validation set (n=49). Factors associated with pathologically complete response (pCR), tumor regression grade (TRG) good response (TRG I and TRG II) and lymph node positivity were analyzed with forward or backward stepwise logistic regression analysis.

Results: In the study set, multivariate analysis revealed the following as predictors of pCR: Endoscopy_grade and MRI_TVRR (magnetic resonance imaging tumor volume reduction rate) more than 83.6 [odds ratio(OR): 29.8; 95% confidence interval(CI), 4.9–181.2; $p < 0.001$ and OR: 7.0; 95% CI, 1.0–46; $p < 0.041$, respectively]. Endoscopy_grade and MRI_DW (OR: 7.6; 95% CI: 1.2–45.9; $p < 0.027$ and OR: 7.8; 95% CI: 1.9–32.3; $p = 0.004$, respectively) were associated with TRG good response. The model for predicting pCR using logistic regression analysis was suggested with tumor location, Endosocpy_grade, MRI_TRG, and MRI_TVRR. The nomogram using these variables showed good discrimination ability in both the study set [AUC (area under the receiver operating characteristic curve) = 0.894] and the validation set (AUC = 0.750). In the experimental set, using the receiver operating characteristic curves of cytotoxicity assay and lymph node status after surgery, the AUC was 0.801. In multivariate analysis, cytotoxicity assay was the only factor that predicted yp node positivity (OR: 13; 95% CI: 1.2–133.2; $p = 0.031$)

Conclusion: This study demonstrated that the combination of endoscopy and MRI parameters could predict good tumor response. Cytotoxicity assay after 3D cell culture with radiation may be a viable option to predict persistent lymph node metastasis after preop-CRT. The clinical impact of this approach should be validated in large patient cohorts.

Key words: rectal cancer, magnetic resonance imaging, 3D cell culture, TRG, pCR

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I. INTRODUCTION

Preoperative chemoradiotherapy (preop-CRT) followed by total mesorectal excision (TME) for locally advanced rectal cancer has been adopted widely due to clinical advantages such as an increased probability of anal sphincter-preservation and decreased local recurrence rate.[1-3] According to the current National Comprehensive Cancer Network clinical guidelines, preop-CRT is highly recommended for patients with T3-4 and/or node positive rectal cancer.[4] Nevertheless, there is some debate on its indications. Some authors have suggested that rectal cancer patients clinically staged as T3N0/N+ or T2N+ with free margin >2 mm from mesorectal fascia could avoid preop-CRT and undergo TME alone without any deterioration of survival outcomes.[5]

A pathologic complete response (pCR) is seen in 10% to 31% of patients, which indicate good oncologic outcomes in terms of recurrence and survival.[6,7] In contrast, 20-25% of patients did not show any regression. A high rate of sphincter preservation can be achieved by shrinking tumor bulk, and, at the same time, unconventional surgical options such as local excision or no surgery at all(the “wait-and-see” policy) can be considered in patients with good clinical tumor response.[8-13] Recent data showed that 74% of patients with clinical complete response (cCR) did not require

salvage surgery on long-term follow up.[8] Maas et al. also reported 5% local recurrence rate after the wait-and-see policy.[9] Local excision is regarded as one possible treatment option after good response following preop-CRT.[11] The main arguments for these alternative modalities are that they can preserve the sphincter, reduce morbidity, and decrease functional impairment of defecation after rectal cancer surgery.[14] Selecting an appropriate approach after preop-CRT requires adequate preoperative staging, and patients must be selected on a highly individualized basis.

However, tumor response after preop-CRT for rectal cancer varies considerably, with some patients showing a complete absence of viable tumor cells whereas others show masses of tumor cells with little or no regressive change. In rare cases, tumor progression during preop-CRT was observed; for these non-responders, preop-CRT did not benefit the patients. Therefore, it is essential for patients' quality of life and prognosis to select the proper treatment modality based on predicted tumor response.

Pretreatment factors that can predict preop-CRT responses are continually being investigated for their applicability to risk-adaptive therapy in locally advanced rectal cancer patients. To assess clinical tumor response, clinicians have used digital rectal examinations, rigid sigmoidoscopy, colonoscopy, transrectal ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET). In addition, researchers have investigated molecular markers and gene analysis to select good responders. Various clinicopathologic parameters, such as carcinoembryonic antigen (CEA)[15], distance from anal verge[16], time interval between the CRT and surgery[17,18], colonoscopy findings[19,20], MRI parameters (3D volumetry[21,22], diffusion weighted image[23,24], MR TRG[25]), and biologic markers[26,27] were suggested as a possible predictors of good response after preop-CRT. Although various parameters have been identified as potential surrogates for response, little has been validated simultaneously using the same cohort of patients.

In clinical practice, it was reported that regional lymph node metastasis (LNM) after rectal cancer surgery is a poor prognostic factor.[28,29] Even after preop-CRT in rectal cancer, persistent LNM was the most indicative poor prognostic factor.[7,30] Another unresolved issue regarding wait-and-see or local excision after good tumor response is the possibility of nodal metastasis. Swellengrebel et al. reported 28.5% of patients with pCR had persistent regional LNM and 41% of near-pCR patients had ypT3 stage.[31] It is difficult to have confidence that even though the patients revealed good clinical response after preop-CRT, the patient's nodal status is as good as the tumor response. Although several researchers investigated predictors of ypN-positive status after preop-CRT, the studies have still some limitations because the factors used in the nomograms are sometimes difficult to obtain before radical resections.[32,33] Considering the low predictive rate of the current imaging modalities for restaging LNM,[34,35] new approaches to predict LNM after preop-CRT are still warranted.

3D cell culture system was widely used because cells in 3D environments might be more similar to cells in living organisms (in vivo) than flat, unnaturally thin, single-layer cells grown in 2D plastic environments. Typical cells in 3D are ellipsoids with dimensions of 10-30 μm; in contrast cells in 2D are flat with typical thickness of 3 μm. With regard to environmental comparison, typical cells in 3D have nearly 100% of their surface area exposed to other cells or matrix whereas cells in 2D have approximately 50% of their surface area exposed to fluid, approximately 50% exposed to the flat culture surface or intermediate, and a very small percent exposed to other cells. In preliminary data analysis of spheroid-based drug screening system, it was reported that the percentages of tumor spheroid size and morphology in a 3D culture system correlated with in vivo efficacy.[36] However, the efficacy of 3D culture after radiation has never been applied in evaluating tumor response after preop-CRT in locally advanced rectal cancer.

Therefore, with this study, we aimed to evaluate the efficacy of clinically available important parameters such as clinicopathologic outcomes, endoscopy findings, MRI

examinations, and 3D culture systems in predicting good tumor response after preoperative chemoradiotherapy in rectal cancer.

II. MATERIALS AND METHODS

1. Inclusion criteria

For this study, we included the following patients: histopathologically confirmed adenocarcinoma of the rectum, tumor distance less than 15 cm from anal verge, age over 20, patients that underwent preop-CRT, radiological stage II or III, and Eastern Cooperative Oncology Group performance status ≤ 2. The exclusion criteria were M1 status at initial diagnosis of rectal cancer, synchronous cancer, previous chemotherapy or radiation therapy, history of hereditary non-polyposis colorectal cancer or familial adenomatous polyposis, or incomplete preop-CRT. All patients underwent preoperative MRIs reviewed by an experienced radiologist. Clinical node was positive if regional lymph nodes were found to be larger than 10 mm or had speculated and indistinct borders.[37]

The included patients were composed of the study/validation set and the experimental set. Between 2012 and 2014, we retrospectively selected 60 patients with locally advanced rectal cancer treated with preop-CRT. All patients had undergone endoscopy and pre- and post-CRT MRI examinations, and these patients constituted the study set. Between August 2015 and March 2017, 49 patients with locally advanced rectal cancer who underwent preop-CRT were prospectively selected, and we assigned them to the validation set. Among these 49 patients in the validation set, we had available results for 26 patients for 3D culture with radiation before preop-CRT, and these patients comprised the experimental set.

2. Treatment of patients

A. Radiation therapy and chemotherapy

Preoperative radiation therapy consisted of a total dose of 45 Gy in 25 fractions delivered to the pelvis, followed by a 5.4 Gy boost to the primary tumor, over a period

of five weeks (1.8 Gy for 5 days). All patients underwent 3D conformal treatment planning using CT simulation. Pelvic irradiation was administered with a 6-MV/10-MV dual-photon linear accelerator. The pelvic radiation volume was as follows: the superior border 1.5 cm above the sacral promontory (L5 level), the inferior border at the inferior margin of the obturator foramen or 3 cm below the lower tumor margin, the lateral border 1.5 cm lateral to the bony pelvis, the anterior border 3 cm anterior to the tumor, and the posterior border 0.5 cm posterior to the sacral surface. The prescription dose was specified at the isocenter; the three-field treatment plan comprised a 6-MV photon posterior-anterior field and 6- or 10-MV photon opposed lateral fields with wedges of 45°. In the boost treatment, five ports were used.

Patients were given combined chemotherapy during preop-CRT with intravenous bolus 5-FU/leucovorin (425/20 mg/m^2 once per day during weeks 1 and 5) or capecitabine (825 mg/m^2) twice daily. Postoperative systemic adjuvant chemotherapy was selectively administered to patients with 5-FU/leucovorin, capecitabine, 5-FU/oxaliplatin, or capecitabine/oxaliplatin according to the yp stage.

B. Surgery

Surgery was scheduled 6 to 12 weeks after the completion of preop-CRT and was performed by expert colorectal surgeons who adhered to the oncologic principles of TME with pelvic autonomic nerve preservation. TME advocates sharp pelvic dissection based on pelvic anatomy under direct vision along the plane of the proper rectal fascia, resulting in en bloc removal of rectal cancer and the surrounding mesorectum containing lymph nodes.

3. Data collection

A. Patient demographics

Patient demographics included clinicopathological parameters such as age, gender, distance from anal verge, carcinoembryonic antigen, body mass index, clinical TNM stage, pathologic TNM stage, cell differentiation, and operation type.

B. Endoscopy

Patients underwent endoscopies 4–6 weeks after completion of preop-CRT. Using endoscopy, we evaluated and recorded variables such as distance from anal verge, size and morphologic categorization, and detection of scar or ulcer lesion (in case of no residual tumor). We scored endoscopic findings as follows: 0-normal mucosa, 1-whitish scar, 2-red scar, 3-ulcer (< 1 cm) without remaining visible tumor, 4-ulcer (≥ 1 cm) without remaining visible tumor, 5-remaining mass (< 2 cm) regardless of ulcer, or 6-remaining mass (≥ 2 cm) regardless of ulcer. We categorized scores of 0–2 into Endoscopy_CR, scores of 3 were Endoscopy_near CR, and scores of 4–6 were Endoscopy_non CR (Figure 1).

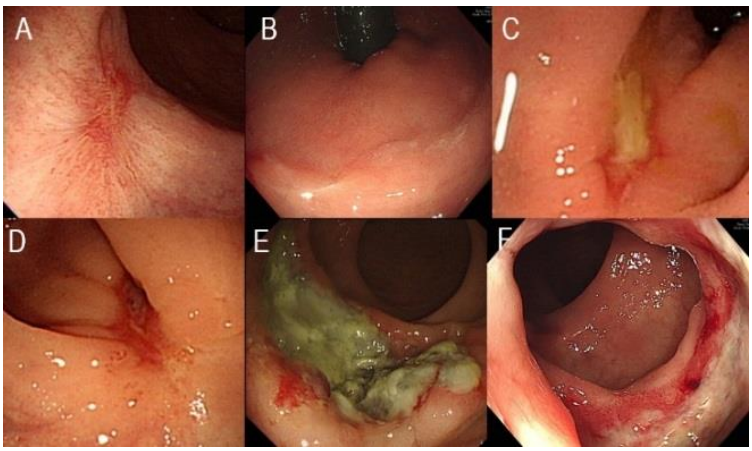

Figure 1. Endoscopic findings of rectal tumors after preoperative chemoradiotherapy (A,B - Endoscopy_CR; C,D - Endoscopy_near CR; E,F - Endoscopy_non CR)

C. MRI evaluation

(A) Image acquisition and analysis

All patients in this study underwent pre-CRT MRI before the start of preop-CRT and underwent post-CRT MRI 4–6 weeks after completion of preop-CRT. All pre- and post-CRT MRI examinations were performed with a 3.0 T scanner (Magnetom Tim Trio, Siemens Medical Solutions, Erlangen, Germany or Ingenia, Philips Medical

Systems), in which a 6-element body phased-array coil was applied to the anterior side of the patient and another 6 elements of the spine coil were applied to the posterior side. To reduce bowel peristalsis, 20 mg of scopolamine butylbromide (Buscopan; Boehringer Ingelheim Korea, Seoul, Republic of Korea) was injected intramuscularly approximately 5 minutes before the MRI examination. T2-weighted MR images were obtained in axial, sagittal, oblique axial, and coronal orientations using a respiratory-triggered echo train spin echo sequence. All MRI studies were analyzed via image archiving/communication system workstation (Centricity, GE Healthcare, Milwaukee, WI, USA). A gastrointestinal radiologist who had more than 5 years of experience in bowel imaging and did not know the tumor response results assessed the images.

(B) MR_TRG

MR_TRG was defined to determine the degree of tumor replacement by fibrotic stroma on MRI based on principles similar to the pathologic TRG system originally described by Dwork.[25,38]

We determined the tumor regression grade by comparing the pre- and post-CRT MRI scans. Using T2-weighted images, we assumed that tumor components with intermediate T2 signal intensity represented soft tissue (viable tumor) and those with dark T2 signal intensity represented fibrosis. We scored each patient as one of the following 5 grades based on the system provided by the MURCURY group.[39] In brief, if fibrosis signal intensity predominated with no or minimal residual intermediate tumor signal intensity, we assigned a TRG of 1 or 2, respectively. If substantial tumor signal intensity was present but did not predominate the fibrosis, the TRG was 3. If there was a predominance of tumor with minimal low signal intensity fibrosis, we assigned a TRG of 4, and if the tumor seemed unchanged after preop CRT, the TRG was 5.

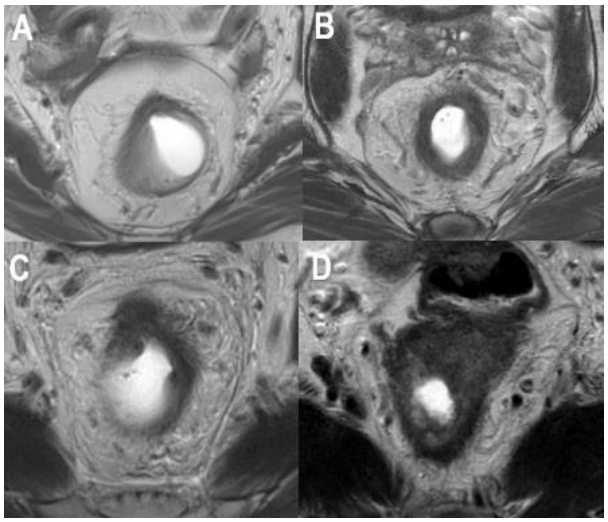

Figure 2. Post-CRT MRI showed different tumor responses. Following the MURCURY group grading system, they were: complete radiologic response (A, grade 1), dense hypointense fibrosis with minimal residual tumor (B, grade 2), ~50% fibrosis/mucin and intermediate signal representing residual tumor (C, grade 3), and minimal fibrosis/mucinous degeneration, mostly tumor (D, grade 4).

(C) Diffusion-weighted image (DWI) of MRI

DWI was defined as tissue characterization based on differences in random movement of water molecules as a surrogate marker for microstructural density (with or without restriction) All DWI studies were generated using a respiratory-triggered echo-planar sequence at three b values, 0, 300, and 1000 sec/mm^2. Axial isotropic DWIs were also acquired using a respiratory-triggered echo-planar sequence. Using post-CRT MRI, we graded the absence or presence of diffusion restriction as negative, equivocal, or positive. When we applied the values in the analysis, we grouped positive and equivocal into one group and negative into another.

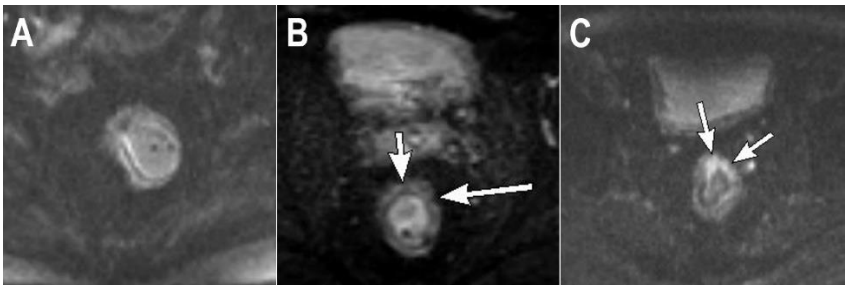

Figure 3. Diffusion-weighted image grading in MRI (A – Negative; B – Equivocal; C – Positive).

(D) MRI tumor volumetry

To measure tumor volume, we archived T2-weighted oblique axial images in Digital Imaging and Communications in Medicine (DICOM) format and stored them on the image archiving/communication system workstation (Centricity, GE Healthcare). We drew a region of interest (ROI) along the margin of the rectal mass on each slice and calculated the total mass was by adding ROI areas in consecutive images. After we manually segmented the tumors on oblique axial T2-weighted MRI images, the tumor volumes were automatically calculated. Tumor volume reduction rate (TVRR) (% decrease) was calculated as follows: ([Pre-treatment tumor volume]-[Post-treatment tumor volume]) × 100 / (Pre-treatment tumor volume).[21]

D. Evaluation of 3D cell culture and radiation

In the experimental set collected prospectively, we performed cytotoxicity assay and morphologic evaluation.

(A) 3D cell culture

Tissue harvest

Tissue was sampled before the start of the preop-CRT. After tissue samples were obtained from the enrolled patients using rigid sigmoidoscopy and forceps biopsy, cancer tissue was put into 25T flask filled with RPMI (Roswell Park Memorial Institute) culture media with 1% penicillin-streptomycin. This medium was delivered to the laboratory facilities at room temperature.

Cancer tissue dissociation & 2D culture

After all media were removed from the 25T flask, the tissue was transferred to fresh 1.5 ml micro tubes, and the weight was measured. The tissue was transferred to 60-mm dishes filled with 5 ml RPMI culture medium with 1% penicillin-streptomycin and washed by pipetting, and this procedure was repeated 2 times. The tissue was transferred to 60-mm dishes filled with 5 ml trypsin EDTA, and the tissue was chopped using a blade (Nopa, Tuttlingen, Germany); this was incubated with trypsin EDTA (Hyclone, Logan, UT, USA) for 20 min in a CO2 incubator. After the chopped tissue was harvested using 5 ml RPMI 1% penicillin-streptomycin 10% FBS, it was transferred to 15-ml conical tubes, and the solution was centrifuged at 1,200 rpm for 2 min. After the supernatant was removed and the pellets were resuspended using 10 ml RPMI 1% penicillin-streptomycin 10% FBS, all the chopped tissue was seeded to 100-mm 2D culture dishes (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C for 2–3 days.

Cancer cell 3D Culture

After the cells were harvested by pipetting, they were put into 15-ml conical tubes. After they were centrifuged at 1,200 rpm for 2 min, the supernatant and resuspended pellets with 5 ml RPMI 1% penicillin-streptomycin were discarded. After centrifuging at 1,200 rpm for 2 min, the 2D cultured cells were detached by Accumax (Millipore, Billerica, MA, USA). We counted the dissociated cells with a C-Chip disposable hemacytometer (INCYTO, Chungnam-do, Korea). We diluted the cells to 1 ml RPMI 1% penicillin-streptomycin 10% FBS to make 5000 cells/100 ul and

seeded the suspended cells in ultra-low attachment 96-well 3D culture plates (Corning, NY, USA).

Radiation treatment

Defining the day on which cells were re-seeded in 3D culture as day 0, we treated 3D cultured cells with 5 Gy radiation at day 3 (Gammacell low dose-rate irradiator, MDS Nordion, Canada). The control group did not receive radiation. The cultured cells were incubated at 37°C for 2 days.

Imaging before and after radiation treatment

Images of 3D cultured cells before and after 2 days of radiation were acquired by Cell Scanner (MBD, Korea) equipped with a 4X objective lens. After the cell and culture media were transferred to 1.5-ml tubes, we centrifuged the tubes at 1,000 rpm for 2 min. We maintained the transferred supernatant and pellets in each tube at -20°C.

(B) Cytotoxicity test

Defining the day on which cells were re-seeded in 3D culture as day 0, the cultured medium was harvested, and lactate dehydrogenase (LDH) enzyme activity was determined to check cytotoxicity at day 5 (2 days of additional incubation after radiation treatment). The cells were separated from the harvested media by centrifugation at 1,000 g for 15 min. The supernatant was collected and the LDH enzyme activity was measured using an LDH-cytotoxicity assay kit (BioVision, USA) following the manufacturer's instruction. To calculate the ratio between LDH enzyme activity in the media and cells, the total cellular LDH enzyme activity from the cells was measured.

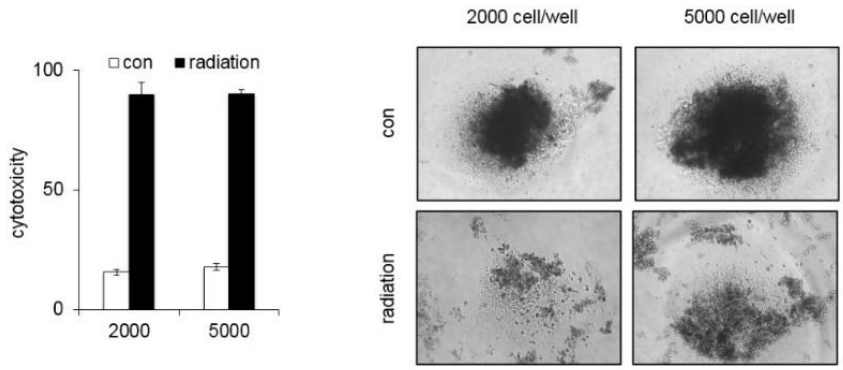

Figure 4. Cytotoxicity assay comparing control and radiation treated cells using LDH enzyme activity

(C) Morphologic classification

We also conducted morphologic assessment and classification before any radiation was applied. We classified the generated spheroids into four distinct groups based on morphology: round, mass, aggregate, and none.[36] In further analysis, we grouped the round, mass, and aggregate types into the category "mass forming type" for efficient statistical analysis. The morphologic classification was completed by the independent researcher who was unaware of the patients' clinicopathological outcomes.

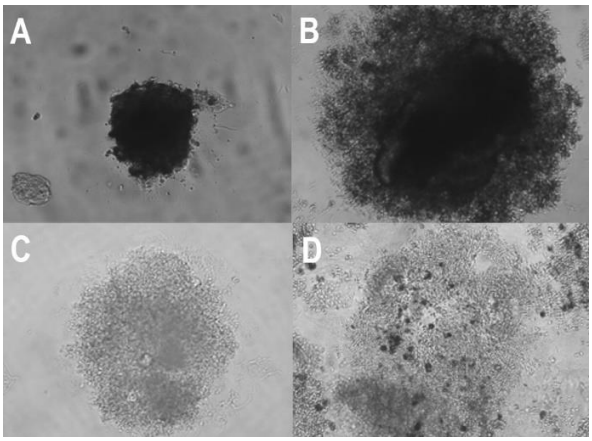

Figure 5. Morphologic classification after 3D cell culture (A – Round; B – Mass; C – Aggregate; D – None).

4. Tumor response assessment after preop-CRT

Pathologic evaluation was completed by a single experienced pathologist who was blinded to the patients' clinical outcomes. Mandard's tumor regression grade was used to evaluate tumor response after preop-CRT as follows: TRG 1: absence of histologically identifiable residual cancer; TRG 2: rare residual cancer cells scattered through the fibrosis; TRG 3: fibrosis outgrowing residual cancer; TRG 4: residual cancer outgrowing fibrosis; TRG 5: absence of regressive change. We considered tumors classified as TRG 1 or TRG 2 to be a good response and tumors staged as TRG 3, 4, or 5 to be TRG non-response. We defined pathologic complete response as no viable tumor cells in the rectal wall (TRG1) with no lymph node metastasis (pN0). Tumor downstaging was assessed by comparing the pretreatment clinical stage (cT and cN) with the post-treatment histopathologic stage (ypT and ypN). T-downstaging was defined as pathologic ypT was smaller compared to cT and N-downstaging was defined clinically positive lymph nodes (cN positive) was converted to pathological lymph node negative status (pN negative).

5. Statistical analysis

We analyzed the categorical variables using the chi-square test or Fisher's exact test and analyzed the continuous variables using Student's t test or analysis of variance. We used receiver operating characteristic(ROC) curves to compare the diagnostic performance of the various parameters for predicting pCR, responders, or lymph node positivity. We obtained the cut-off values for the variables that provided the best separation between the groups using ROC curve analyses. Specifically, we performed forward or backward stepwise logistic regression analysis of factors associated with pCR, good TRG response, and lymph node positivity; all variables with $p < 0.2$ in univariate analysis were entered into multivariate logistic regression analysis, and we considered $p < 0.05$ to indicate significance. We performed all statistical analyses using SPSS version 23.0 (IBM Corp., Armonk, NY, USA).

III. RESULTS

In this study, there were 60 and 49 patients were included in the study and validation sets, respectively; patient demographics are listed in Table 1. There were no differences in sex, age, BMI (kg/m^2), distance from anal verge, tumor location, initial CEA (μg/L), cT stage, or cN stage between the two groups. However, capecitabine instead of IV 5FU/LV during radiation therapy was more frequent in the validation set (41.7% in the study set vs. 98% in the validation set, $p < 0.001$). The rate of time interval to surgery more than 9 weeks was significantly higher in the validation set (18.3 % in the study set vs. 53.1% in the validation set, $p < 0.001$).

Table1. Patient demographics of the study set (n = 60) and the validation set (n = 49)

		Study set n (%)	Validation set n (%)	P
Sex	Male	38 (63.3)	29 (59.2)	0.696
	Female	22 (36.7)	20 (40.8)	
Age	Mean ± SD	58.8 ± 11.8	56.8 ± 13.6	0.421
BMI (kg/m^2)	Mean ± SD	23.3 ± 3	23.5 ± 3.3	0.812
Distance from anal verge (cm)		5.4 (2.3)	6.0 (2.2)	0.153
Tumor location	Low (< 5cm)	35 (58.3)	20 (40.8)	0.084
	Middle (5-10cm)	25 (41.7)	29 (59.2)	
CEA(initial) (μg/L)	Mean ± SD	7.2 ± 9.4	6.2 ± 7.4	0.556
cT stage	cT2	2 (3.3)	2 (4.1)	0.907*
	cT3	51 (85)	43 (87.8)	
	cT4	7 (11.7)	4 (8.2)	
cN stage	Node negative	11 (18.3)	6 (12.2)	0.436
	Node positive	49 (81.7)	43 (87.8)	
Chemotherapy agent during radiotherapy	IV 5FU/LV	35 (58.3)	1 (2)	< 0.001
	Capecitabine	25 (41.7)	48 (98)	
Time interval to surgery (weeks)	< 9	49 (81.7)	23 (46.9)	< 0.001
	≥ 9	11 (18.3)	26 (53.1)	

* Fisher's exact test

Abbreviations: BMI: Body mass index; CEA: Carcinoembryonic antigen

SD: Standard Deviation

Table 2 shows the endoscopic and MRI response after preop-CRT. There were no differences in endoscopy grade, pre-CRT MR tumor volume, post-CRT tumor volume, MR_TRG, or MR_DWI between the two groups. However, the study set showed larger MR_TVRR than the validation set (mean ± standard deviation, 72.8 ± 15.2 vs. 63.5 ± 17.1, p = 0.004).

Table 2. Endoscopy and MRI response after preoperative chemoradiotherapy in study/validation set

		Study set n (%)	Validation set n (%)	P
Endoscopy_grade	Endoscopy_CR	6 (10)	5 (10.2)	1.0*
	Endoscopy_near CR	14 (23.3)	11 (22.4)	
	Endoscopy_non CR	40 (66.7)	33 (67.3)	
MR_Tumor volume (cm^3)	Pre_CRT, Mean ± SD	19.5 ± 15.2	19.8 ± 14.3	0.926
	Post_CRT, Mean ± SD	5.5 ± 5.5	7.2 ± 5.8	0.114
MR_TVRR (%)	Mean ± SD	72.8 ± 15.2	63.5 ± 17.1	0.004
MR_TRG	Grade I	3 (5)	5 (10.2)	0.135*
	Grade II	10 (16.7)	14 (28.6)	
	Grade III – V	47 (78.3)	30 (61.2)	
MR_DWI	Negative	30 (50)	16 (31.7)	0.081
	Equivocal & Positive	30 (50)	33 (67.3)	

* Fisher's exact test

Abbreviations: CR: Complete response; CRT: Chemoradiotherapy; TVRR: Tumor volume reduction rate; TRG: Tumor regression grade; DWI: Diffusion weighted image

SD: Standard Deviation

Pathologic outcomes after preop-CRT are listed in Table 3. There were no differences in ypT, ypN, distal resection margin, tumor size, circumferential resection margin involvement rate, number of positive and total lymph nodes, histology grade, or TRG between the two groups.

Table 3. Pathologic outcomes after preoperative chemoradiotherapy

		Study set n (%)	Validation set n (%)	P
ypT	ypT0	18 (30)	12 (24.5)	0.876*
	ypT1	4 (6.7)	5 (10.2)	
	ypT2	13 (21.7)	10 (20.4)	
	ypT3	24 (40)	22 (44.9)	
	ypT4	1 (1.7)	0	
ypN	Node negative	46 (76.7)	38 (77.6)	1.0
	Node positive	14 (23.3)	11 (22.4)	
Resection margin (cm)	Proximal, Mean ± SD	13.6 ± 6	16.7 ± 7.4	0.018
	Distal, Mean ± SD	1.7 ± 1.6	1.3 ± 1.0	0.161
Tumor size (cm)	Mean ± SD	1.8 ± 1.1	2.2 ± 1.1	0.103
CRM involvement		1 (1.7)	3 (6.1)	0.324*
No. of positive lymph nodes	Mean ± SD	0.4 ± 1.2	0.5 ± 1.2	0.744
No. of total lymph nodes	Mean ± SD	14.4 ± 7.5	13.2 ± 7.7	0.412
Histology grade	High	56 (93.3)	47 (95.9)	0.689*
	Low	4 (6.7)	2 (4.1)	
TRG grade (Mandard grade)	1	18 (30)	12 (24.5)	0.642
	2	13 (21.7)	10 (20.4)	
	3	22 (36.7)	17 (34.7)	
	4	7 (11.7)	10 (20.4)	
	5	0	0	

* Fisher's exact test

Abbreviations: TRG: Tumor regression grade

SD: Standard Deviation

Using the study set, we analyzed factors associated with pCR and good tumor response (TRG 1 and 2) and multivariate analysis revealed the following as predictive factors for pCR: Endoscopy_grade and MR_TVRR more than 83.6 [odds ratio (OR): 29.8; 95% confidence interval (CI): 4.9–181.2; $p < 0.001$ and OR: 7.0; 95% CI: 1.0–46; $p < 0.041$, respectively] (Table 4-A). Endoscopy_grade and MR_DWI (OR: 7.6; 95% CI: 1.2–45.9; $p < 0.027$ and OR: 7.8; 95% CI: 1.9–32.3; $p = 0.004$, respectively) were associated with good tumor response (TRG 1 or 2) in multivariate analysis (Table 4-B).

Table 4. Univariate and multivariate analysis of factors associated with tumor response in the study set (n = 60)

4–A) Factors associated with pathologic complete response (pCR)

		Univariate analysis		Multivariate analysis	
		pCR n (%)	P	OR (95% CI)	P
Sex	Male	9 (23.7)	0.161		
	Female	9 (40.9)			
Age (year)	< 70	14 (31.1)	1.0*		
	≥ 70	4 (26.7)			
BMI (kg/m^2)	< 25	11 (26.8)	0.547		
	≥ 25	7 (36.8)			
Tumor location	Middle	3 (12)	0.021	1	N.S.
	Low	15 (42.9)		7.9 (0.9 – 64.2)	
CEA(initial) (μg/L)	< 3	10 (38.5)	0.261		
	≥ 3	8 (23.5)			
cT stage	cT2	1 (50)	0.351*		
	cT3	14 (27.5)			
	cT4	3 (42.9)			
cN stage	Node negative	5 (45.5)	0.279*		
	Node positive	13 (26.5)			
Time interval to surgery	< 9 weeks	13 (26.5)	0.279*		
	≥ 9 weeks	5 (45.5)			
Chemotherapy agent	IV 5FU/LV	12 (33.3)	0.368		
	Capecitabine	18 (24.7)			
Endoscopy_grade	Endoscopy_non CR	4 (10)	< 0.001	1	< 0.001
	Endoscopy_CR & near CR	14 (70)		29.8 (4.9 – 181.2)	
MR_TRG	Grade III – V	12 (25.5)	0.181*	1	N.S.
	Grade I & II	6 (46.2)		8.6 (0.8 – 64.2)	
MR_TVRR	TVRR < 83.6	9 (20)	0.007*	1	0.041
	TVRR ≥ 83.6	9 (60)		7.0 (1.0 – 46.0)	
MR_DWI	Equivocal & Positive	4 (13.3)	0.010		
	Negative	14 (46.7)			

Abbreviations: pCR: pathologic complete response; CR: Complete response; TVRR: Tumor volume reduction rate; TRG: Tumor regression grade; DWI: Diffusion weighted image

* Fisher's exact test

N.S: Not Significance

4–B) Factors associated with TRG good response (TRG I & II)

		Univariate analysis		Multivariate analysis	
		TRG 1&2 n (%)	P	OR (95% CI)	P
Sex	Male	18 (47.4)	0.431		
	Female	13 (59.1)			
Age (year)	< 70	24 (53.3)	0.769		
	≥ 70	7 (46.7)			
BMI (kg/m^2)	< 25	20 (48.8)	0.585		
	≥ 25	11 (57.9)			
Tumor location	Middle	8 (32)	0.018		
	Low	23 (65.7)			
CEA(initial) (μg/L)	< 3	16 (61.5)	0.203		
	≥ 3	15 (44.1)			
cT stage	cT2	2 (100)	0.217*		
	cT3	24 (47.1)			
	cT4	5 (71.4)			
cN stage	Node negative	7 (63.6)	0.509		
	Node positive	24 (49)			
Time interval to surgery	< 9 weeks	24 (49)	0.379		
	≥ 9 weeks	7 (63.6)	0.419		
Chemotherapy agent	IV 5FU/LV	19 (52.8)			
	Capecitabine	32 (43.8)			
Endoscopy_grade	Endoscopy_non CR	13 (32.5)	< 0.001	1	0.027
	Endoscopy_CR & near CR	18 (90)		7.6 (1.2 – 45.9)	
MR_TRG	Grade III - V	19 (40.4)	0.001*	1	N.S.
	Grade I & II	12 (92.3)		7.6 (0.7 – 83.4)	
MR_TVRR	TVRR < 83.6	20 (44.4)	0.075		
	TVRR ≥ 83.6	11 (73.3)			
MR_DWI	Equivocal & Positive	7 (23.3)	< 0.001	1	0.004
	Negative	24 (80)		7.8 (1.9 – 32.3)	

Abbreviations: CR: Complete response; pCR: pathologic complete response; TVRR: Tumor volume reduction rate; TRG: Tumor regression grade; DWI: Diffusion weighted image

* Fisher's exact test

N.S: Not Significance

Our prediction model for pCR using logistic regression analysis was suggested with tumor location, Endosocpy_grade, MR_TRG, and MR_TVRR, and this model was visually represented by a nomogram to predict the probability of pCR (Figure 6). Figure 7 presents the ROC curve and calibration plot of the study set. The predictive accuracy measured by the c-index was 0.894 (95% CI: 0.793–0.996). The nomogram was well calibrated, and there was good correlation between predicted and actual probability. In the validation set, the predictive accuracy measured by the c-index was 0.750 (95% CI: 0.553–0.946). However, the calibration plots for predicted and actual probability appeared not to show good correlations because the intermediate probability could be ambiguous for binary variables (Figure 8).

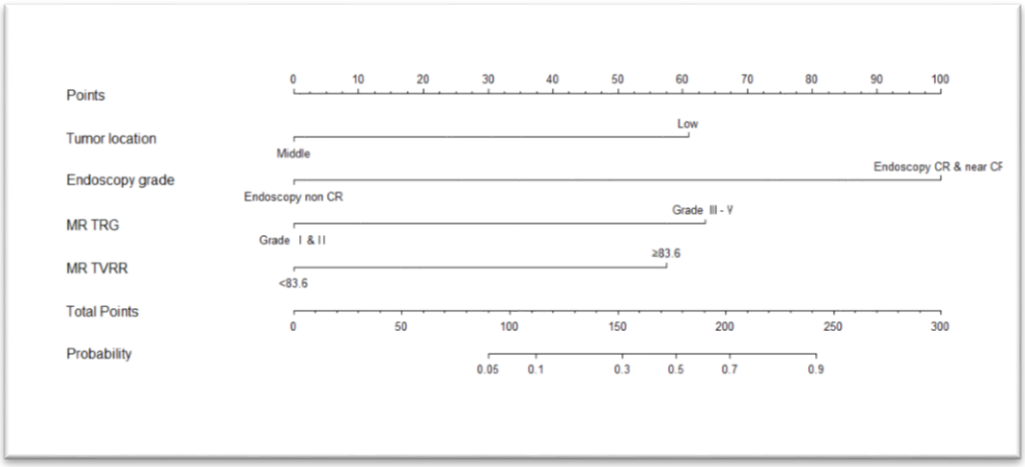

Figure 6. Nomogram predicting the probability of pathologic complete response after preoperative chemoradiotherapy. The nomogram is based on totaling the points identified on the top scale for each of the four variables. The total points projected to the bottom scale indicate the % probability of pCR.

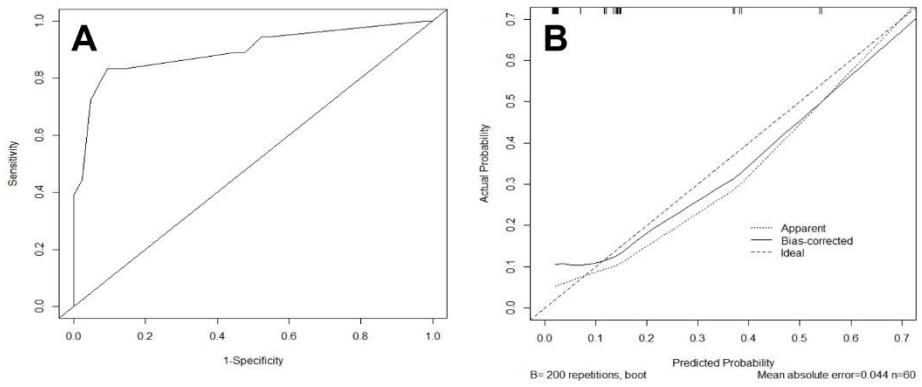

Figure 7. Discrimination and calibration of the nomogram in the study set (n = 60). A. ROC curve from the multiple logistic model. The predictive accuracy measured by the c-index was 0.894 (95% CI: 0.793–0.996). B. Calibration plot for probability of pCR. Predicted and actual pCR probabilities are plotted as logistic calibration (bootstrap 200 repetitions).

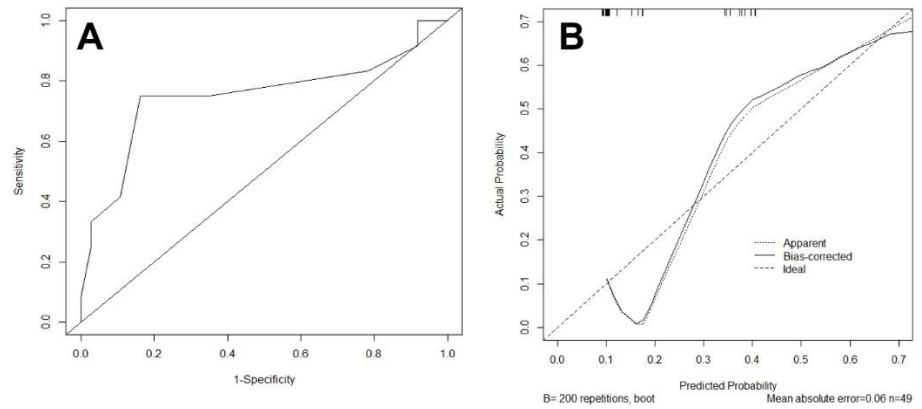

Figure 8. Discrimination and calibration of the nomogram in the validation set (n = 49). A. ROC curve from the multiple logistic model. The predictive accuracy measured by the c-index was 0.750 (95% CI: 0.553–0.946). B. Calibration plot for

probability of pCR. Predicted and actual pCR probabilities are plotted as logistic calibration (bootstrap 200 repetitions).

We performed cytotoxicity assay using 3D cell culture and radiation in the 49 patients in the validation set, but the cultures and assays were successful in only 26 patients (53%), who then constituted the experimental set. The reasons for failure consisted of cell contamination (65%) and insufficient biopsy specimens (35%). By morphologic classification, the aggregate type was most common (57.7%), followed by none (26.9%). Cytotoxicity ranged from 25.5 to 72.6 (median: 47.6; Table 5).

Table 5. Cytotoxicity assay using 3D cell culture and radiation in the experimental set (n = 26)

		n (%)
Results of 3D culture	Success	26 (53)
	Failure	23 (47)
Reason of 3D culture failure	Cell contamination	15 (65)
	Insufficient specimen from the biopsy	8 (35)
3D morphologic classification*		
	Round	1 (3.8)
	Mass	3 (11.5)
	Aggregate	15 (57.7)
	None	7 (26.9)
Cytotoxicity (%)	Median (Range)	47.6 (25.5 – 72.6)

*: morphologic classification was analyzed before radiation treatments

We assessed correlations between cytotoxicity assay and 3D morphologic classification, and there were no differences in cytotoxicity among the 4 groups. When we grouped the round, mass, and aggregate types into “mass forming type,” there were no differences in the cytotoxicity assay between the mass forming type and none (Table 6).

Table 6. Correlation of cytotoxicity assay with 3D morphologic classification before radiation in the experimental set (n = 26)

Parameters		n	Cytotoxicity Mean ± SD	P
3D morphologic classification	Round	1	50.9	0.490
	Mass	3	51.2 ± 10.7	
	Aggregate	15	52.4 ± 16.8	
	None	7	41.9 ± 9.3	
Subgroup analysis	Mass forming type*	19	52.1 ± 15.3	0.113
	None	7	41.9 ± 9.3	

*Mass forming type included morphologic classification of "Round", "Mass", and "Aggregate".

We also correlated the morphologic classification with pathologic tumor response in the experimental set (Table 7). T downstaging was marginally higher with none than with the mass forming type (p = 0.069), and the yp node positive rate was marginally higher in the none type (p = 0.057).

Table 7. Correlation of 3D morphologic classification with pathologic tumor response in the experimental set (n = 26)

Parameters		N	Mass forming type (n = 19) n (%)	None (n = 7) n (%)	P
TRG grade (Mandard grade)	1	6	6 (31.6)	0	0.292*
	2	4	2 (10.5)	2 (28.6)	
	3	11	7 (36.8)	4 (57.1)	
	4	5	4 (21.1)	1 (14.3)	
	5	0	0	0	
TRG response	TRG 1 & 2	10	8 (42.1)	2 (28.6)	0.668*
	TRG 3 – 5	16	11 (57.9)	5 (71.4)	
pCR	Yes	6	6 (31.6)	0	0.146*
	No	20	13 (68.4)	7 (100)	
T downstaging	Yes	16	14 (73.7)	2 (28.6)	0.069*
	No	10	5 (26.3)	5 (71.4)	
N downstaging	Yes	17	14 (73.7)	3 (42.9)	0.188
	No	9	5 (26.3)	4 (57.1)	

ypT Stage	0	6	6 (31.6)	0	0.252*
	1	1	1 (5.3)	0	
	2	8	6 (31.6)	2 (28.6)	
	3	11	6 (31.6)	5 (71.4)	
ypN Stage	Node negative	19	16 (84.2)	3 (42.9)	0.057*
	Node positive	7	3 (15.8)	4 (57.1)	

* Fisher's exact test

Abbreviations: TRG: Tumor regression grade; pCR: pathologic complete response

When we compared cytotoxicity assay with pathologic outcomes in the experimental set, there was a significant difference in cytotoxicity between the yp node positive and negative groups (node negative group: 53.2 ± 14.1 vs. node positive group: 38.7 ± 10.1, p = 0.021; Table 8)

Table 8. Correlation of cytotoxicity assay with pathologic tumor response in the experimental set (n = 26)

Parameters		N	Cytotoxicity Mean ± SD	P
TRG grade (Mandard grade)	1	6	50 ± 18.6	0.940
	2	4	45.2 ± 8.9	
	3	11	50.8 ± 12.8	
	4	5	48.8 ± 19.8	
	5	0		
TRG response	TRG 1 & 2	10	48.1 ± 15	0.729
	TRG 3 – 5	16	50.1 ± 14.6	
pCR	Yes	6	50 ± 18.6	0.909
	No	20	49.2 ± 13.6	
T downstaging	Yes	16	52.3 ± 14	0.199
	No	10	44.6 ± 14.7	
N downstaging	Yes	17	52.8 ± 14.3	0.096
	No	9	42.8 ± 13.2	
ypT Stage	0	6	50 ± 18.6	0.895
	1	1	53.8	
	2	8	51.8 ± 11.5	
	3	11	46.8 ± 15.7	
ypN Stage	Node negative	19	53.2 ± 14.1	0.021
	Node positive	7	38.7 ± 10.1	

Abbreviations: TRG: Tumor regression grade; pCR: pathologic complete response

Thus, we calculated ROC curves for cytotoxicity assay and lymph node status after surgery in the experimental set (n = 26); the AUC was 0.801.

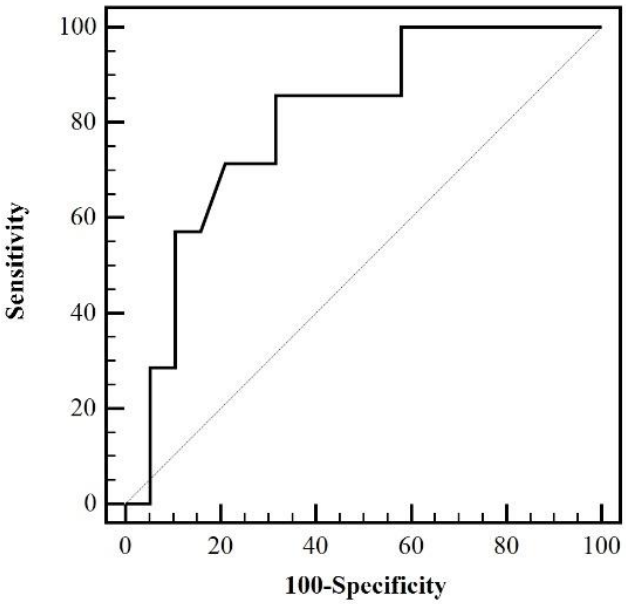

Figure 9. ROC curves of cytotoxicity assay and lymph node status after surgery in the experimental set (n = 26) by the multiple logistic model. AUC is 0.801 (95% CI: 0.599–0.930).

Using the ROC curves, we calculated Youden's index to be 45.05. We dichotomized the experimental set using this number into low versus high cytotoxicity. There were no differences in the clinicopathologic and radiologic parameters between the two groups except the ypN stage (Table 9).

Table 9. Clinicopathological characteristics and MRI parameters according to the low and high cytotoxicity groups in the experimental set (n = 26)

		Low cytotoxicity group (< 45) (n = 12) n (%)	High cytotoxicity group (≥ 45.1) (n = 14) n (%)	P
Sex	Male	6 (50)	8 (57.1)	1.0
	Female	6 (50)	6 (42.9)	
Age	Mean ± SD	51.4 ± 10.8	54.9 ± 16.2	0.530
BMI (kg/m^2)	Mean ± SD	23.7 ± 3.5	24.5 ± 4	0.575
Distance from anal verge (cm)	Mean ± SD	6.1 ± 2.1	6.2 ± 2.2	0.875
CEA (initial) (μg/L)	Mean ± SD	6.5 ± 7	5.6 ± 8	0.759
yp T stage	T0	3 (25)	3 (21.4)	0.933*
	T1	0	1 (7.1)	
	T2	3 (25)	5 (35.7)	
	T3	6 (50)	5 (35.7)	
yp N stage	Node negative	6 (50)	13 (92.9)	0.026*
	Node positive	6 (50)	1 (7.1)	
pCR	Yes	3 (25)	3 (21.4)	1.0*
	No	9 (75)	11 (78.6)	
Endoscopy	Endoscopy_non CR	7 (58.3)	12 (85.7)	0.190*
	Endoscopy_CR & Endoscopy_near CR	5 (41.7)	2 (14.3)	
MR_TRG	Grade I & II	7 (58.3)	10 (71.4)	0.683*
	Grade III - V	5 (41.7)	4 (28.6)	
MR_Tumor volume (cm^3)	Pre_CRT, Mean ± SD	17.2 ± 10.0	25.6 ± 14.1	0.098
	Post_CRT, Mean ± SD	6.9 ± 5.4	8.6 ± 6.0	0.467
	TVRR (%), Mean ± SD	62.4 ± 20.9	66.1 ± 14.3	0.603
MR_DWI	Negative	4 (33.3)	4 (28.6)	1.0*
	Equivocal & Positive	8 (66.7)	10 (71.4)	

* Fisher's exact test

Abbreviations: CEA: Carcinoembryonic antigen; BMI: Body mass index; pCR: pathologic complete response; CR: Complete response; TVRR: Tumor volume reduction rate; TRG: Tumor regression grade; DWI: Diffusion weighted image

SD: Standard Deviation

We analyzed factors associated with lymph node positivity by logistic regression analysis with forward stepwise selection of variables. We entered into the multivariate logistic regression analysis all lymph node positivity variables with $p < 0.2$ in univariate analysis. In this model, we entered the 3D morphologic classifications and cytotoxicity assay findings into the final multivariate analysis, and the assays were the only factors that predicted node positivity in multivariate analysis (OR: 13; 95% CI: 1.2–133.2; $p = 0.031$).

Table 10. Univariate and multivariate analysis of factors correlated with lymph node status after surgery in the experimental set (n = 26)

			Univariate analysis		Multivariate analysis	
Parameters		No.	n (%)	P	OR (95% CI)	P
Sex	Male	14	4 (28.6)	1.0*		
	Female	12	3 (25)			
Age	< 65	21	7 (33.3)	0.278*		
	≥ 65	5	0			
cT	T3	23	6 (26.1)	1.0*		
	T4	3	1 (33.3)			
cN	Node negative	2	0	1.0*		
	Node positive	24	7 (29.2)			
Endoscopy_grade	Endoscopy_non CR	19	6 (31.6)	0.629*		
	Endoscopy_CR & Endoscopy_near CR	7	1 (14.3)			
MR_TRG	Grade I&II	9	3 (33.3)	0.661*		
	Grade III – V	17	4 (23.5)			
MR_TVRR (%)	< 83.6	23	7 (30.4)	0.540*		
	≥ 83.6	3	0			
MR_DWI	Negative	8	1 (12.5)	0.375*		
	Equivocal & Positive	18	6 (33.3)			
3D morphologic classification	Mass forming type	19	3 (15.8)	0.057*		
	None	7	4 (57.1)			
Cytotoxicity assay	High (> 45.1)	14	1 (7.1)	0.026*	1	0.031
	Low (< 45)	12	6 (50)		13 (1.2 – 133.2)	

* Fisher's exact test

Abbreviations: CR: complete response; pCR: pathologic complete response; TRG: Tumor regression grade; TVRR: Tumor volume reduction rate; DWI: Diffusion weighted image

IV. DISCUSSION

Our present study showed that the combination of endoscopy and MRI parameters could predict good tumor response and cytotoxicity assay using 3D cell culture with radiation could be a novel option for predicting persistent lymph node metastasis after preoperative chemoradiotherapy in rectal cancer.

The pathologic complete response after preop-CRT in rectal cancer showed good oncologic outcomes and rate ranges from 10 to 30 %.[6,40,41] The patients' excellent positive outcomes increased our desire to predict pCR using clinical parameters. Investigating such variables could enable physicians to offer patients different treatment options according to the risk-adaptive model.

Among these, both pre- and post-CRT CEA levels and the ratio of the two have been suggested as easily obtainable blood markers for predicting pCR.[42-44] It has been reported that delayed surgery following preop-CRT (more than 6–8 weeks) showed significantly higher pCR rates than those in an early group.[45,46] In contrast, a recent randomized control trial that compared delays of 7 versus 11 weeks showed no difference in pCR rate and only showed higher morbidity in the 11-week delayed surgery group.[47] The efficacy of endoscopic assessment has recently been investigated for predicting tumor response after preop-CRT.[20,44,48] Several endoscopic features that suggest pCR have been reported such as non-macroscopic ulceration, fibrotic scar, whitening of the mucosa, flat scar, disappearance of the neoplastic fit pattern, and neoplastic nodules.[19,44,48] However, some authors have asserted that although endoscopic assessment correlated with tumor regression grading, it cannot fully discriminate pCR.[49]

The role of MRI in staging or restaging rectal cancer after preop-CRT has been well defined, and many variables have already been investigated in the field of response evaluation after preop-CRT. With regard to MR volumetry, previous studies investigated the relationship between the volume or volume reduction rate and tumor response.[21,22] Study authors used different parameters (tumor length, 3D tumor size, whole volume) and different study end points (complete response [ypT0] versus residual tumor; tumor regression grade 1 to 2 versus 3 to 5; T downstaging)[50]. In comparing these different MRI parameters, Martens et al. reported that validating the whole-volume measurements with pCR was mainly accurate.[50] mrTRG has been used to define tumor regression grading after preop-CRT, and it was reported that mrTRG can discriminate survival outcomes between poor and good responders.[38,51] Diffusion weighted imaging has demonstrated better predictive power than morphologic T2-weighted imaging.[52] It was reported that quantitative DWI post-CRT had better sensitivity and specificity than pre-CRT quantitative DWI in detecting pCR.[53]

Although various parameters including clinical, endoscopy, and MRI findings have been identified as potential surrogates for tumor response, little has been validated simultaneously using the same cohort of patients. Our study set included and measured the impact of these clinicopathologic, endoscopic, and MRI parameters in the same cohort, and our results showed that endoscopy assessment and MRI volumetry were independently associated with pCR. Regarding good tumor response (TRG I and II), endoscopy and MR_DWI are correlated with good tumor response. These results re-emphasize the clinical utility of endoscopy and MRI examinations in predicting tumor regression. However, endoscopic grading systems have some limitations. Although several features such as whitening of mucosa, presence of telangiectasias, and loss of flexibility were suggested as indicators of pCR, lack of interobserver concordance was inevitable due to the subjective interpretation of the gross findings.[44] Second, the time intervals between the completion of preop-CRT and endoscopic evaluation varied in the different studies. Although all endoscopic examinations in our study were completed between 4 and 6 weeks after completion

of preop-CRT, there is no standard period that is best for evaluating tumor response using endoscopy.

Increasing data suggest that good tumor response after preop-CRT may warrant decrease of radical resection and increasing the wait-and-see policy or local excision.[12,13] Li et al. compared the short- and long-term outcomes between 251 rectal cancer patients who achieved clinical complete response and 344 who achieved pathologic complete response through radical resection in a systematic review and meta-analysis. Although the non-surgical group had a higher rate of local recurrence, the authors concluded that for patients who achieved cCR after preop-CRT, a wait-and-see strategy expect to produce good outcomes provided with adequate selection criteria and appropriate follow-up.[10] However, the basic prerequisite for local excision or wait-and-see is no or very little possibility of regional lymph node metastasis(LNM). Accurate preoperative assessment of LNM is crucial for planning to wait-and-see or for radical resection in patients with suspect cCR. Nevertheless, it was reported that the current imaging tools have low sensitivity or specificity for defining LNM after preop-CRT.[34] The reliability of imaging modalities for evaluating lymph node positivity after preop-CRT in rectal cancer is known to be poor, and there are no standard guidelines to define LN positivity.[54]

To overcome these inherent limitations of imaging-based detection of lymph node positivity after preop-CRT, a clinical outcome-based prediction model was introduced. Jwa et al. reported that patient age, yp T stage, tumor differentiation, cN stage, lymphovascular invasion (LVI), and perineural invasion (PNI) could reliably predict LNM after preop-CRT. The authors of that study developed a nomogram using these parameters and showed good agreement between nomogram-predicted and real lymph node positivity after preop-CRT.[32] Although this study's value is that the authors suggested a nomogram of the LNM after preop-CRT using a relatively large and homogeneously treated patient group from a single center, this nomogram included pathologic parameters such as ypT stage, LVI, and PNI that can be more accurately derived from resected rectum after definite total mesorectal excision than

from preoperative biopsy specimens. The use of this nomogram thus might be limited in deciding on radical surgery. Considering this limitation, another study consisted of 8,984 patients from the National Cancer Database of the United States for predicting rates of lymph node positivity after preop-CRT.[33] The authors of that study reported 70.9% predictive accuracy of the nomogram using young age, low Charlson score, mucinous histology, poorly differentiated and undifferentiated tumors, LVI, elevated CEA, and clinically positive lymph node as significant predictive factors of persistent LNM following preop-CRT.[33] Although that study included parameters that were possibly available before definite surgery, as the authors stated, clinically available variables such as MRI, endorectal ultrasonography, endoscopy findings, and genetic mutations were not included in developing the nomogram.

In our experimental set, we evaluated the effect of the cytotoxicity assay in predicting tumor response after preop-CRT, but the assay could not predict postoperative pCR or good tumor response. In contrast, univariate analysis showed that low cytotoxicity, dichotomized by 45% in the cytotoxicity assay, was associated with LNM, and morphologic classification before radiation showed trends of lymph node positivity. Other factors known to be associated with pCR in our study cohort did not have any impact on predicting lymph node positivity; in multivariate analysis, only cytotoxicity level was correlated. The underlying mechanism of correlation between ypN positivity and cytotoxicity level cannot be fully accounted for by the result of this study; however, it might be associated with different radiation responses between primary tumors and metastatic lymph nodes, especially considering the lack of correlation between cytotoxicity level and tumor regression grade. The current practice of local excision after good clinical response indicates that metastatic lymph nodes might regress at the same level as the primary tumor after preop-CRT, which was supported by the clinical observations.[55,56] In contrast, some authors reported 28.5% lymph node positivity in patients who showed pCR (ypT0).[31] In a recent study, Choi et al. analyzed the prognostic impact of the lymph node regression grade (LRG) after preop-CRT and found that LRG was the only independent factor associated with

relapse-free survival in ypN-positive rectal cancer patients who underwent preop-CRT.[57] One of the interesting findings from that study was that the distribution of LRG was not associated with the TRG of the primary tumor and the LRGs of each metastatic lymph node differed even in the same patients. These findings meant that radiation's effects could differ between the primary tumor and metastatic lymph nodes. These discrepancies might be one possible reason for the different prediction power between primary tumor cells and lymph node positivity in our cytotoxicity assays.

The cytotoxicity assay using 3D cell culture and radiation has some merits. First, we believe that the 7-day turnaround time for this procedure is acceptable because unduly long turnaround times could hinder clinical decision making. Second, the assay could predict patients' tumor response before starting preop-CRT. Although preop-CRT reduced the local recurrence rate more than postoperative adjuvant CRT, overall survival gain is not anticipated. Rather, preop-CRT may be associated with late adverse effects on long-term anorectal, sexual, and urinary dysfunction.[58] In this regard, some researchers are conducting studies to confirm the efficacy of induction chemotherapy with selective radiation treatments.[59] One reason for this new approach is the expectation that radiation-induced complications may be reduced. Many of the tumor response assays in current practice are regarded as response evaluation tools rather than prediction models. MRI-based tumor volumetry evaluation, DWI, mrTRG, endoscopy grading, delay of surgery, and other variables can be evaluated after preop-CRT is completed but cannot predict tumor response before preop-CRT. In this regard, our preliminary results might be useful for counseling patients on whether or not to undergo preop-CRT, although the clinical benefits should be evaluated in further investigation.

Nevertheless, there are several potential disadvantages with this approach. Appropriate fresh tissue stored immediately in 25T flasks filled with RPMI culture medium with 1% penicillin-streptomycin is mandatory for the analysis. Although board-certified surgeons performed the pre-treatment biopsies, it was not always easy

to obtain adequate tissue using rigid sigmoidoscopy. Another limitation is cell contamination during cell culture process. It is well-known that nearly all intestinal and feces flora are composed of obligate anaerobes such as bacteroides and bifidobacterium, and the accompanied bacteria during the biopsy process might be included in the culture process. The cell contamination rate was 30% in our study, which might be an obstacle with this procedure, and another protocol is needed to overcome this problem.

The limitations of this study derive from its small sample size and retrospective design. In our study set, the pCR rate was 30%, which was higher than that in other studies. Although we randomly selected patients from our prospectively collected database, selection bias may have been possible due to the retrospective design in the study set.

There are potential differences according to the definitions of ypT stage and TRG. Even with the same ypT3, some patients have small islets of viable cancer cells scattered in the subserosa layer that show predominant fibrosis (good TRG), whereas other patients have ypT3 tumors in which most cancer cells remain viable (poor TRG).[60] In this regard, although it is debatable whether the TRG differences by same ypT stage correlate with the cytotoxicity assay, subgroup analysis was not possible in our study because of the small sample size. However, it is also possible that the cytotoxicity assay might not accurately reflect humans' radio-resistance because it does not accurately reproduce the surrounding environments of rectal cancer patients. This needs to be validated in further research.

V. CONCLUSION

Endoscopic regression grade and MRI parameters such as tumor volume reduction rate and MR_DWI might predict ypCR or good tumor response after preop-CRT, and cytotoxicity assay after 3D cell culture and radiation could predict lymph node metastasis. These factors might be useful for investigating clinical response after preop-CRT and for patient stratification in clinical trials.

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ABSTRACT (IN KOREAN)

직장암에서 3D 세포 배양을 포함한 다차원 접근법을 이용한 수술전 화학방사선 치료 후 병리학적 완전 관해의 예측

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강정현

연구의 배경: 국소진행성 직장암에서 수술전 화학방사선 치료의 반응을 예측할 수 있는 치료 전 요인들을 이용하여 환자의 맞춤 치료에 사용하기 위한 노력들이 많이 있었으나, 이러한 여러 요인들의 유용성이 동일한 환자군에서 비교 분석된 연구는 드물다. 본 연구의 목적은 수술전 화학방사선 치료를 시행 받은 직장암 환자에서 임상요인, 내시경, 자기공명영상 및 3D 세포 배양 등의 다중요인을 적용하여 화학방사선 치료 예측인자를 확인하는 것이다.

재료 및 방법: 본 연구의 환자들은 study/validation 및 experimental 군으로 구성되어 있다. 2012 년부터 2014 년까지 60 명의 환자를 후향적으로 선택하였다. 모든 환자들에서 화학방사선 치료 전후에 MRI 검사 및 화학방사선 치료 후 내시경 검사를 시행하였고 이 환자군이 study 군을 이룬다. 2015 년 8 월부터 2017 년 4 월까지 49 명의 국소 진행성 직장암 환자들에 대하여 전향적인 자료를 수집하였고, 이 환자군들이 validation

set 를 형성한다. 49 명의 환자군에서 3D 세포 배양결과가 확인 가능한 26 명의 환자들을 experimental 군으로 분류하였다. 임상소견, 내시경 및 MRI 소견을 이용하여 단변량 및 다변량 로지스틱 회귀 분석을 시행하여 우수한 종양반응을 예측하는 요인을 확인하였다. 노모 그램은 study 군에서 개발되었고 validation 군에서 검증되었다. pCR, good TRG 반응 및 림프절 양성과 관련된 인자는 전방 또는 후방 단계별 변수 선택에 의해 수행된 로지스틱 회귀 분석에 의해 분석되었다.

결과: study 군에서 83% 이상의 종양감축과 Endoscopy_grade 가 독립적으로 pCR 을 예측할 수 있는 것으로 확인되었다. 양호한 종양 반응 (TRG I 및 TRG II)과 관련하여 Endoscopy_grade 및 MR_DWI (확산 가중 이미지)가 의미 있는 인자로 분석되었다. 종양 위치, Endosocpy_grade, MR_TRG 및 MR_TVRR 을 이용하여 logistic regression analysis 를 이용한 pCR 의 예측모델을 만들었다. 이 변수를 사용하는 노모 그램은 study 군 (AUC = 0.894)과 validation 군 (AUC = 0.750)에서 우수한 차별 능력을 보였다. Experimental 군에서, 수술 후 세포 독성 분석 과 림프절 전이 여부에 관한 ROC 곡선을 이용한 분석에서 AUC 값은 0.801 이었다. 다변량 분석에서 세포 독성 분석이 림프절 양성을 예측하는 유일한 인자로 확인되었다 (OR: 13; 95% CI: 1.2–133.2; p = 0.031).

결론: 본 연구는 내시경 검사와 MRI 측정값의 조합이 좋은 종양 반응을 예측할 수 있음을 보여 주었다. 3D 세포배양 및 방사선치료를 시행 후 세포 독성 분석을 통해 preop-CRT 후 림프절 전이 여부 예측가능성이 있었다.

핵심되는 말: 직장암, 자기공명 영상, 3D 세포배양, 종양반응등급, 병리학적 완전관해