

Prediction of tumor response of rectal cancer cells via 3D cell culture and *in vitro* cytotoxicity assay before initiating preoperative chemoradiotherapy

JEONGHYUN KANG¹, MIN CHUL PARK², JINA KIM², HYUK HUR¹, BYUNG SOH MIN¹,
SEUNG HYUK BAIK¹, KANG YOUNG LEE¹ and NAM KYU KIM¹

¹Department of Surgery, Yonsei University College of Medicine, Seoul 03722;

²CureBio Co., Ltd., Suwon, Gyeonggi 16229, Republic of Korea

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Abstract. The aim of the present study was to investigate the utility of 3D cell culture and *in vitro* cytotoxicity assays, performed using cells derived from biopsies obtained prior to the initiation of preoperative chemoradiotherapy (preop-CRT), in predicting tumor response to chemoradiotherapy following preop-CRT in rectal cancer. Biopsies were obtained from 49 patients with locally advanced rectal cancer that underwent preop-CRT between August 2015 and March 2017. Tumor tissue was obtained before initiating preop-CRT. The response to chemoradiation was assessed by *in vitro* cytotoxicity assay following 3D cell culture and radiation treatment. The associations between the results from the cytotoxicity assay, and tumor regression grade (TRG) and yp node (ypN) positivity were investigated. Among 49 patients, 26 patients were available for analysis. Cytotoxicity ranged from 25.5-72.6% (median, 47.6%). There was no difference in cytotoxicity according to the TRGs 1-5 ($P=0.940$), or good tumor response (TRGs 1-2 vs. TRGs 3-5; $P=0.729$). However, there was a significant difference in cytotoxicity between the ypN-negative and -positive groups (53.2 ± 14.1 and 38.7 ± 10.1 , respectively; $P=0.021$). Following dichotomization of patients with 45% cut-off value, the cytotoxicity assay was the only factor that predicted ypN positivity in multivariate analysis (odds ratio, 13; 95% confidence interval, 1.2-133.2; $P=0.031$). In conclusion, the cytotoxicity assay using the 3D cell culture method can be used to predict tumor response, particularly ypN positivity, in patients with rectal cancer who are scheduled for preop-CRT.

Introduction

Preoperative chemoradiotherapy (preop-CRT) is considered as the standard treatment option for locally advanced rectal cancer (1). Following reports on the favorable long-term oncological outcome of patients with pathologic complete response (pCR) (2-4), investigation into potentially useful clinicopathological factors for predicting pCR was conducted, with various factors proposed to be associated with pCR (5,6). Delaying surgery for patients who presented with good clinical outcomes has gained popularity recently (7,8). It was reported that following preop-CRT in rectal cancer, persistent lymph node metastasis (LNM) was the most indicative factor of poor prognosis (9,10). In addition, yp node (ypN) positivity (metastatic lymph node detection after surgery) was revealed to be the most important prognostic factor to predict the survival of patients harboring no residual tumor (ypT0) following surgical resection (2). There is still some controversy regarding this topic, and one of the main concerns with delaying the surgery or the local excision approach is the difficulty of predicting LNM even in the case of good clinical response. Swellengrebel *et al* (11) reported that 28.5% of patients with no residual tumor in the rectal wall had persistent regional LNM and 41% of near-complete response patients had ypT3 stage. Although several researchers have investigated predictors of ypN-positive status following preop-CRT, the studies are limited, as the factors used in the nomograms are often difficult to obtain prior to radical resections (12-14). Considering the low predictive rate of the current imaging techniques for restaging LNM (15,16), novel strategies to predict LNM more accurately following preop-CRT are still warranted.

The 3D cell culture system has been widely used, as cells in 3D environments may be more similar to cells in living organisms (*in vivo*) compared to the flat, unnaturally thin and single-layer cells grown in 2D environments (17). Typical cells in 3D systems are ellipsoids with dimensions of 10-30 μm ; in contrast, cells in 2D are flat with a typical thickness of 3 μm (18). With regards to environmental comparison, typical cells in 3D have ~100% of their surface area exposed to other cells or matrix, whereas cells in 2D have ~50% of their surface area exposed to fluid, ~50% exposed to the flat culture

Correspondence to: Professor Nam Kyu Kim, Department of Surgery, Yonsei University College of Medicine, 50 Yonsei-ro, Seodaemun, Seoul 03722, Republic of Korea
E-mail: namkyuk@yuhs.ac

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surface or intermediate, and very little surface area exposed to other cells. A previous data analysis of spheroid-based drug screening systems revealed that tumor spheroid size and morphology in a 3D culture system was associated with *in vivo* drug sensitivity (19). Previous studies demonstrated significantly increased resistance to radiation in tumor cells in 3D cell culture, in comparison with that of 2D culture (20,21). Storch *et al* (21) reported increased levels of heterochromatin in 3D cell cultures that led to increased survival, and a decreased number of DNA double-strand breaks and lethal chromosome aberrations in response to radiation, compared with those in monolayer cell cultures. These data suggest potential clinical benefits of 3D cell culture for predicting tumor responses to radiation. Nevertheless, the predictive potential of 3D cell culture and cytotoxicity analysis has not been widely assessed in patients with rectal cancer who have undergone preop-CRT.

Thus, the aim of the present study was to evaluate the clinical potential of the 3D cell culture method for predicting tumor response to preoperative chemoradiotherapy in patients with rectal cancer.

Materials and methods

Patient enrolment. A total of 49 individuals (29 males, aged 22-82 years) suffering from locally advanced rectal cancer that underwent preop-CRT were prospectively enrolled at the Severance Hospital, Yonsei University College of Medicine (Seoul, Republic of Korea) between August 2015 and March 2017. Patients with histopathologically confirmed adenocarcinoma of the rectum at clinical stage II or III were enrolled for the present study (22). All enrolled patients underwent preoperative biopsy, for the cytotoxicity assay, prior to the initiation of preop-CRT. The protocol used for the preop-CRT was described in previous studies (23,24). Briefly, the 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 5 weeks (1.8 Gy for 5 days). 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°. For the boost treatment, five ports were used. Intravenous bolus injections of 5-fluorouracil/leucovorin (425/20 mg/m² once per day during weeks 1 and 5) or capecitabine (825 mg/m², twice daily during the radiation therapy period) were administered to the patients during preop-CRT. Total mesorectal excision (TME) was performed 6-12 weeks after the completion of preop-CRT. The protocol of the present study was approved by the institutional review board of the Severance Hospital, Yonsei University College of Medicine (Seoul, Republic of Korea) (approval no. 4-2011-0445). Written informed consent was obtained from all participants.

3D cell culture

Tissue harvest. Tissue was collected prior the initiation of preop-CRT using rigid sigmoidoscopy and forceps biopsy. The tissues were placed into 25-cm² flasks, filled with RPMI culture medium supplemented with 1% penicillin-streptomycin (HyClone; GE Healthcare Life Sciences). Before tissue dissociation, harvested tissues were maintained at 37°C in a 5% CO₂ incubator.

Cancer tissue dissociation and 2D culture. Following the removal of media from the 25-cm² flasks, the tissue samples were transferred to 1.5-ml micro tubes, and the weight was measured. The samples were subsequently transferred to 60-mm² dishes filled with 5 ml RPMI culture medium supplemented with 1% penicillin-streptomycin and washed by pipetting; this procedure was repeated twice. The tissue was then transferred to 60-mm² dishes filled with 5 ml trypsin EDTA (HyClone; GE Healthcare Life Sciences), cut into smaller pieces using a blade (Nopa Instruments), and incubated for 20 min at 37°C in a 5% CO₂ incubator. Subsequently, the tissue was harvested using 5 ml RPMI medium supplemented with 1% penicillin-streptomycin and 10% FBS (HyClone; GE Healthcare Life Sciences), transferred to 15-ml conical tubes, and the solution was centrifuged at 260 g for 2 min at room temperature. The supernatant was removed, and the pellets were resuspended in 10 ml RPMI supplemented with 1% penicillin-streptomycin and 10% FBS, followed by transferring the tissues to 100-mm² 2D culture dishes (Thermo Fisher Scientific, Inc.) and incubating at 37°C for 2-3 days.

Cancer cell 3D culture. Cells were harvested by pipetting and transferred to 15-ml conical tubes. The tissue was then transferred to 60-mm² dishes filled with 5 ml trypsin EDTA (HyClone; GE Healthcare Life Sciences), cut into smaller pieces using a blade (Nopa Instruments Medizintechnik GmbH), and incubated for 20 min at 37°C in a 5% CO₂ incubator. Subsequently, the tissue was harvested using 5 ml RPMI medium supplemented with 1% penicillin-streptomycin and 10% FBS (HyClone; GE Healthcare Life Sciences), transferred to 15-ml conical tubes, and the solution was centrifuged at 260 g for 2 min at room temperature. The 2D-cultured cells were detached using Accumax (EMD Millipore). The dissociated cells were counted using a C-Chip disposable hemocytometer (INCYTO). The cells were diluted in 1 ml RPMI medium supplemented with 1% penicillin-streptomycin and 10% FBS to a concentration of 5,000 cells/100 µl and seeded onto ultra-low attachment 96-well 3D-culture plates (Corning Inc.).

Radiation treatment. Defining the day on which the cells were reseeded for 3D culture as day 0, the 3D-cultured cells were treated with 5 Gy radiation at day 3 (Gammacell low dose-rate irradiator; Nordion, Inc.). The control group did not receive any 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 2 days after radiation treatment were acquired by Cell Scanner (MBD), equipped with a x4 objective lens. The cells and culture media were transferred to 1.5-ml tubes, and centrifuged at 95 g for 2 min. The supernatant and pellets in each tube were stored at -20°C.

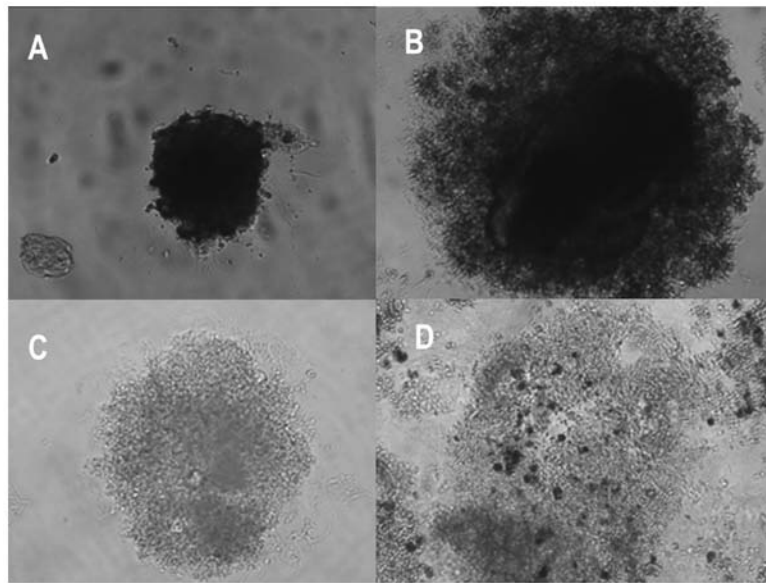


Figure 1. Four types of morphology of 3D spheroids formed in 3D cell cultures. The 3D spheroids formed by 3D cell culture of rectal cancer tissues, obtained before preoperative chemoradiotherapy, were grouped as (A) 'round', (B) 'mass', (C) 'aggregate' and (D) 'none' types.

Evaluation of 3D cell culture

Morphological classification. Morphological assessment and classification were performed prior to radiation treatment. The resulting spheroids were classified into four distinct groups based on morphology as 'round', 'mass', 'aggregate' and 'none' (Fig. 1) (19). For further analysis, the 'round', 'mass', and 'aggregate' types were grouped into the category 'mass-forming group' for efficient statistical analysis. The morphological classification was completed by an independent researcher, who was unaware of the patients' clinicopathological outcomes.

Cytotoxicity analysis. Defining the day on which the cells were reseeded for 3D culture as day 0, the culture medium was harvested and lactate dehydrogenase (LDH) enzyme activity was determined to check cytotoxicity levels at day 5 (2 days of additional incubation following radiation treatment). The cells were separated from the harvested media by centrifugation at 1,000 g for 15 min at room temperature. The supernatant was collected and the LDH enzyme activity was measured using an LDH-cytotoxicity assay kit (BioVision, Inc.) following the manufacturer's protocol. In order to calculate the ratio between LDH enzyme activity in the media and cells, the total cellular LDH enzyme activity from the cells was also measured.

Measurements of endoscopy and magnetic resonance imaging (MRI) parameters. All patients underwent endoscopies from 4-6 weeks following completion of preop-CRT. All endoscopic regression grading was estimated by endoscopists. Endoscopic grading was classified into endoscopy_CR (normal mucosa, whitish scar only), endoscopy_near CR (<1 cm ulcer without remaining visible tumor), and endoscopy_non CR (ulcer >1 cm without remaining visible tumor, remaining mass regardless of ulcer).

Patients underwent two stages of MRI examinations. The first examination was performed before the initiation of preop-CRT (pre-CRT MRI) and the MRI second was

performed from 4-6 weeks following the completion of preop-CRT (post-CRT MRI). A 3.0 T scanner [(Magnetom Tim Trio; Siemens Medical Solutions) or (Ingenia; Philips Medical Systems)] was used for pre- and post-CRT MRI examinations. In the present study, magnetic resonance (MR) tumor regression grade (TRG), diffusion-weighted image (DWI) and MR tumor volume reduction rate (TVRR) were evaluated and recorded for each enrolled patient. These MRI-based parameters were evaluated based on protocols from previous studies (23,25,26).

Tumor response assessment following preop-CRT. The Mandard's classifications were used to evaluate TRG (27). pCR was defined as no viable tumor cells in the rectal wall along with no LNM. According to the TRG classification, TRG 1 and TRG 2 were classified as 'good tumor response', while TRG 3-5 were classified as 'poor tumor response'. With respect to tumor downstaging, T downstaging was defined as smaller pathologic ypT compared with clinical T stage, and N downstaging was defined as conversion of clinically positive lymph nodes to pathologically negative status.

Statistical analysis. All statistical analyses were performed using SPSS version 23.0 (IBM Corp.). Categorical variables were analyzed using the χ^2 test or Fisher's exact test, and continuous variables were analyzed using Student's t-test or ANOVA. Receiver operating characteristic (ROC) curve analysis was used to compare the diagnostic performance of the parameter for predicting ypN positivity. The cut-off values for the variables that provided the best classification between the groups were determined. Factors associated with ypN positivity were analyzed by logistic regression analysis with forward stepwise selection of variables. All variables with $P < 0.2$ on univariate analysis were initially entered into the multivariate analysis. $P < 0.05$ was considered statistically significant.

Table I. Comparison of clinicopathological factors according to the success or failure of patients that were included in the cytotoxicity assay.

Clinicopathological factor	Success group (n=26), n (%)	Failure group (n=23), n (%)	P-value
Sex			0.562
Male	14 (53.8)	15 (65.2)	
Female	12 (46.2)	8 (34.8)	
Age, years			0.332
<65	21 (80.8)	15 (65.2)	
≥65	5 (19.2)	8 (34.8)	
BMI, kg/m ²	24.1±3.7 ^a	22.8±2.6 ^a	0.167
Distance from anal verge, cm	6.2±2.1 ^a	5.9±2.3 ^a	0.661
Tumor location			0.394
Low (<5 cm)	9 (34.6)	11 (47.8)	
Middle (5-10 cm)	17 (65.4)	12 (52.2)	
CEA (initial), μg/l	6.0±7.4 ^a	6.5±7.5 ^a	0.796
cT stage			0.286 ^c
cT2	0	2 (8.7)	
cT3	23 (88.5)	20 (87)	
cT4	3 (11.5)	1 (4.3)	
cN stage			0.4 ^c
Node-negative	2 (7.7)	4 (17.4)	
Node-positive	24 (92.3)	19 (82.6)	
Cytotoxicity, % (range)	47.6 ^b (25.5-72.6)	N/A	

^aMean ± standard deviation. ^bMedian. ^cFisher's exact test. BMI, body mass index; CEA, carcinoembryonic antigen; N/A, not available; cT, clinical tumor; cN, clinical node.

Table II. Association of cytotoxicity assay with 3D morphologic classification before radiation.

Parameters	n=26, n (%)	Cytotoxicity, mean ± SD	P-value
3D morphologic classification ^a			0.490 ^c
Round	1 (3.8)	50.9	
Mass	3 (11.5)	51.2±10.7	
Aggregate	15 (57.7)	52.4±16.8	
None	7 (26.9)	41.9±9.3	
Subgroup analysis			0.113
'Mass-forming group' ^b	19 (73)	52.1±15.3	
'None group'	7 (26.9)	41.9±9.3	

^a3D morphologic classification was analyzed before radiation treatment. ^bMorphologic classification of 'round', 'mass' and 'aggregate' were classified into 'mass forming' group and 'none' was classified into the 'none' group. ^cAnalyzed by ANOVA test.

Results

A total of 49 patients were initially enrolled in the present study for performing a cytotoxicity assay on 3D-cultured cells exposed to radiation. Finally, the results from 26 patients (53%) were available for further analysis. The reasons for failure in obtaining results from the remaining patients comprised cell culture contamination for 15 (65%) patients and insufficient specimens from the biopsy for 8 (35%) patients. There was

no difference in clinicopathological characteristics between the success group (n=26) and the failure group (n=23; Table I). Among the 26 patients included in the final analysis, cytotoxicity ranged from 25.5-72.6% (median, 47.6%).

By morphologic classifications, the 'aggregate' type was the most common (57.7%), followed by the 'none' type (26.9%). Mean and the standard deviation of cytotoxicity were 50.9 in 'round' type, 51.2±10.7 in mass type, 52.4±16.8 in aggregate type and 41.9±9.3 in none type respectively. With respect to

Table III. Association of cytotoxicity with pathologic tumor response.

Parameters	N	Cytotoxicity, mean \pm SD	P-value
TRG grade			0.940 ^a
1	6	50 \pm 18.6	
2	4	45.2 \pm 8.9	
3	11	50.8 \pm 12.8	
4	5	48.8 \pm 19.8	
5	0		
TRG response			0.729
TRG 1-2	10	48.1 \pm 15	
TRG 3-5	16	50.1 \pm 14.6	
pCR			0.909
Yes	6	50 \pm 18.6	
No	20	49.2 \pm 3.6	
T downstaging			0.199
Positive	16	52.3 \pm 14	
Negative	10	44.6 \pm 14.7	
N downstaging			0.096
Positive	17	52.8 \pm 14.3	
Negative	9	42.8 \pm 13.2	
yp T stage			0.895 ^a
0	6	50 \pm 18.6	
1	1	53.8	
2	8	51.8 \pm 11.5	
3	11	46.8 \pm 15.7	
yp N stage			0.021
Node-negative	19	53.2 \pm 14.1	
Node-positive	7	38.7 \pm 10.1	

^aAnalyzed by ANOVA test. TRG, tumor regression grade; pCR, pathologic complete response; T, tumor; N, node.

associations between cytotoxicity and 3D morphologic classification, there were no differences in cytotoxicity among the 4 groups. Following the grouping of the 'round', 'mass' and 'aggregate' types into the 'mass-forming' group, there were no differences in the cytotoxicity between the 'mass-forming group' and the 'none group' (Table II).

With respect to the association between cytotoxicity and pathologic tumor response (Table III), there was no difference in cytotoxicity based on either the TRGs 1-5, ($P=0.940$) or good tumor response (TRGs 1-2 vs. TRGs 3-5; $P=0.729$). There was a weak association between cytotoxicity and N downstaging status (52.8 \pm 14.3 in the N downstaging-positive group vs. 42.8 \pm 13.2 in the N downstaging-negative group; $P=0.096$). In contrast, there was a significant difference in cytotoxicity between the ypN-positive and the ypN-negative groups (53.2 \pm 14.1 vs. 38.7 \pm 10.1, respectively; $P=0.021$).

The morphological classifications were associated with pathologic tumor response. The rate of T downstaging was marginally higher in the 'mass-forming group' compared

to that of the 'none group' (73.7% vs. 28.6%, respectively; $P=0.069$). Although the ypN-positive rate was lower in the 'mass-forming group' (15.8%) compared with that in the 'none group' (57.1%), the difference was not statistically significant ($P=0.057$; Table IV). The ROC curves were generated for cytotoxicity in patients with ypN-positive status and the area under the curve was 0.801. Using the ROC curves, the Youden's index was calculated to be 45.05 (Fig. 2).

Using the Youden's index value as the cut-off value, the patients were dichotomized into low-cytotoxicity and high-cytotoxicity groups. There were no differences in the clinicopathological and radiological parameters between the high- and low-cytotoxicity groups, except for the ypN-positivity (Table V). The high-cytotoxicity group showed significantly lower ypN-positivity (7.1%) than that of the low-cytotoxicity group (50%; $P=0.026$).

3D morphologic classification and cytotoxicity assay data were considered in the final multivariate analysis to predict the ypN-positivity (Table VI). The cytotoxicity (dichotomized as low vs. high) was the only factor that predicted ypN-positivity in the multivariate analysis (high-cytotoxicity vs. low-cytotoxicity; odds ratio, 13; 95% confidence interval, 1.2-133.2; $P=0.031$).

Discussion

The present study demonstrated that the cytotoxicity assay, using 3D cell culture with radiation, could be a novel alternative for predicting lymph node positivity following preop-CRT in rectal cancer.

Increasing evidence has suggested that good tumor response following preop-CRT may warrant a decrease in radical resection and promotion of an approach that involves either delaying the surgery or local excision (28,29). However, the basic prerequisite for these approaches is no or minimal possibility of regional LNM. Accurate preoperative assessment of LNM is crucial for effectively employing the delay in surgery or radical resection approaches in patients suspected of clinical complete response. Nevertheless, it was reported that current imaging tools have low sensitivity or specificity for defining LNM following preop-CRT (15). The reliability of imaging techniques for evaluating lymph node positivity following preop-CRT in rectal cancer is known to be poor, and there are no standard guidelines to define lymph node positivity (30).

In order to overcome these inherent limitations of imaging-based detection of lymph node positivity following preop-CRT, a clinical outcome-based prediction model was introduced. Jwa *et al* (12) reported that patient age, ypT stage, tumor differentiation, clinical N stage, lymphovascular invasion (LVI) and perineural invasion (PNI) could reliably predict LNM following preop-CRT. A nomogram using these parameters was developed and showed high similarity between nomogram-predicted and real lymph node positivity following preop-CRT. Although the nomogram was based on a relatively large and homogeneously treated patient group from a single center, this nomogram included pathological parameters such as ypT stage, LVI and PNI that can be more accurately derived from resected rectum, following definite TME, compared to preoperative biopsy specimens. The use of this nomogram

Table IV. Association of 3D morphologic classification with pathologic tumor response.

Parameters	N	Mass-forming group (n=19), n (%)	None group (n=7), n (%)	P-value
TRG grade				0.292 ^a
1	6	6 (31.6)	0	
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				0.668 ^a
TRG 1-2	10	8 (42.1)	2 (28.6)	
TRG 3-5	16	11 (57.9)	5 (71.4)	
pCR				0.146 ^a
Yes	6	6 (31.6)	0	
No	20	13 (68.4)	7 (100)	
T downstaging				0.069 ^a
Positive	16	14 (73.7)	2 (28.6)	
Negative	10	5 (26.3)	5 (71.4)	
N downstaging				0.188
Positive	17	14 (73.7)	3 (42.9)	
Negative	9	5 (26.3)	4 (57.1)	
yp T stage				0.252 ^a
0	6	6 (31.6)	0	
1	1	1 (5.3)	0	
2	8	6 (31.6)	2 (28.6)	
3	11	6 (31.6)	5 (71.4)	
yp N stage				0.057 ^a
Node-negative	19	16 (84.2)	3 (42.9)	
Node-positive	7	3 (15.8)	4 (57.1)	

^aFisher's exact test. TRG, tumor regression grade; pCR, pathologic complete response; T, tumor; N, node.

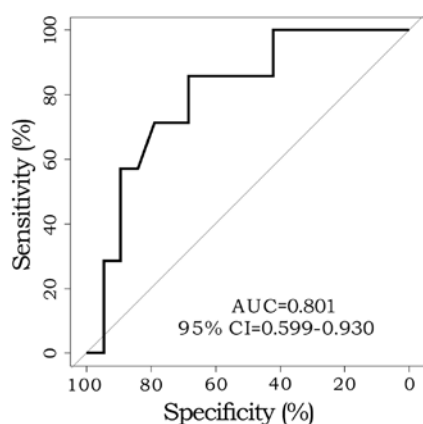


Figure 2. Receiver-operating characteristic curve of 3D cell culture cytotoxicity following radiation and yp node positivity following surgery. The AUC was 0.801 (95% CI, 0.599-0.930). The optimal cut-off point was calculated as 45.05%, according to Youden's index. AUC, area under the curve; CI, confidence interval.

may therefore be limited in deciding on proceeding for radical surgery.

In the present study, a cytotoxicity assay was conducted to predict tumor response following preop-CRT. However, the assay could not predict postoperative pCR or good tumor response (TRGs 1-2). In contrast, low cytotoxicity, dichotomized by a 45% cut-off in the cytotoxicity assay, was associated with ypN positivity. Notably, most of the well-known possible predictors of pCR, such as endoscopy grade, MR TRG, MR TVRR and MR DWI demonstrated no associations with ypN positivity, which confirms the difficulties in predicting lymph node status using clinical variables. Nevertheless, the underlying mechanism of the association between ypN positivity and the cytotoxicity assay cannot be elucidated from the findings of the present study. It may be associated with the difference between the radiation responses of primary tumors and metastatic lymph nodes, considering the lack of association between the cytotoxicity level and TRG. The current practice of local excision following good clinical response indicated that metastatic lymph nodes may regress at the same level as the primary tumor following preop-CRT, which was supported by the clinical observations (31,32). In contrast, a study reported 28.5% lymph node positivity in patients who exhibited pCR (ypT0) (11). Moreover, Choi *et al* (33) demonstrated that lymph

Table V. Clinicopathological characteristics and magnetic resonance parameters according to the low- and high-cytotoxicity groups.

	Low-cytotoxicity group (≤45; n=12), n (%)	High-cytotoxicity group (>45; n=14), n (%)	P-value
Sex			>0.999
Male	6 (50)	8 (57.1)	
Female	6 (50)	6 (42.9)	
Age, years	51.4±10.8 ^a	54.9±16.2 ^a	0.530
BMI, kg/m ²	23.7±3.5 ^a	24.5±4 ^a	0.575
Distance from anal verge, cm	6.1±2.1 ^a	6.2±2.2 ^a	0.875
CEA (initial), μg/l	6.5±7 ^a	5.6±8 ^a	0.759
yp T stage			0.933 ^a
T0	3 (25)	3 (21.4)	
T1	0	1 (7.1)	
T2	3 (25)	5 (35.7)	
T3	6 (50)	5 (35.7)	
yp N stage			0.026 ^b
Node-negative	6 (50)	13 (92.9)	
Node-positive	6 (50)	1 (7.1)	
pCR			>0.999 ^b
Yes	3 (25)	3 (21.4)	
No	9 (75)	11 (78.6)	
Endoscopy			0.190 ^b
Endoscopy_non CR	7 (58.3)	12 (85.7)	
Endoscopy_CR and endoscopy_near CR	5 (41.7)	2 (14.3)	
MR TRG			0.683 ^b
Grade I and II	7 (58.3)	10 (71.4)	
Grade III-V	5 (41.7)	4 (28.6)	
MR Tumor volume, cm ³			
Pre-CRT	17.2±10.0 ^a	25.6±14.1 ^a	0.098
Post-CRT	6.9±5.4 ^a	8.6±6.0 ^a	0.467
TVRR, %	62.4±20.9 ^a	66.1±14.3 ^a	0.603
MR DWI			1.0 ^b
Negative	4 (33.3)	4 (28.6)	
Equivocal and positive	8 (66.7)	10 (71.4)	

^aMean ± standard deviation. ^bFisher's exact test. CEA, carcinoembryonic antigen; BMI, body mass index; T, tumor; N, node; pCR, pathologic complete response; CR, complete response; MR, magnetic resonance; TVRR, tumor volume reduction rate; CRT, chemoradiotherapy; TRG, tumor regression grade; DWI, diffusion weighted image; SD, standard deviation.

node regression grade (LRG) following preop-CRT was the only independent factor associated with relapse-free survival in patients with ypN-positive rectal cancer, who underwent preop-CRT. One of the interesting findings of this study was that the distribution of LRG was not associated with the TRG of the primary tumor, and that the LRG of each metastatic lymph node differed from the other within the same patient. These findings meant that the effects of radiation could differ between the primary tumor and metastatic lymph nodes. These discrepancies may be a possible reason for the different prediction powers of primary tumor cells and lymph node positivity in the cytotoxicity assays described in the present study. However, there was no ypN positivity in patients with

ypT0; thus, considering the relatively low rate of ypN-positive results in patients with ypT0 in a previous study (2), further large-scale investigation may be required to elucidate the true mechanisms underlying the observations of the present study.

The cytotoxicity assay, following 3D cell culture and radiation, had certain advantages. Firstly, the 7-day turnaround time for this procedure was acceptable, as long turnaround times could hinder clinical decision-making. Secondly, the assay can predict patients' tumor response before initiating preop-CRT. Although preop-CRT decreased the local recurrence rate more than postoperative adjuvant CRT, overall survival gain was not anticipated. Rather, preop-CRT may be associated with delayed adverse effects on long-term anorectal, sexual and

Table VI. Univariate and multivariate analysis of factors associated with yp node positivity.

Parameters	Univariate analysis			Multivariate analysis	
	N	N (%)	P-value	OR (95% CI)	P-value
Sex			>0.999 ^a		
Male	14	4 (28.6)			
Female	12	3 (25)			
Age, years			0.278 ^a		
<65	21	7 (33.3)			
≥65	5	0			
cT			>0.999 ^a		
T3	23	6 (26.1)			
T4	3	1 (33.3)			
cN			>0.999 ^a		
Node negative	2	0			
Node positive	24	7 (29.2)			
Endoscopy grade			0.629 ^a		
Endoscopy_non-CR	19	6 (31.6)			
Endoscopy_CR and endoscopy_near CR	7	1 (14.3)			
MR TRG			0.661 ^a		
Grade I and II	9	3 (33.3)			
Grade III-V	17	4 (23.5)			
MR TVRR (%)			0.540 ^a		
<83.6	23	7 (30.4)			
≥83.6	3	0			
MR DWI			0.375 ^a		
Negative	8	1 (12.5)			
Equivocal and positive	18	6 (33.3)			
3D morphologic classification			0.057 ^a		
'Mass-forming group'	19	3 (15.8)			
'None group'	7	4 (57.1)			
Cytotoxicity assay			0.026 ^a		0.031
High (>45)	14	1 (7.1)		1	
Low (≤45)	12	6 (50)		13 (1.2-133.2)	

^aFisher's exact test. CR, complete response; pCR, pathologic complete response; TRG, tumor regression grade; TVRR, tumor volume reduction rate; DWI, diffusion weighted image; MR, magnetic resonance; T, tumor; N, node.

urinary dysfunction (34). In this regard, a study demonstrated the efficacy of initiating chemotherapy in conjugation with selective radiation treatments (35). One reason for employing this new approach is the speculation that radiation-induced complications may be decreased. Several tumor response assays currently used in practice were considered as response evaluation tools rather than prediction models. MRI-based tumor volumetric evaluation, DWI, MR TRG, endoscopy grading, delay of surgery and other variables can be evaluated post-preop-CRT; however, these cannot predict tumor response before initiating preop-CRT. In this regard, the cytotoxicity assay may be useful for predicting whether a patient should undergo preop-CRT, although the actual clinical benefits should be evaluated with further investigations.

Nevertheless, there were several potential limitations of the present study. Immediate storage of fresh tissue in 25-cm² flasks, filled with RPMI culture medium supplemented with 1% penicillin-streptomycin is mandatory for the analysis. Although board-certified surgeons performed the pre-treatment biopsies, it is not always possible to obtain adequate tissue using rigid sigmoidoscopy. Another limitation was cell contamination during the cell culture process. It is well known that the intestinal and feces flora is mostly composed of obligate anaerobes such as *Bacteroides* and *Bifidobacterium*, and the accompanying bacteria during the biopsy process may contaminate the culture. The failure rate, due to cell contamination, cannot be ignored in the present study, which may hinder this procedure and therefore, a modified protocol is required to overcome this issue.

The main limitation of the present study was its small sample size. In a previous study concerned with permeability, based on the spheroid 'round'-type morphological characteristic, increased resistance to drug therapy was observed compared with 'aggregate', 'mass' or 'none' types, which was associated with the Janus kinase-STAT signaling pathway (19). However, in the present study, the 'mass', 'aggregate' and 'round' types were grouped into the 'mass-forming' group, as the mean cytotoxicity level following radiation was similar among the 'round', 'mass' and 'aggregate' types. Although the response of the 3D spheroids to radiation treatment may be entirely different from the response to chemotherapy, the limited number of enrolled patients may hinder the detailed comparison. There are potential differences according to the definitions of ypT stage and TRG. Even with the same ypT3, some patients had small islets of viable cancer cells scattered in the subserosa layer that showed predominant fibrosis (good TRG), whereas other patients have ypT3 tumors in which most cancer cells remain viable (poor TRG) (36). Due to the small sample size in the present study, whether the differences in TRG of the same ypT stage were associated with the cytotoxicity assay could not be analyzed. Finally, it was also possible that the cytotoxicity assay may not accurately reflect humans' resistance to radiation and it does not accurately reproduce the surrounding environments of patients with rectal cancer. This needs to be validated in further studies.

In conclusion, a cytotoxicity assay following 3D cell culture and radiation predicted ypN positivity in patients with locally advanced rectal cancer that underwent preop-CRT. The preliminary findings of the present study may be useful in conducting further studies on this matter, which can potentially lead to clinical application in decision-making prior to initiating preoperative chemoradiotherapy.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JKa provided the concept and idea of this study, analyzed and interpreted the experimental and patients' clinical data, and was a major contributor in writing the manuscript. MCP, JKi, HH, BSM, SHB, KYL and NKK participated in the data acquisition. NKK provided the concept and idea of the present study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol of the present study was approved by the institutional review board of the Severance Hospital, Yonsei University College of Medicine (Seoul, Republic of Korea) (approval no. 4-2011-0445). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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