

# Effect of Long Interval Between Hyperthermochemoradiation Therapy and Surgery for Rectal Cancer on Apoptosis, Proliferation and Tumor Response

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**Abstract.** Neoadjuvant chemoradiotherapy is commonly used to improve the local control and resectability of locally advanced rectal cancer, with surgery performed after an interval of a number of weeks. We have been conducting a clinical trial of preoperative chemoradiotherapy in combination with regional hyperthermia (hyperthermo-chemoradiation therapy; HCRT) for locally advanced rectal cancer. In the current study we assessed the effect of a longer (>10 weeks) interval after neoadjuvant HCRT on pathological response, oncological outcome and especially on apoptosis, proliferation and p53 expression in patients with rectal cancer. Forty-eight patients with proven rectal adenocarcinoma who underwent HCRT followed by surgery were identified for inclusion in this study. Patients were divided into two groups according to the interval between HCRT and surgery,  $\leq 10$  weeks (short-interval group) and >10 weeks (long-interval group). Patients in the long-interval group had a significantly higher rate of pathological complete response (pCR) (43.5% vs. 16.0%) than patients of the short-interval group. Patients of the long-interval group had a significantly higher rate of down-staging of T-stage (78.3% vs. 36.0%) and relatively higher rate of that of N-stage (52.2% vs. 36.0%) than patients of the short-interval group. Furthermore, apoptosis in the long-interval group was relatively higher compared to that of the short-interval group,

without a significant difference in the Ki-67 proliferative index and expression of p53 in the primary tumor. In conclusion, we demonstrated that a longer interval after HCRT (>10 weeks) seemed to result in a better chance of a pCR, a result confirmed by the trends in tumor response markers, including apoptosis, proliferation and p53 expression.

Neoadjuvant chemoradiotherapy (CRT) is commonly used to improve the local control and resectability of locally advanced rectal cancer, with surgery performed after an interval of several weeks (1-7). Current guidelines from the National Comprehensive Cancer Network recommend that patients with clinical stage II/III rectal cancer should be treated with preoperative chemoradiation followed by total mesorectal excision (TME) (8). We have been conducting a clinical trial of preoperative chemoradiotherapy in combination with regional hyperthermia (hyperthermo-chemoradiation therapy; HCRT) for locally advanced rectal cancer (9). The advantages of preoperative HCRT include tumor down-staging, improved resectability and overall survival, and increased anal sphincter preservation (9-11). However, the optimal interval between CRT or HCRT and surgery is still unclear.

In 1999, Francois *et al.* advocated the adoption of an interval between chemoradiation and surgery of six to eight weeks (12). Since the Lyon trial, this 6- to 8-week interval has become part of the standard protocol for neoadjuvant CRT of rectal cancer (7, 12). However, some studies recently suggested that increased intervals could potentially increase the tumor down-staging effect because radiation-induced necrosis appears to be a time-dependent phenomenon (13).

Therefore, in the current study we assessed the effect of a long-term interval after neoadjuvant HCRT on pathological response, oncological outcome and, worthy of special mentioning, apoptosis, proliferation and p53 expression in

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patients with rectal cancer. The present study was designed to determine whether an interval time between chemoradiation and surgery of more than 10 weeks affects tumor response because surgery was performed at a median of 68 days after completion of HCRT interval in our retrospective analysis.

## Patients and Methods

**Patients' characteristics.** Forty-eight patients with proven rectal adenocarcinoma who underwent HCRT followed by surgery in the Department of General Surgical Science, Graduate School of Medicine, Gunma University, from April 2004 to December 2009 were identified for inclusion in this study. During the diagnostic work-up, all patients underwent staging for distant metastasis and lymph node metastasis with computed tomography (CT) of the abdomen and thorax. T-Stage was determined by magnetic resonance imaging (MRI) and colonoscopy with transrectal ultrasonography. The staging work-up was repeated before surgery. The extent and location of the tumor were classified according to the TNM.

**Preoperative HCRT.** All patients received preoperative HCRT. The radiation treatment was delivered by 10-MV X-rays through a three-field box technique. The clinical target volume encompassed the primary tumor and the entire mesorectal tissue. The total radiation dose was 50 Gy, with daily fractions of 2.0 Gy on five consecutive days per week. Chemotherapy consisted of 5-FU (250 mg/m<sup>2</sup> per day) and leovorin calcium (25 mg/m<sup>2</sup> per day) administered by continuous infusion at night for five days a week in the first, third, and fifth weeks of radiation. Two to five hyperthermia sessions were performed once a week with 8-MHz radiofrequency capacitive heating equipment (Thermotron-RF 8; Yamamoto Vinita Co., Ltd., Japan).

**Surgical resection and postoperative follow-up.** Rectal resection was performed using the principles of TME with pelvic autonomic nerve preservation eight weeks after the completion of HCRT but because of logistics, scheduling, and other clinical factors, the practical interval varied from five to 34 weeks and the median interval was 68 days. The median interval obtained in cases with incision was adopted as the cut-off value: 70 days (10 weeks): ≤10 weeks (short-interval group) and >10 weeks (long-interval group). A complete 6-month course of adjuvant 5-FU-based chemotherapy was typically recommended for all medically-fit patients completing HCRT and curative surgery. The majority of patients received oral 5-FU/leucovorin. All patients were closely followed-up by surgeons. Postoperative follow-up on all patients was conducted every three or six months for 2 years. After 2 years, patients underwent follow-up examinations every six months. CT of the abdomen and thorax was first done at the sixth postoperative month and then yearly thereafter.

**Pathology and immunohistochemistry.** Histopathological examination and evaluation of the pathological response to HCRT were performed by experienced pathologists according to the histological criteria of the Japanese Classification of Colorectal Carcinoma (14). Grades were assigned according to the amount of necrosis, degeneration, and lytic change of the tumor in the estimated total amount of the lesion (15). Apoptosis of tumor cells was quantified by immuno-

histochemistry with M30-CytoDEATH antibody (Roche, Mannheim, Germany). The cell cycle and apoptosis control gene *p53* may play a major role in the tumor response to cytotoxic agents such as radiation and chemotherapy. Immunohistochemistry was performed using formalin-fixed and paraffin-embedded tissue sections with the following antibodies, according to the standard streptavidin-biotin complex technique: anti-human M30-CytoDEATH (mouse monoclonal, 1:6; Histofine) and anti-human *p53* antibody. Antigen retrieval was performed in an autoclave (citrate buffer, pH 6.0). Isotype-matched non-immune IgG was used as a negative control instead of the primary antibody, and showed negative results (data not shown). Immunohistochemical staining for Ki-67 was performed according to a previously described method (19, 20). Antigen retrieval was performed in an autoclave (citrate buffer, pH 6.0). The sections were incubated with anti-Ki-67 monoclonal antibody (DAKO, Glostrup, Denmark) at a dilution of 1:100. The Ki-67 proliferative index (Ki index) was defined as the percentage of nuclear-stained tumor cells among more than 1000 cells counted (16, 17). Five high-power fields were evaluated for each of three different regions.

**Statistical analysis.** Patients were divided into two groups according to the interval between HCRT and surgery, ≤10 weeks (short-interval group) and >10 weeks (long-interval group). A univariate analysis was conducted using Fisher's exact test or the  $\chi^2$  test with or without Yates' correction. To compare the two groups, Student's *t*-test was used. To test the independence of the risk factors, the variables were entered into a multivariate logistic regression model with a likelihood of  $p < 0.05$ . The recurrence-free interval and overall survival was calculated by the Kaplan-Meier method. The log-rank test was used to evaluate differences between overall survival and recurrence-free intervals. A value of  $p < 0.05$  was considered to be statistically significant.

## Results

**Patients' and tumor characteristics.** We analyzed the cases of 48 patients with rectal cancer who underwent HCRT followed by rectal resection. The mean age of the patients was 61.4±9.4, with an age distribution from 33-75 years, and 37 patients (77.1%) were male. Patients' characteristics are shown in Table 1. All patients tolerated this regimen without hematological toxicities. The practical interval from completion of HCRT to surgery ranged from 5 to 34 weeks, and the median time was 68 days. Out of the 48 patients, 25 (52.1%) underwent surgery less than 10 weeks after HCRT completion (short-interval group), whereas 23 patients (47.9%) underwent surgery 10 weeks or more after HCRT (long-interval group). Table I summarizes not only the patient characteristics, but also the results of the univariate analysis conducted to determine the relationship between the short- and long-interval groups. The two groups did not differ in age, gender ratio, or distribution of clinical T-stage and N-stage.

**Pathological and tumor response.** When the clinical pre-treatment stage was compared with the pathological results, the overall down-staging rate, including both the T- and N-

Table 1. Patients' characteristics and clinicopathological features associated with interval between HCRT and surgery. Values are expressed as the mean±SD.

	Short interval (≤10 weeks) N=25	Long interval (>10 weeks) N=23	p-Value
Age (years)	63.0±8.6	59.7±10.2	0.228
Gender (male/female, n)	18/7	15/8	0.422
Preoperative T-stage (n)			
cT2	4	3	0.580
cT3	18	19	
cT4	3	1	
Preoperative N-positive (n)	14	13	0.601
Anal preserving surgery (n)	21	21	0.375
Adjuvant therapy (n)	17	9	0.031
pCR (n)	4	10	0.037
Down-staging T-stage (n)	9	18	0.004
Down-staging N-stage (n)	9	12	0.201
Ki-67 (%)	47.6±37.1	54.7±35.9	0.586
M30-CytoDEATH (%)	1.46±1.34	3.46±4.37	0.058
p53 (%)	47.4±37.1	34.4±36.8	0.353
Local recurrence (n)	2	1	0.532
Distant recurrence (n)	6	4	0.419

stage, was 72.9% (35 patients). Down-staging of the T- and N-stages was possible in 27 (56.3%) and 21 patients (43.8%), respectively. Patients of the long-interval group had a significantly higher rate of down-staging of the T-stage (36.0% *vs.* 78.3%) and a relatively higher rate of that of N-stage (36.0% *vs.* 52.2%) than patients of the short-interval group, respectively. Fourteen patients (29.2%) achieved pathological complete response (pCR) of the primary tumor. There was a significant difference in the pCR rate (short-interval group: 16.0% *vs.* long-interval group; 43.5%;  $p=0.037$ ). No significant difference in the Ki-67 proliferative index (Figure 1b) and expression of p53 (Figure 1c) of the primary tumor was observed. Although the difference was not statistically significant, the expression of M30-CytoDEATH in the long-interval group tended to be higher than that of the short-interval group ( $p=0.058$ ) (Figure 1a).

**Oncological outcome.** The median follow-up time from surgery was 32 months (range=4 to 102 months). The local recurrence rate was 6.3% (short 8.0% *vs.* long 4.3%;  $p=0.532$ ) and that for total metastasis was 25% (short 28.0% *vs.* long 21.7%;  $p=0.435$ ). The rates of local and distant metastases were not significantly different between the two groups. Disease-free survival and overall survival by Kaplan–Meier curves differed between the two groups, but the difference was not statistically significant (Figure 2).

## Discussion

Locally advanced rectal cancer is usually treated with preoperative CRT followed by surgery. This strategy has been shown to achieve pathological down-staging and improve local control. We previously showed the advantage of preoperative HCRT, or regional hyperthermia in combination with CRT, for locally advanced rectal cancer, including tumor down-staging, improved resectability and overall survival, and increased anal sphincter preservation (9, 10). Although an interval of 6-8 weeks from CRT to surgery is historically considered the standard, the optimal interval has not yet been determined. The aim of this study was therefore to assess the effect of a longer (>10 weeks) interval after neoadjuvant HCRT on pathological response, oncological outcome, and especially apoptosis, proliferation and p53 expression in patients with rectal cancer.

The key observations made in this study can be summarized as follows: i) Patients of the long-interval group had significantly higher rate of pCR (43.5% *vs.* short interval group 16.0%) than patients of the short-interval group; ii) patients of the long-interval group had significantly higher rate of T-staging than patients of the short-interval group, respectively; and iii) apoptosis in the long-interval group tend to be higher than that of the short-interval group without significant difference in the Ki-67 proliferative index and expression of p53 in primary tumor. These findings provide clear evidence that a longer interval after HCRT (>10 weeks) seems to result in a higher chance of a pCR, likely due the tumor response, including effects on apoptosis, proliferation and p53 expression.

The rate of pCR after preoperative CRT ranged from 10% to 16% in various series examined in a previous review (18). Recently, some studies have demonstrated that longer intervals between neoadjuvant CRT and surgery are associated with favorable pathological findings (3, 19). In a retrospective study, Tulchinsky *et al.* showed a longer interval from preoperative CRT to surgery (>7 weeks) was associated with higher rates of pCR or near-pCR than a shorter interval (≤7 weeks) (35% *vs.* 17%) (20). Several studies also reported that an extended time interval of more than eight weeks between the end of preoperative therapy and surgery led to a higher pCR rate (7). Some studies have also shown that an interval longer than 10 weeks between completion of CRT and surgery is more effective (7, 19). In the present study, the pCR rate of patients with longer interval to surgery (>10 weeks) was significantly higher than that of patients with a short interval (43.5% *vs.* 16.0%), which compares favorably with findings in other reports. The favorable results of our current study might be due to additional HCRT. Further investigations to improve the pCR rate of HCRT for rectal cancer are required.



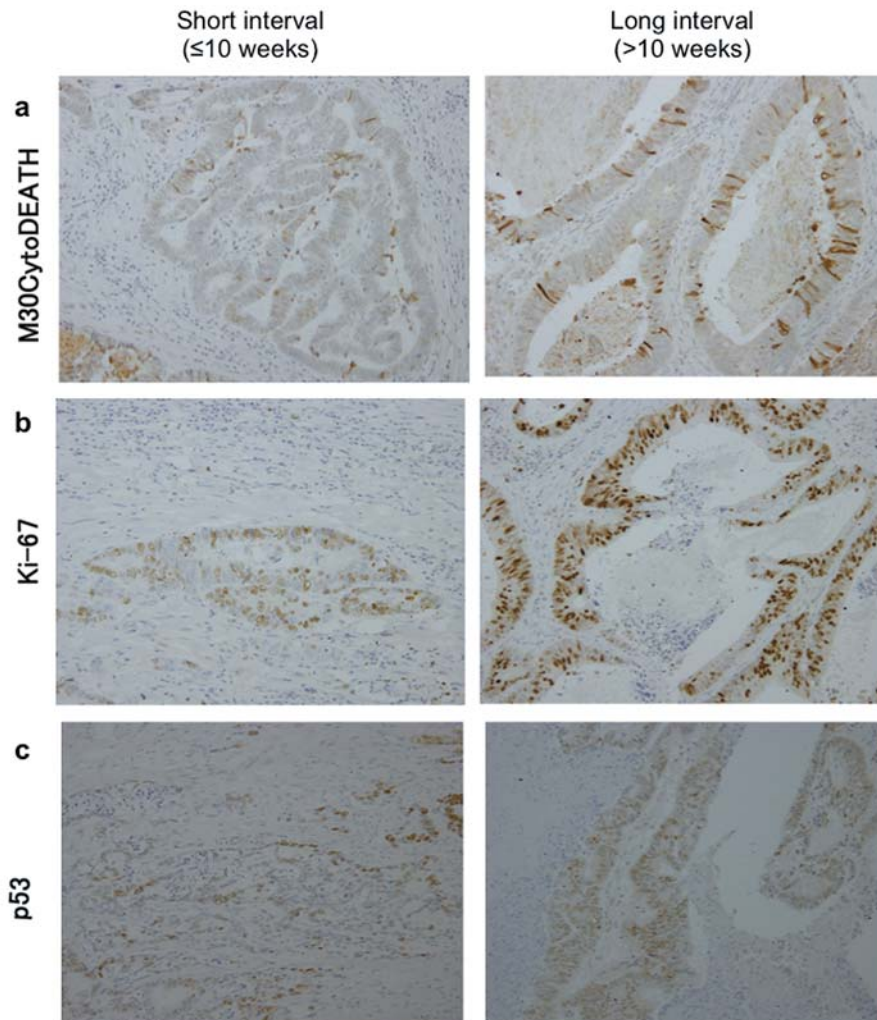


Figure 1. Immunohistochemical finding of M30-cytoDEATH (a), Ki-67 (b) and p53 (c) after hyperthermochemoradiation therapy (H&E,  $\times 200$ ). No significant difference in the Ki-67 proliferative index and expression of p53 in the primary tumor was observed. Although the difference was not statistically significant, the expression of M30-CytoDEATH in the long-interval group tended to be higher than that of the short-interval group.

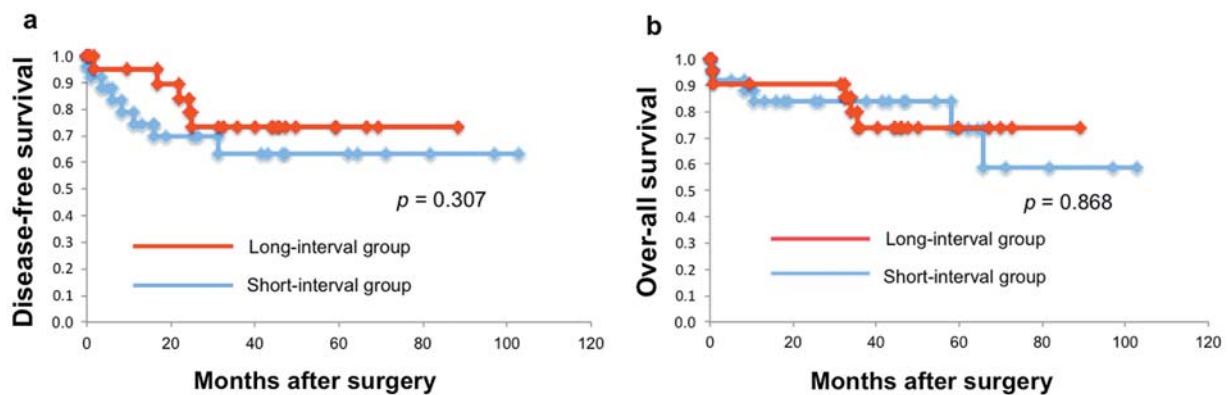


Figure 2. Time to tumor recurrence and overall survival by the Kaplan–Meier curves differ among patients of the longer- and the shorter-interval groups, but no statistically significant differences were observed.

In addition, our study showed a significantly increased rate of down-staging and relatively better oncological results in patients of the long-interval group (>10 weeks). In our previous study, down-staging was a good predictor of oncological outcome (10), and several other studies have also shown that pathological down-staging is an important prognostic factor (4). These findings reflect the possibility that a longer interval from HCRT to surgery is more effective for oncological outcome. The fact that oncological outcomes were not significantly different in the two groups in our study might be due to the small sample size. Thus, further investigations are needed in order to better document oncological results.

Not all tumors respond to preoperative CRT. As the interval to surgery increases, the tumor may in fact progress, with detrimental effects on resectability; eventually, this could negatively impact the oncological outcome (21). There is no evidence-based protocol to follow if the tumor fails to regress or increases in size after HCRT. Therefore, in the current study, we assessed the effect of a longer-term interval after neoadjuvant HCRT on tumor response, including apoptosis, proliferation and p53 expression, in addition to pathological response, or oncological outcome, in patients with rectal cancer. In the current study, no significant difference in the Ki-67 proliferative index and expression of cell cycle and apoptosis control gene *p53* in primary tumor after a long interval (>10 weeks) from HCRT were observed (Figure 1). The expression of M30-CytoDEATH, an apoptotic gene, in the long-interval group tended to be higher than that of the short-interval group, but the difference was not statistically significant (Figure 1a). Tumor regression and radiation-induced necrosis are a time-dependent phenomenon (13). These data emphasize that a longer interval, beyond 10 weeks, is safe and tolerable from the viewpoint of tumor behavior after HCRT.

This study showed that a longer interval (>10 weeks) was effective with a significantly higher pCR rate. However, the important question regarding the optimal timing from HCRT to surgery remains unanswered. Furthermore, controversy exists about the surgical treatment of patients with pCR, compared to more conservative approaches such as a wait-and-see strategy (22). The downside and danger is the potential effect of tumor progression during this therapy-free interval. Major challenges still remain with regard to the optimal timing for surgery and patient selection for the conservative strategy. Predictors of response to HCRT and implementation of individualized strategies are necessary to extend the interval between HCRT and surgery even more.

This study has potential limitations. It was a retrospective analysis and the number of cases in our study was relatively small. However, the results of our study are encouraging in terms of the rate of pCR, down-staging, and tumor response

after HCRT. Further studies are required to determine strategies for optimizing the oncological outcome on an individual basis.

In conclusion, we have demonstrated that a longer interval after HCRT (>10 weeks) resulted in a significant better chance of a pCR, likely due to tumor response, including apoptosis, proliferation and p53 expression. This study showed the effectiveness and safety of a longer interval (>10 weeks) after HCRT; however, this interval should be examined in the context of a randomized control trial.

## Competing Interests Statement

The Authors declare that they have no competing financial interests.

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