J Gastrointest Surg (2007) 11:520–528
DOI 10.1007/s11605-007-0127-x

# Bromodeoxyuridine Labeling Index as an Indicator of Early Tumor Response to Preoperative Radiotherapy in Patients with Rectal Cancer

**Anna Gasinska · Jan Skolyszewski · Tadeusz Popiela · Piotr Richter · Zbigniew Darasz · Krystyna Nowak · Joanna Niemiec · Beata Biesaga · Agnieszka Adamczyk · Krzysztof Bucki · Krzysztof Malecki · Marian Reinfuss · Teresa Kowalska**

Published online: 2 March 2007


**Abstract**
*Purpose* Assessment of tumor proliferation rate using Bromodeoxyuridine labeling index (BrdUrdLI) as a possible predictor of rectal cancer response to preoperative radiotherapy (RT).
*Methods and material* Ninety-two patients were qualified either to short RT (5 Gy/fraction/5 days) and surgery about 1 week after RT (schedule I), or to short RT and 4–5 weeks interval before surgery (schedule II). Tumor samples were taken twice from each patient: before RT and at the time of surgery. The samples were incubated with BrdUrd for 1 h at 37°C, and the BrdUrdLI was calculated as a percentage of BrdUrd-labeled cells.
*Results* Thirty-eight patients were treated according to schedule I and 54 patients according to schedule II. Mean BrdUrdLI before RT was 8.5% and its value did not differ between the patients in the two compared groups. After RT tumors showed statistically significant growth inhibition (reduction of BrdUrdLI). As the pretreatment BrdUrd LI was not predictive for early clinical and pathologic tumor response, prognostic role of the ratio of BrdUrdLI after to BrdUrdLI before RT was considered. The ratios were calculated separately for fast (BrdUrd LI>8.5%) and slowly (BrdUrd LI≤8.5%) proliferating tumors and correlated with overall treatment time (OTT, i.e., time from the first day of RT to surgery). One month after RT, accelerated proliferation was observed only in slowly proliferating tumors.
*Conclusions* Pretreatment BrdUrdLI was not predictive for early clinical and pathologic tumor response. The ratio after/before RT BrdUrdLI was correlated to inhibition of proliferation in responsive tumors.

**Keywords** BrdUrdLI · Proliferation rate · Early tumor response · Rectal cancer · Radiotherapy

The paper was presented at ECCO 13, October 30 to November 03, 2005 in Paris, France

A. Gasinska (✉) · J. Niemiec · B. Biesaga · A. Adamczyk
Department of Applied Radiobiology, Center of Oncology, Garncarska 11, 31-115 Krakow, Poland
e-mail: z5gasins@cyf-kr.edu.pl

J. Skolyszewski · T. Kowalska
Department of Gastrointestinal and Urological Cancer Unit, Center of Oncology, Krakow, Poland

T. Popiela · P. Richter · K. Bucki
Collegium Medicum, Jagiellonian University, Kopernika 40, Krakow, Poland

Z. Darasz
Department of Surgery, Center of Oncology, Krakow, Poland

K. Nowak
Department of Pathophysiology, Collegium Medicum, Jagiellonian University, Krakow, Poland

K. Malecki
Breast and Thoracic Cancer Unit, Center of Oncology, Krakow, Poland

M. Reinfuss
Department of Radiation Oncology, Center of Oncology, Krakow, Poland

## Introduction

In specialized centers, a refined surgical technique has resulted in high local control figures in rectal cancer. However, local recurrence rates after "standard" surgery are generally high, with figures ranging between 20 and 40%[1,2], although after adopting the total mesorectal excision (TME) concept they fell down to 10–12%[3,4]. Radiotherapy in addition to surgery significantly diminishes the risk of local failure by more than half, from 8 to 2% after 2 years[3]. Therefore, combined treatment: radiotherapy (RT) and surgery in the treatment of patients with resectable rectal cancer has been proposed in many trials using either preoperative[5,6] or postoperative irradiation[7,8]. Better results of preoperative RT for 5 days (25 Gy in five fractions) in comparison with postoperative 60 Gy in 30 fractions were achieved by a Swedish group[4,9,10], with respect to the local recurrence rate[11] and overall survival[11,12].

A corresponding improvement in overall survival has not been demonstrated after postoperative radiotherapy alone[13]. Graf's[12] study provided a clinically significant biologic effect of a short preoperative course of radiotherapy on the tumor size and on the incidence of nodal metastases; however, this effect was minimized if surgery was performed immediately after radiotherapy. The effect is most likely caused by death of tumor cells in the primary tumor and in the involved nodes. A short treatment course of radiotherapy, i.e., 5×5 Gy is desirable, and this regimen is currently considered as the gold standard in many centers. However, using this schedule it is difficult to observe a down-staging and/or downsizing of the tumor, which is of importance for the selection of patients for sphincter-preserving surgery (anterior resection).

In clinical practice there are no certain methods able to predict tumor response to preoperative radiotherapy (RT). The optimal timing of surgery after preoperative radiotherapy in rectal cancer is unknown. However, it was shown that a long interval (6–8 weeks) between preoperative radiotherapy (39 Gy in 13 fractions) and surgery was associated with a significantly greater clinical tumor volume reduction than a short interval (2 weeks)[14]. On the other hand, it was shown that subclinical pelvic deposits of rectal cancer could grow rapidly during preoperative radiation therapy and during the radiotherapy–surgery interval, with an adverse influence on the rate of pelvic tumor control from protracting the overall treatment time[15]. Graf et al[12] showed that low doses in short RT only offer clinically relevant reduction in the risk of pelvic relapses if the overall radiation treatment time is short. Thus, the rate of cancer cell proliferation seems to be a very important prognostic factor.

The aim of this study is to evaluate BrdUrd LI and S-phase fraction (SPF) as the possible indicators of tumor proliferation rate and predictors of the tumor response to neoadjuvant RT in patients with rectal cancer, and to suggest an optimal interval between short RT course and surgery.

## Methods and Materials

### Patients

Between November 2003 and January 2006 we recruited 92 patients with resectable rectal carcinoma for whom curative surgery was planned. Patients were eligible for the trial if they were less than 75 years old, had a histopathologically proved adenocarcinoma (T2/T3)[16] situated less than 12 cm from the verge of the anus, and gave informed consent for their participation. The protocol was approved by the Ethical Committee of the Center of Oncology, and each patient gave written consent.

The criteria for exclusion were: locally nonresectable tumor; plan to perform only local tumor excision; known metastatic disease; previous radiotherapy of pelvis region; other malignant disease; and patient's refusal.

### Preoperative Radiotherapy

The patients assigned to preoperative radiotherapy received a total tumor dose of 25 Gy. The treatment was given in five fractions over 5 days, one posterior and two lateral wedged fields were irradiated with photons of maximum 6 MV energy. According to the random selection surgery was performed the following week (schedule I) or after longer interval of 4–5 weeks (schedule II).

### Surgery

Anterior resection of rectum or abdominoperineal excision was performed within a week or a month after the completion of RT. Type of surgery was resection of the rectum and lower sigmoid with involved adjacent tissue and regional lymph nodes up to or above the origin of inferior mesenteric artery. A minimal touch technique was used with high tight ligation of the inferior mesenteric artery. The decision whether the patient should have an abdominoperineal resection or a sphincter-preserving surgery was made by the surgeon during the operation.

An abdominoperineal resection of rectum was performed in 41 (44.6%) of the patients, and sphincter preserving surgery was performed in 51 (55.4%).

### Biological Assessment of Tumor Response

Tumor samples were taken twice: before radiotherapy (through a rectoscope) and during surgery from the same

place, i.e., at the lowest edge of the tumor mass. Each biopsy was divided into two parts: one was used for BrdUrd LI assessment, and the second was used for immunohistochemical analysis (these results will be the subject of a separate study).

### Bromodeoxyuridine Labeling Index

Incorporation of BrdUrd in tumor samples from a biopsy (0.3–0.5 $cm^3$) was carried out *in vitro* according to the high-pressure oxygen method. The BrdUrd staining procedure and flow cytometry have been described in detail elsewhere[17]. The stained preparations were analyzed with a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, Sunnyvale, CA, USA) by one coauthor (AG) and $20\times10^3$ events were collected in each histogram. The BrdUrdLI was calculated as a percentage of BrdUrd-labeled cells in a sample, which incorporated BrdUrd during 1 h of incubation at 37°C (with discrimination of diploid subpopulation in aneuploid tumors). The tumor ploidy and SPF were calculated from the DNA profile with ModFit software running on a MacIntosh computer. Apoptotic cells were identified as objects with a fractional DNA content not less than 20% of the 2n DNA content. Cell death was calculated as the sum of apoptosis and debris. The tumor ploidy was estimated by evaluating the DNA index, i.e., the ratio of the modal DNA fluorescence of abnormal to normal $G_{1/0}$ cells. Aneuploidy was assessed in cases in which the normal and neoplastic cell populations gave two separate peaks. Human lymphocytes were used for the reference peak. Tumors with BrdUrdLI >8.5% (median value) were considered as fast, and those with BrdUrdLI ≤8.5% were considered as slowly proliferating tumors.

### Clinical Assessment of Tumor Response

Tumor size before RT was assessed basing on measures taken during rectoscopy, and endorectal sonography. Tumor regression after RT was assessed at the time of operation by surgeons according to the following Response Evaluation Criteria in Solid Tumors (RECIST)[18]:

Complete response (CR): 100% disappearance; partial response (PR): 30–99% decrease; stable disease (SD): neither CR, PR or PD criteria met; progression of disease (PD): 20% increase in sums of tumor longest diameters.

### Pathological Assessment of Tumor Response

Tumor regression after RT was evaluated by a pathologist on the excised tumor mass. The following criteria of tumor regression assessed by Dworak et al.[19] were applied:

D0—no regression; D1—dominant tumor mass with obvious fibrosis and/or vasculopathy; D2—dominantly fibrotic changes with few tumor cells or groups; D3—very few (difficult to find microscopically) tumor cells in fibrotic tissue with or without mucous substance; D4—no tumor cells, only fibrotic mass (total regression or response).

### Statistical Methods

Statistical analysis was performed with STATISTICA vs.5. Intergroup differences in the ordinal data were tested with ANOVA test or Student's *t* test. *P* values of less than 0.05 were considered to indicate statistical significance. Linear regression was applied for assessing differences between fast and slowly proliferating tumors in relation with OTT, and its significance was determined by testing the difference between two correlation coefficients. Stratification by BrdUrd LI level was introduced and tested by the inclusion of dummy variable in the regression model.

## Results

### Patients

A total of 92 patients were included in the study. Twenty-eight (23.3%) out of 120 patients initially qualified for this study were excluded from the analysis because of discontinuation of treatment, metastatic tumor noticed at operation, or no tumor samples taken for biological assessment during surgery. Mean age for the entire group of patients was 61.6 years (range 30–75). There were 68 men and 24 women. There were no statistical differences between the two groups at the time of recruitment for prognostic factors such as: sex, age, histologic grade, or tumor stage (Table 1).

In our series of patients, there were 27 stage 1 (29.3%), 55 were T2 (59.8%), and 10 were T3 (10.9%). In 26 patients, tumor cells well differentiated (G1), 63 moderately differentiated (G2), and three poorly differentiated (G3) (Table 1). Thirty-eight patients were treated according to schedule I, in which time interval between end of irradiation and surgery averaged 8.8 days (range 2–14; Table 1). In 54 patients, schedule II was applied, in which mean break was 32.9 days (range 17–45). Because the interval between RT and surgery appeared to be longer than planned, overall treatment time (OTT), e.g., time from the beginning of RT to surgery, was calculated and it appeared to be 7–50 days (Table 1).

### Biologic, Pathologic, and Clinical Assessment of Tumor Response

Mean BrdUrd LI before RT was 8.5% (range 1.0–24.2%) and SPF was 22.0% (range 3.8–49.9%) and the mean values did not differ between the two schedules (Table 2).

**Table 1** Selected Characteristics of Patients and Treatment Parameters

| Characteristics | Schedule I | Schedule II | Total |
|---|---|---|---|
| Age mean (±SD) years | (38)[a] 61.2± 12.0 | (54) 61.9± 9.5 | (92) 61.6± 10.6 |
| Sex | | | |
| Male | 30 | 38 | 68 |
| Female | 8 | 16 | 24 |
| Histological grade | | | |
| G1 | 6 | 20 | 26 |
| G2 | 29 | 34 | 63 |
| G3 | 3 | 0 | 3 |
| Tumor stage | | | |
| T1 | 8 | 19 | 27 |
| T2 | 25 | 30 | 55 |
| T3 | 5 | 5 | 10 |
| PTNM | | | |
| 1 | 16 | 25 | 41 |
| 2 | 8 | 6 | 14 |
| 3 | 13 | 17 | 30 |
| 4 | 1 | 2 | 3 |
| Interval between RT and surgery | | | |
| Mean (range) days | (38)[a] 8.8 (2–14) | (54) 32.9 (17–45) | (92) 22.9 (2–45) |
| OTT mean (range) days | (38) 13.8 (7–19) | (54) 37.9 (22–50) | (92) 27.9 (7–50) |
| Surgery | | | |
| Sphincter-preserving | 20 (52.6 %) | 31(57.4 %) | 51 |
| Abdominoperineal resection | 18 | 23 | 41 |

[a] Number of patients

Poorly differentiated tumors showed statistically significant higher BrdUrd LI than grades 1 and 2 tumors ($P$=0.015; Table 3). After RT, tumors treated according to both schedules showed statistically significant growth inhibition (reduction of BrdUrd LI and percentage of SPF cells) in comparison with the values obtained before RT (Table 2). Radiation induced inhibition of tumor proliferation was expressed as a percentage of the after RT to before RT BrdUrd LI, and SPF as after/before RT percentage. This ratio ranged from 2.5 to 514% for BrdUrd LI (Fig. 1) and from 5.8 to 522.2% for SPF. When we stratified patients into two groups according to their biological RT response, those radioresponsive with reduction of pretreatment values after radiotherapy above 50% and those less responsive with reduction below 50%, it appeared that the mean values (of the after/before RT ratios of BrdUrd LI and SPF) for the more radioresponsive tumors were significantly higher than for the less responsive ones. Therefore, these ratios were presented separately for fast (BrdUrd LI >8.5%, SPF >22.0%) and slowly (BrdUrd LI ≤8.5%, SPF ≤22.0%) proliferating tumors. Mean BrdUrd LI value after RT for fast proliferating tumors (41 cases) showed statistically significant ($P$=0.027) reduced pretreatment percentage (46.8%) in comparison with slowly proliferating tumors (85.3%, 51 cases). The same was true for SPF of fast (56.4%, 55 cases) and slowly (113.8%, 37) proliferating tumors ($P$=0.006).

Next, the after/before RT ratios for BrdUrd LI and SPF were correlated with OTT. For SPF, statistical difference between linear regression coefficients for fast and slowly proliferating tumors was not obtained ($P$=0.446), therefore the data for BrdUrd LI only are shown (Fig. 1). Insert on Fig. 1 shows a significant ($P$=0.033) difference in proliferation rate between fast and slowly proliferating tumors treated within OTT >30 days. At that time slowly proliferating tumors, contrary to fast proliferating ones, show no inhibition but accelerated proliferation of tumor cells. This phenomenon was also confirmed by increased fraction of S-phase cells in tumors treated with longer RT schedule (Table 2). The influence of BrdUrdLI level has been also tested by the extended regression model between OTT and the percentage of after/before RT BrdUrd LI. BrdUrd LI level higher than 8.5% has been coded as dummy variable. It appeared to be significant ($P$=0.025) in the relation between OTT and the percentage of after/before RT BrdUrd LI. The partial regression coefficient indicates that the average decrease of the percentage after/before RT BrdUrdLI for fast proliferating tumors (BrdUrd LI >8.5%) equals 39%.

All 92 irradiated rectal tumors were reviewed by the same pathologist (KN). The tumors were classified according to the World Health Organization classification of

**Table 2** Status of Biological Parameters Before and After RT

| Group | BrdUrd LI (%) Mean (range) | S-phase fraction (%) Mean (range) | Apoptosis (%) Mean (range) |
|---|---|---|---|
| All patients | | | |
| Before RT | 8.5 (1.0–24.2) | 22.0 (3.8–49.9) | 5.9 (0–52.8) |
| After RT | 4.1* (0.4–18.3) | 16.8** (1.5–101.0) | 9.8*** (0–45.9) |
| RT schedule I | | | |
| Before RT | 8.4 (1.1–24.2) | 21.5 (6.1–49.2) | 6.6 (0–32.4) |
| After RT | 3.8*(0.8–12.6) | 14.1**** (1.5–47.9) | 10.5 (0–43.3) |
| RT schedule II | | | |
| Before RT | 8.6 (1.0–20.0) | 22.3 (3.8–49.9) | 5.4 (0–52.8) |
| After RT | 4.5* (0.4–18.3) | 17.2 (2.6–101.0) | 9.5***** (0–45.9) |

*$P$=0.000
**$P$=0.015
***$P$=0.010
****$P$=0.002

**Table 3** The Relationship Between Tumor Biological Parameters and Histological Grade

| Histological grade | *N* | BrdUrd LI (%) Mean (range) | S-phase fraction (%) Mean (range) | Apoptosis (%) Mean (range) |
|---|---|---|---|---|
| G1 | 26 | 8.5 (1.1–17.1) | 23.7 (5.8–49.9) | 3.4 (0–32.4) |
| G2 | 61 | 8.2 (1.0–20.0) | 21.0 (3.8–45.6) | 7.2 (0–52.8) |
| G3 | 3 | 16.2*, ** (9.5–24.2) | 27.7 (18.9–34.7) | 2.1 (0.4–4.8) |

*$P$=0.015, difference between G1 and G3
**$P$=0.013, difference between G2 and G3

intestinal carcinoma[16] and staged according to the TNM classification[20]. Of the 92 rectal tumors four had no pTNM classification, 41 were pT1 (46.6%), 14 were pT2 (15.9%), 30 were pT3 (34.1%), and three were pT4 (3.4%). Regional lymph node metastases were found in 27 (30.7%) patients, and 27 (30.7%) patients had their tumor down-staged.

Pathologic assessment of tumor regression after RT according to classification described by Dworak[19] was performed in 90 out of 92 patients (for two patients the assessment was impossible). The analysis showed no tumor regression (D0) in 18 (20.0%) tumors, dominant tumor mass (D1) in 46 (51.1%) tumors, a few tumor cells in fibrotic mass (D2) in 18 (20.0%) tumors, single tumor cells (D3) in four (4.4%), and no tumor cells were observed in four (4.4%) of the examined tumors (Fig. 2a). In 25 (27.8%) out of 90 patients marked pathologic down-staging (no residual tumor confined to the rectal wall) was visible. Pretreatment BrdUrdLI and SPF were not correlated with early clinical and pathologic tumor response. However, patients having tumors with LI >8.5% were more radio-responsive (showed significant reduction in proliferative rate after radiotherapy) than patients with BrdUrdLI ≤8.5% tumors, although statistically significant difference between the two tumor subgroups was seen only for D0–D1 grade (Fig. 2b).

In the clinical assessment of tumor mass resected during surgery, 34 (36.9%) tumors showed stable disease, 12 (13.0%) showed progressive disease, 41 (44.6%) showed partial response, and four (4.3%) showed complete response (Fig. 3a). And again, in fast proliferating tumors, greater inhibition in tumor proliferation rate (reduction of pretreatment BrdUrd LI value >50%) was observed in fast than in slowly proliferating tumors; however, this difference was not statistically significant (Fig. 3b). As the observed correlation between clinical assessment and SPF was weaker than for BrdUrd LI, the data were not shown. Partial and total tumor regression was observed in 45 (48.9%) tumors. However, tumor proliferation status was not in agreement with the kind of surgery. Sphincter-preserving surgery was performed in 51 out of 92 patients: in

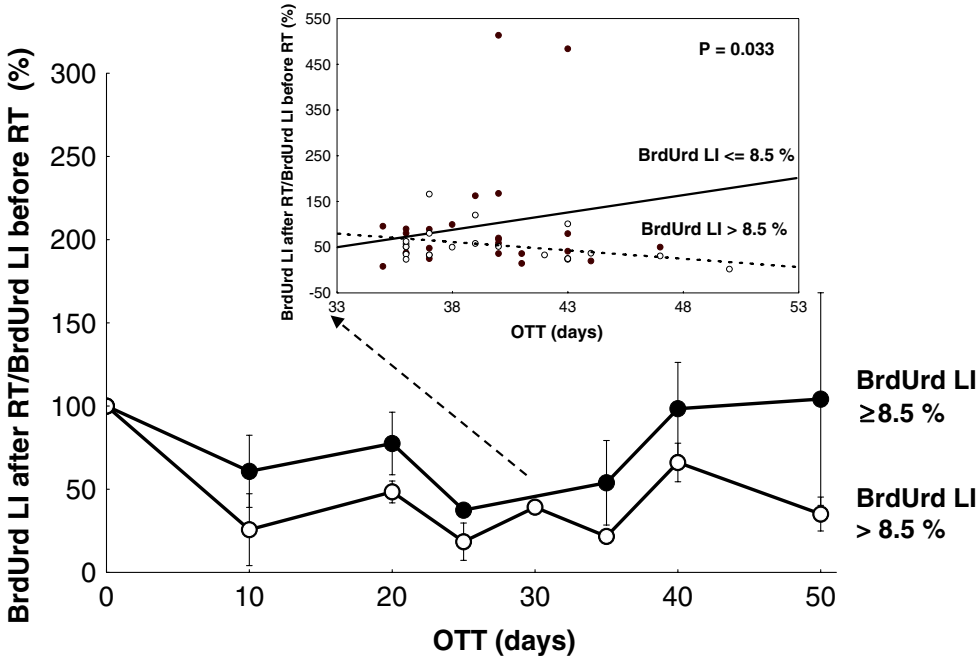


**Figure 1** The association between biological tumor response for slowly (BrdUrd LI≤8.5%; closed symbol) and faster proliferating tumors (BrdUrd LI>8.5%; open symbol) and overall treatment time. Insert shows linear regression performed separately for each of the tumor subgroups for OTT>30 days. *P* value shows difference between two correlation coefficients.

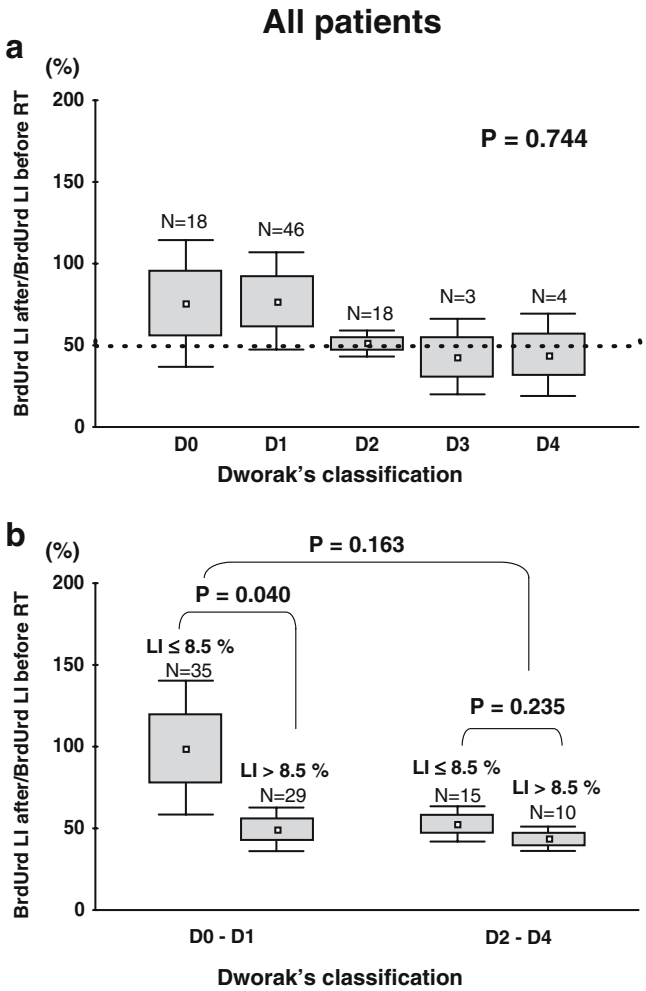


**Figure 2** Association between biological and pathological assessment (Dworak classification) of early tumor regression for (a) total group of patients and (b) for slowly (BrdUrdLI≤8.5%) and fast proliferating (BrdUrdLI>8.5%) tumors. Mean values±SE are shown. For stages D0–D1, statistically significant lower inhibition of tumor cell proliferation after RT was observed for slowly than fast proliferating tumors.

22 (23.9%) fast proliferating and in 29 (31.5%) slowly proliferating tumors.

## Discussion

This study provides evidence of a clinically significant biological effect of a short preoperative course of RT on tumor proliferation rate. The impact of irradiation on biological tumor response was assessed by BrdUrd LI, SPF, and the degree of subsequent pathologic and clinical down-staging of the tumors after surgery. The study showed differences in the pretreatment proliferation rate of the tumor. Mean BrdUrd LI before RT was equal to 8.5% and ranged from 1 to 24.2%. Mean SPF was 22.0% and ranged from 3.8 to 49.9%. The proportion of cells in S-phase as estimated by the DNA content overestimates the labeling index determined by the uptake of BrdUrd. This may be so because the exposure time is quite short and there may be subpopulations in the tumors that are synthesizing DNA at a very slow rate, or there may indeed be cells with an S-phase DNA content that are not synthesizing DNA (as a result of nutrient or oxygen supply, lack of growth factors, inadequate vascularity).

Mean value of the BrdUrd LI obtained in this study was lower than the one estimated by Bergstrom et al.[21], Palmqvist et al.[22], and Terry et al.[23], and can be explained by a different method used by these authors: in vivo incorporation of iodouridine/bromodeoxyuridine, which can cause longer exposure of the tracers to S-phase cells. The differences in the LI value might be caused also by heterogeneity in proliferation within the tumor. It was shown by Bergstrom et al.[21] that rectal tumors are polarized, having the superficial surface toward the lumen of the gut and the other toward deep structures facing

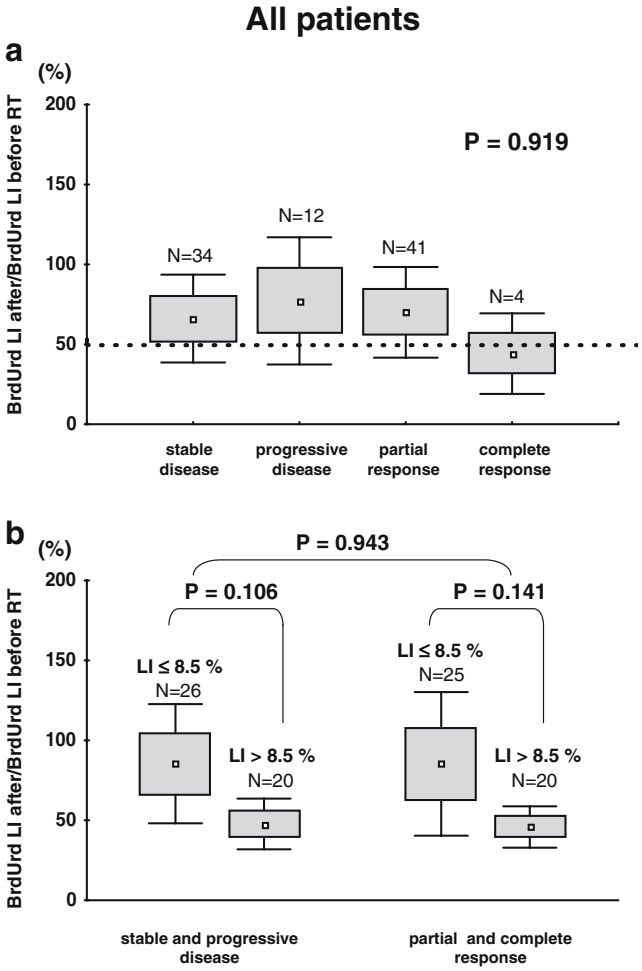


**Figure 3** Association between biological and clinical assessment of early tumor regression after RT for all tumors (a) and separately for slowly and fast proliferating tumors (b). Mean value±SE are shown.

totally different environments. Apart from Bergstrom et al., none of the above-mentioned authors gave account of site from where the tumor samples were taken. In each tumor analyzed by us, all the samples were taken from the same region, i.e., the bottom part of the mass.

In our study, pretreatment BrdUrd LI or SPF was not predictive for early clinical and pathologicl tumor response, probably because of different tumor microenvironment. However, BrdUrd LI after/before RT ratio gave information on the different significant biological processes that take place after irradiation, and have impact on cell death like redistribution, repopulation, and reoxygenation.

BrdUrd LI after RT decreased to mean 4.1% independently of the time interval between RT and surgery. Magnitude of LI reduction after RT was correlated with tumor proliferation rate. Greater reduction of BrdUrd LI value was observed in fast proliferating (LI >8.5%) tumors (to mean 46% of the pretreatment value) than in slowly (LI ≤8.5%) proliferating tumors (to mean 85.3% of pretreatment value). What then is the justification for better RT response of fast proliferating tumor cells? According to current knowledge on tumor proliferation, radiation therapy should preferentially inactivate rapidly dividing cells, leaving behind a population biased toward slow proliferation. However, recruitment is a known effect of cytotoxic treatment, and new cells from quiescent cell populations are recruited into active proliferation after irradiation. Probably, slowly proliferating tumors might have greater propensity to recruit cells into rapid cycle in response to treatment than fast proliferating tumors, which might have little reserve capacity for further accelerating their cell cycle[24]. That might be why we observed acceleration of proliferation rate in slowly proliferating tumors from 5 weeks after RT (basing on after/before RT BrdUrd LI ratio), which followed temporary reduction of the number of DNA-synthesizing cells, 4–5 weeks after the start of RT. Accelerated proliferation was confirmed by increased S-phase fraction. However, better biological tumor responsiveness of fast proliferating tumors on cellular level did not find confirmation on tissue level that is in surgery because a fewer number of sphincter saving resections were performed in patients with fast (22) than those with slowly proliferating tumors (29).

Regression of rectal carcinoma after preoperative irradiation varies, likely reflecting differences in the physical and biologic properties of these tumors. Apart from biological characteristics discussed here, tumor down-staging depends on the total irradiation dose, the fractionation, and the interval between irradiation and surgery[25]. We showed association of tumor proliferation rate after RT with tumor response basing on BrdUrd LI. SPF, considered as a less sensitive method of tumor proliferation, did not show such a correlation. The after/before radiotherapy BrdUrd LI ratios correlated, however nonsignificantly, with the degree of pathologic and clinical down-staging, which indicates that more radiation-induced cell death occurred in tumors that expressed high levels of BrUrd LI, or that an increased rate of tumor clearance occurred in more rapidly proliferating tumors. This effect was reflected by significantly higher incidence of apoptosis observed after RT only in fast proliferating tumors (4.1% vs 11.1%; $P$=0.000). However, patients having tumors with LI >8.5% did not show higher rate (11.2%) of tumor pathological down-staging (D2–D4) than patients with BrdUrd LI ≤8.5% (16.8%) tumors, which may be suggestive of significant impact on tumor response also by biological processes other than proliferation. In the Spanish study[26], high proliferative activity of rectal cancer, as determined by PCNA immunostaining, was predictive of response to preoperative chemoradiotherapy. Willett et al.[27], in the same tumor type treated with higher RT dose (47–52 Gy) and 5 FU, showed that patients having tumors with extensive Ki-67 staining had also a higher rate of tumor down-staging (36%) 4–6 weeks after treatment than patients with minimal to moderate Ki-67 staining tumors (22–23%). These authors show that elevated postirradiation tumor proliferative activity correlated strongly with improved survival[28]. These authors, in contrast to our study, did not consider the proliferation profile of pre- and postirradiation for individual patients. The correlation of down-staging and higher survival rates was also found by other authors[29,30].

In our study, even in totally regressed tumors (D4), the percentage of the after/before radiotherapy BrdUrd LI was about 50%, which may not indicate tumor but normal cell proliferation, mainly a fraction of activated fibroblasts or cycling endothelial cells in capillaries high in colorectal carcinoma[31]. Our study showed complete pathologic response (D4) similar to that in a Norwegian study (4.5%)[32,] where histological tumor slides were analyzed after treatment with a dose of 31.5 Gy in 18 fractions and 2–3 weeks interval between RT and surgery. However, it should be stressed that in this study, a high incidence (31.3%) of recurrences was observed at late follow-up. Our analysis showed that patients having fast proliferating tumors, as assessed by BrdUrdLI, experienced higher rates of regression than patients with slowly proliferating tumors, which could suggest a more frequent possibility of performing sphincter-preserving procedures in these tumors. However, this was not confirmed in surgical procedures. Therefore, we do not know yet if pretreatment BrdUrd LI assessment will be a good predictor for a locoregional failure. Berger et al.[25], analyzing tumor sterilization after preoperative RT for rectal cancer, did not find a predictive factor for complete pathological response among such factors as age, sex, tumor stage, and pathologic grade. However, they found favorable influence of higher doses (>44 Gy) on pathologic stage.

There is no known optimal time for the interval between RT and surgery. The Swedish group keeps the interval at about a week; however, in other institutions, using longer RT treatments and higher total dose, longer intervals—4 to 6 weeks were adopted[14,25]. The main reason for a longer interval is tumor regression, which makes sphincter preservation possible. Similar to Francois et al[14], we observed higher clinical and pathologic response rate after longer interval between RT and surgery. However, these authors[14] showed nonsignificantly better overall survival for patients treated with shorter interval. Withers and Haustermans[33] estimated the interval between long course of fractionated RT (40–54 Gy) and surgery and stated that the interval is not critical to either local recurrence or distant metastases. The authors offered the following arguments: the tumor cells do not disseminate until the primary tumor is large enough to be clinically detectable (probably 80% of patients whose rectal tumors have not metastasized to lymph nodes will be free of metastases). Irradiation with a dose of 40 Gy in 2 Gy fractions (equivalent to 25 Gy in five fractions) reduces tumor cell survival by about six decades, e.g., from $10^{10}$ to $10^{4}$ cells. However, we have to remember that although the short overall treatment duration in the 25 Gy in five-fraction regimen provides a radiobiological advantage, this is a relatively low dose[34], which causes about a 66% reduction in the rate of local recurrence[11]. A retrospective analysis of published results of preoperative radiation therapy for rectal cancer showed that local control probability curves were displaced toward higher doses as the overall duration of preoperative radiation therapy was increased[15]. Therefore, longer intervals between short RT schedule (25 Gy) and surgery may be inappropriate in case of patients with incomplete resection (cut-through) of primary tumor, in whom the average subclinical cancer cell burden increases during long interval. Also, subclinical disease beyond the future surgical margins, may be a potential target for future recurrences. Longer intervals after short RT can be dangerous because of potential subclinical tumor, which may grow more quickly than primary tumor[15,33], the and risk of developing distant metastases. If we imply that moderately differentiated adenocarcinoma cells have different metastatic and proliferative activities from poorly differentiated cancer cells, which was shown by Taniyama et al[35], then we could have an indication to adjuvant chemotherapy for patients with differentiated tumors. The authors[35] indicated that moderately differentiated cancer cells are associated with hematogenous metastases to the liver, and the loss of tubular formation of cancer cells in poorly differentiated tumors may be fundamentally related to lymph node metastases and infiltrative growth. Therefore, particularly in patients with moderately differentiated and slowly proliferating tumors, adjuvant chemotherapy could be suggested after OTT shorter than 4 weeks, to prevent developing metastases to the liver.

In conclusion, our study shows that pretreatment BrdUrd LI or SPF were not predictive for early clinical and pathologic tumor response. After/before BrdUrd LI ratios showed inhibition of proliferation in responsive tumors, but this was not reflected in the number of sphincter preserving procedures performed. As 1 month after RT, accelerated proliferation of tumor cells is observed only in slowly proliferating tumors, we think that longer interval between RT and surgery is inadvisable.

If late tumor response confirms that patients having tumors with increased proliferative activity have statistically significantly less recurrences and improved survival rates compared with patients with less proliferative tumors, then we will be able to suggest a prognostic factor for individual rectal cancer patient, and a basis for selection to postoperative adjuvant chemotherapy.

**Acknowledgments** The authors would like to thank Anna Cichocka, M.Sc. for her valuable assistance. This work was supported by a grant of the State Committee for Scientific Research No. PBZ-KBN-091/P05/2003.

**Conflicts of Interest** None.

## References

1. Gerard A, Buyse M, Nordlinger B, et al. Preoperative radiotherapy as adjuvant treatment in rectal cancer: final results of a randomized study of the European Organization for Research and Treatment of Cancer (EORTC). Ann Surg 1988;208:606–614.
2. Pahlman L, Glimelius B. Local recurrences after surgical treatment for rectal carcinoma. Acta Chir Scand 1984;150:331–335.
3. Kapiteijn E, Marijnen CA, Nagtegaal ID, et al. Preoperative radiotherapy combined with total mesorectal excision for respectable rectal cancer. N Engl J Med 2001;345:638–646.
4. Glimelius BL. The role of preoperative and postoperative radiotherapy in rectal cancer. Clin Colorectal Cancer 2002;2:82–92.
5. Allal AS, Bieri S, Brundler MA, et al. Preoperative hyperfractionated radiotherapy for locally advanced rectal cancers: a phase I-II trial. Int J Radiat Oncol Biol Phys 2002;54:1076–1081.
6. Stockholm Rectal Cancer Study Group. Preoperative short-term radiation therapy in operable rectal carcinoma: a prospective randomized trial. Cancer 1990;66:49–55.
7. Gastrointestinal Tumor Study Group. Prolongation of the disease-free interval in surgically treated cancer carcinoma. N Engl J Med 1985;312:1465–1472.
8. Pahlman L, Glimelius B. Pre-or postoperative radiotherapy in rectal and rectosigmoid carcinoma: report from a randomized multicenter trial. Ann Surg 1990;211:187–195.
9. Frykholm G, Glimelius B, Pahlman L. Preoperative or postoperative radiation in adenocarcinoma of the rectum. Final treatment results of a randomized trial and an evaluation of late secondary effects. Dis Colon Rectum 1993;36:564–572.
10. Pahlman L, Glimelius B. Pre-operative and post-operative radiotherapy and rectal cancer. World J Surg 1992;16:858–865.

11. Swedish Rectal Cancer Trial. Improved survival with preoperative radiotherapy in resectable rectal cancer. New Engl J Med 1997;336:980–987.
12. Graf W, Dahlberg M, Osman MM, et al. Short-term preoperative radiotherapy results in down-staging of rectal cancer: a study of 1316 patients. Radiother Oncol 1997;43:133–137.
13. Glimelius B, Isacsson U, Jung B, et al. Radiotherapy in addition to radical surgery in rectal cancer. Acta Oncol 1995;34:565–570.
14. Francois Y, Nemoz CHJ, Vignal J, et al. Influence of the interval between preoperative radiation therapy and surgery on down-staging and on the rate of sphincter-sparing surgery for rectal cancer: The Lyon R90-01 randomized trial. J Clin Oncol 1999;17:2396–2402.
15. Suwinski R, Taylor JMG, Withers HR. Rapid growth of microscopic rectal cancer as a determinant of response to preoperative radiation therapy. Int J Radiat Oncol Biol Phys 1998;42:943–951.
16. World Health Organization. International histological classification of tumours. Histological typing of intestinal tumours. In Jass JR, Sobin LH, eds. Berlin Heidelberg New York: Springer, 1989.
17. Gasinska A, Urbanski K, Jakubowicz J, et al. Tumour cell kinetics as a prognostic factor in squamous cell carcinoma of the cervix treated with radiotherapy. Radiother Oncol 1999;50:77–84.
18. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumours. J Nat Cancer Inst 2000;92:205–216.
19. Dworak O, Keilholz L, Hoffmann A. Pathological features of rectal cancer after preoperative radiochemotherapy. Int J Colorect Dis 1997;12:19–23.
20. American Joint Commission on Cancer. AJCC Cancer Staging Manual, 5th ed. Philadelphia: Lippincott-Raven Publishers, 1997.
21. Bergstrom Ch, Palmqvist R, Denekamp J, et al. Factors influencing the estimates of proliferative labelling indices in rectal cancer. Radiother Oncol 1998;46:169–177.
22. Palmqvist R, Oberg A, Bergstrom C, et al. Systematic heterogeneity and prognostic significance of cell proliferation in colorectal cancer. Br J Cancer 1998;77:917–925.
23. Terry NHA, Meistrich ML, Roubein LD, et al. Cellular kinetics in rectal cancer. Br J Cancer 1995;72:435–441.
24. Wilson GD. A new look at proliferation. Acta Oncol 2001;40: 989–994.
25. Berger Ch, Muret A, Garaud P, et al. Preoperative radiotherapy (RT) for rectal cancer: predictive factors of tumor downstaging and residual tumor cell density (RTCD): prognostic implications. Int J Radiat Oncol Biol Phys 1997;37:619–627.
26. Diez M, Ramos P, Medrano MJ, et al. Preoperative irradiated rectal carcinoma: analysis of the histopathological response and predictive value of proliferating cell nuclear antigen immunostaining. Oncology 2003;64:213–219.
27. Willett ChG, Warland G, Coen J, et al. Rectal cancer: the influence of tumour proliferation on response to preoperative irradiation. Int J Radiat Oncol Biol Phys 1995;32:57–61.
28. Willett ChG, Warland G, Hagan MP, et al. Tumor proliferation in rectal cancer following preoperative irradiation. J Clin Oncol 1995;13:1417–1424.
29. Bouzourene H, Bosman FT, Seelentag W, et al. Importance of tumour regression assessment in predicting the outcome in patients with locally advanced rectal carcinoma who are treated with preoperative radiotherapy. Cancer 2002;94:1121–1130.
30. Kaminsky-Forrett MCh, Conroy Th, Luporsi E, et al. Prognostic implications of downstaging following preoperative radiation therapy for operable T3–T4 rectal cancer. Int J Radiat Oncol Biol Phys 1998;42:935–941.
31. Vermeulen PB, Verhoeven D, Hubens G, et al. Microvessel density, endothelial cell proliferation and tumour cell proliferation in human colorectal adenocarcinomas. Ann Oncol 1995;6:59–64.
32. Horn A, Morild I, Dahl O. Tumour shrinkage and down staging after preoperative radiation of rectal adenocarcinoma. Radiother Oncol 1990;18:19–28.
33. Withers HR, Haustermans K. Where next with preoperative radiation therapy for rectal cancer? Int J Radiat Oncol Biol Phys 2004;58:597–602.
34. Gerard JP, Romestaing P, Chapet O. Radiotherapy alone in the curative treatment of rectal carcinoma. Lancet 2003;4:158–166.
35. Taniyama K, Sasaki N, Wada S, et al. Comparison of proliferative activities and metastases between two subtypes classified at the deeply infiltrating sites of colorectal moderately differentiated adenocarcinomas. Pathol Int 1996;46:195–203.