High Ki67, Bax, and thymidylate synthase expression well correlates with response to chemoradiation therapy in locally advanced rectal cancers: proposal of a logistic model for prediction

M Kikuchi*1,2, T Mikami1, T Sato3, W Tokuyama1, K Araki1, M Watanabe3, K Saigenji2 and I Okayasu1

1Department of Pathology, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan; 2Department of Gastroenterology, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan; 3Department of Surgery, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan

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BACKGROUND: Recently, preoperative chemoradiation therapy (CRT) for rectal cancer has been increasingly used as a neoadjuvant treatment. In the present study, the relation between histological response to CRT and immunohistochemical markers in biopsy specimens was investigated.

METHODS: Biopsy specimens from a total of 60 patients were collected before preoperative CRT with S-1 and irinotecan, and liniac 45 Gy. Immunohistochemical staining for Ki67, Mm3, Bax, Bcl-2, ssDNA, Grp78, thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), CD34, vascular endothelial growth factor, nestin, and L-type amino-acid transporter 1 was performed to allow comparison of the Ki67 labelling index (LI), Bax score, TS score, DPD score, microvessel density by CD34, and Grp78 score with cancer regression.

RESULTS: When the cases were divided into responders (Dworak grades 3 and 4) and non-responders (grades 1 and 2) groups, good correlations were evident with Ki67 LI, Bax, Grp78, and TS expression. On multiple logistic regression analysis, Ki67 LI, Bax, and TS scores were found to be independent factors. With their use in a logistic model, P-values could predict responder cases with a sensitivity of 82.8% and a specificity of 83.9%.

CONCLUSION: Using this system, treatment strategy for locally advanced rectal cancers can be determined before chemoradiation.

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conversion to its active metabolite, 5-fluoro-2'-deoxuryridine-5'-monophosphate, which inhibits DNA synthesis by forming a stable complex with TS in presence of folates (Pinedo and Peters, 1988), and then initiates cell-cycle arrest or cell death. In general, high expression of thymidine phosphorylase and low expression of DPD in tumours are considered to result in higher intratumoural expression of thymidine phosphorylase and low expression of DPD and then initiates cell-cycle arrest or cell death. In general, high expression of thymidine phosphorylase and low expression of DPD in tumours are considered to result in higher intratumoural concentration of 5-FU (Jakob et al, 2005).

Glucose-regulated protein 78 is a member of the Hsp70 superfamily of heat-shock proteins whose increased expression is part of a coordinated protein response required to alleviate endoplasmic reticulum stress (Misra et al, 2005), maintain endoplasmic reticulum function, and protect cells against cell death. Glucose-regulated protein 78 may confer resistance against adriamycin- and etoposide-mediated apoptosis in cancer cells through inhibition of Bax and caspase-7 activation (Reddy et al, 2003; Davidson et al, 2005; Ermakova et al, 2006; Lee et al, 2006; Ranganathan et al, 2006).

Recently, the concept of cancer stem cells has attracted increasing attention with regard to human cancers (Wang and Dick, 2005). Minichromosome maintenance (Mcm) proteins 2–7 are present through all phases of the proliferative cell cycle, but are absent in ‘out-of-cycle’ states, suggesting functions as replication licensing factors (Stoeber et al, 2001), and Dudderidge et al (2005) have proposed that the Mcm2-Ki67 labelling index (LI) reflects the presence of non-proliferating dormant ‘cancer stem’ cells, associated with reduced disease-free survival in renal cell carcinoma cases.

It was reported that high intratumoural microvessel density (MVD) and vascular endothelial growth factor (VEGF) were correlated with poor prognosis of colorectal cancer (Des Guetz et al, 2006). It has been also reported that VEGF-positive rectal cancer was resistant to radiotherapy (Zlobec et al, 2008). Nestin, a class VI intermediate filament protein, has recently received attention as a marker for detecting newly formed endothelial cells (Teranishi et al, 2007).

As for apoptosis, cancer cell apoptosis in biopsy before CRT was correlated with tumour regression whereas apoptosis inhibitory protein Bcl-2 expression indicated no correlation with regression (Rödel et al, 2002). In addition, L-type amino-acid transporter 1 (LAT1) is highly expressed in malignant tumours to support growth and proliferation, and the inhibition of LAT1 activity led to cancer cell apoptosis (Kim et al, 2008).

Using these parameters, multiple logistic regression analysis was adopted to generate a model for predicting response to preoperative CRT.

MATERIALS AND METHODS

A total of 60 cases of rectal cancer treated with preoperative CRT were collected. The patients’ clinical criteria were previously reported (Sato et al, 2007).Briefly, all had previously untreated locally advanced distal rectal cancer T3 or T4, N0–2, and M0 (UICC classification) (Sobin and Wittekind, 2002), with an Eastern Cooperative Oncology Group performance status of 0–2 (Oken et al, 1982). Aged were 20–80 years at enrolment.

Biopsy materials of the 60 cases were endoscopically obtained from the rectal cancers before the initiation of therapy, at least two pieces of carcinoma being sampled for each case. The histological typing was in accordance with the WHO classification (Hamilton and Aaltosen, 2000). Tumour size was measured using double-contrast barium enema in 56 cases, but no X-ray photographs were available for 4 cases. Clinical tumour node metastasis (TNM) stage was judged with computed tomographic scans and/or magnetic resonance images. Because images were not available for two cases, clinical TNM stage could not be determined for these. A summary of clinical data of the cases is shown in Table 1. All patients received preoperative chemoradiation as follows: radiotherapy was administered in lienic fractions of 1.8 Gy per day, 5 days per week. The total dose of radiation was 45 Gy. S-1 (80 mg m⁻² per day) and was given orally after breakfast and dinner on days 1–5, 8–12, 22–26, and 29–33. Irinotecan (80 mg m⁻² per day) and 5-FU were given orally after breakfast and dinner on days 1–5, 8–12, 22–26, and 29–33. Irinotecan (80 mg m⁻²) was administered as a continuous i.v. infusion for 90 min on days 0, 8, 22, and 29. Radical surgery was performed at least 4–6 weeks after the completion of 5 weeks of chemoradiation. The dose of S-1 was in accordance with the manufacturer’s guideline (Taiho Pharmaceuticals Co. Ltd, Tokyo, Japan). The recommended dose of irinotecan was examined in our previous study (Sato et al, 2007). This protocol was started in 2004 in approval with the ethics committee of Kitasato University Hospital. All patients gave written informed consent.

Pathological evaluation

Therapeutic responses to preoperative CRT were evaluated with the surgically resected specimens. The excised tissues were fixed in buffered formalin and embedded in paraffin. In each case, the entire lesion was serially sliced at 4 mm for routine processing and was stained with haematoxylin and eosin, and examined by light microscopy. Amounts of residual tumour mass, fibrotic changes, radiation vasculopathy, and peritumoural inflammatory reaction were checked, and therapeutic effects were assessed using Dworkar grades (Dworkar et al, 1997) as follows:

- grade 0: no regression;
- grade 1: dominant tumour mass with obvious fibrosis and/or vasculopathy;
- grade 2: dominant fibrotic changes with few tumour cells or groups (easy to find);
- grade 3: very few tumour cells in fibrotic tissue with or without mucous substance;
- grade 4: no tumour cells, only fibrotic mass (total regression).

Immunohistochemistry of Ki67, Mcm3, Bax, Bcl-2, ssDNA, Grp78, TS, DPD, CD34, VEGF, nestin, and LAT1

Formalin fixed, paraffin-embedded histological sections (4 μm in thickness) in tumour biopsies before CRT were immunostained for 12 antigens (Ki67, Mcm3, Bax, Bcl-2, ssDNA, Grp78, TS, DPD, CD34, VEGF, nestin, and LAT1). The antibodies used and methods

| Characteristic | Mean ± SD | Range |
|---------------|----------|-------|
| Age (year)    | 63.9 ± 10.6 | 32–81  |
| Sex (male/female) | 44 (73.3%)/16 (26.7%) | |
| Tumour size (mm) | 47.4 ± 17.2 | 20–95  |
| Clinical T stage | cT3 56 |       |
|                | cT4 2   |       |
| Clinical N stage | cN0 32  |       |
|                | cN1/cN2 26 |     |
| Histological type (biopsy) | Well 36 |   |
|                | Moderate 23 |      |
|                | Poor 1 |      |
| CEA (mg/100 ml) | 8.9 ± 12.7 |       |
| CA19-9 (ng/ml) | 19.7 ± 27.2 |       |

s.d., standard deviation. Normal ranges of CEA and CA19-9 were <5 mg/100 ml and <37 ng/ml, respectively.
for antigen retrieval are listed in Table 2. Endogenous peroxidase was blocked with 3.0% hydrogen peroxide for 10 min, and incubation with Protein block serum-free solution (DakoCytomation, Glostrup, Denmark) for 10 min. Sections were incubated with the anti-Ki67, Bax, Grp78, TS, DPD, and CD34 primary antibodies for 60 min at room temperature, and with the anti-Mcm3, Bcl-2, ssDNA, VEGF, and nestin antibodies overnight at 4°C. After incubation with either labelled polymer, anti-mouse, or anti-rabbit (EnVision+ System HRP; DakoCytomation) for 60 min at room temperature, 3,3′-diaminobenzidine was used as the chromogen. Nuclei were counterstained with methyl green solution to facilitate histopathological assessment. The immunohistochemical protocol with the LAT1 staining kit (Fuji Biomedix, Tokyo, Japan) was according to the manufacturer’s manual.

### Evaluation of immunohistochemical staining

Ki67 and Mcm3 LI were determined as percentage values counting at least 1000 tumour cells in high-power fields \((\times 400)\). With ssDNA staining, immunohistochemically positive cells were so few in number that at least 5000 nuclei were counted. ssDNA indices also were determined as percentage values.

Immunoreactivity for Bax, Bcl-2, Grp78, TS, DPD, and VEGF was evaluated using a score based on the classification of Sinicrope et al (1995). The staining intensity was scored as follows: none, 0; weak, 1; moderate, 2; intense, 3. If heterogeneity of staining intensity existed in a section, the staining intensity was scored based on that which was predominantly observed. The percentages of positive cells were assigned to one of five categories of protein expression: 0, <5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, >75%. The two scores were then multiplied to produce a weighted score for each tumour specimen. Two pathologists (MK and TM) independently scored the lesions and determined the final scores by discussion when they differed.

CD34-expressing capillaries were counted to give the MVD. Nestin-examined capillaries were considered as capillaries consisting of newly formed endothelial cells (Teranishi et al, 2007). Areas of highest neovascularisation were found by scanning tumour sections at low power \((\times 100)\). The highest vascular counts of two different fields were averaged and used to calculate numbers of microvessels per mm², defined as MVD with both stainings.

For LAT1, a biomarker for high-grade malignancy, staining intensity was scored according to a previous report (Sakata et al, 2009): none, 0; weak, 1; moderate, 2; intense, 3. The percentages of positive cells were assigned to one of four categories: 0, <0%; 1, 1–10%; 2, 10–30%; 3, >30%. The values for the two variables were then multiplied, resulting in a scoring from 0 to 9. Two pathologists (MK and TM) independently scored the lesions.

### Statistical analysis

Data were analysed using Dr. SPSS II (SPSS, Chicago, IL, USA) and Statview 5.0 (SAS Institute Inc., Cary, NC, USA) software. Immunohistochemically labelled and scores were compared using the Kruskal-Wallis test and the Mann-Whitney U-test. Logistic regression analysis was performed with a stepwise method. 

### RESULTS

#### Pathological response to CRT

Pathological evaluation of responses to preoperative CRT in resected rectum revealed radiation effects in all cases, with fibrosis and vascular changes. All 60 cases were classified into Dworak regression grades 1–4. Of these, 15 (25.0%) showed complete pathological responses (regression grade 4) and 14 (23.3%) showed microscopic residual tumours (regression grade 3), whereas 21 (35.0%) and 10 (16.7%) showed moderate (regression grade 2) or minimal (regression grade 1) responses to preoperative CRT, respectively.

Ki67, Mcm3, and ssDNA expression was confined to tumour cell nuclei, and Bax, Bcl-2, Grp78, and VEGF immunoreactivity to the tumour cell cytoplasm. TS and DPD were expressed in both nuclei and cytoplasm. LAT1 was confined to cell membranes, and CD34 and nestin were expressed in endothelium of intratumoural microvessels (Figure 1).

Figure 2 demonstrates the relationship between each immunohistochemical marker of the tumour biopsies before CRT and the pathological tumour response. A high Ki67 LI, Bax score, TS score, DPD score, MVD by CD34, and a low Grp78 score correlated with regression on univariate analysis. Recent studies have revealed that...
patients with pathological CR and greater than 95% pathological response groups achieve a significantly improved overall survival and recurrence-free survival when compared with less than 95% pathological response groups (Ruo et al., 2002; Guillem et al., 2005). Therefore, we divided the cases into two groups: Dworak grades 1 and 2, and grades 3 and 4 (Gavioli et al., 2005). The latter were considered as responders to CRT. A high Ki67, Bax score, and TS score and a low Grp78 score were well correlated with response. On the other hand, there were no associations with the other immunohistochemical factors, as well as clinicopathological factors (Table 3).

### Multiple logistic regression analysis

Multiple logistic regression analysis was performed with a stepwise method (Tanaka et al., 1999). Independent variables were the data for Ki67 LI, Bax score, TS score, and Grp78 score, and dependent variables were no-response (0; Dworak regression grades 1 and 2) or response (1; Dworak regression grades 3 and 4). Other immunohistochemical markers and clinicopathological factors were not used. By the logistic regression analysis, we detected the Ki67 LI, Bax score, and TS score as independent factors (Table 4). The Bax score (odds ratio 18.1) had the strongest influence. The logistic regression formula was as follows:

$$\log_e \left( \frac{p}{1 - p} \right) = -24.0 + 0.15 \times [\text{Ki67 LI}] + 2.90 \times [\text{Bax score}] + 0.60 \times [\text{TS score}]$$

### Receiver-operating characteristic curve

A receiver-operating characteristic curve was generated by plotting the true-positive rate (sensitivity) on the y axis and the
false-positive rate \((1 - \text{specificity})\) on the x axis (Figure 3) (Tanaka et al., 1999).

Although the \(P\)-value at the point closest to the left upper corner on the curve is generally considered to represent the best balance of both sensitivity and specificity in distinguishing between response and no-response, we determined four points of \(P\) as the cut-off values \((0.90, 0.50, 0.40, \text{ and } 0.20)\) to construct practical criteria for the five categories ‘responder’, ‘probable responder’, ‘unknown’, ‘probable non-responder’, and ‘non-responder’ (Table 5). The points of \(P = 0.90\) and \(0.20\) meant the points of specificity 100% and sensitivity 100%, respectively. The point of \(P = 0.50\) meant the point at which the specificity was maximum and the sensitivity was more than 80%. The point of \(P = 0.40\) meant the point at which the specificity for prediction of non-responder was maximum and the sensitivity more than 80%.

**Sensitivity and specificity**

A \(P\)-value for each case was calculated with three immunohistochemical markers examined in 60 sets of biopsy specimens. Using
the calculated P-value, we classified the 60 patients into one of the above five categories with criteria distinguishing between responder and non-responder. Sensitivities and specificities of the criteria are shown in Table 5.

**DISCUSSION**

In this study, we sought clinicopathological factors and immuno- histochemical markers that might contribute to prediction of chemoradiation effects on locally advanced rectal cancer. Our conclusion is that it is possible to predict a responder to preoperative CRT, with 82.8% sensitivity and 83.9% specificity, using the value calculated with the three elements of the Ki67 LI, the Bax score, and the TS score in biopsy specimens before CRT. In fact, high expression of Ki67, Bax, and TS was positively correlated with therapeutic effects.

The first factor, high proliferative activity with Ki67 as the marker, was earlier found to correlate with PCNA immunostaining, and mitotic counts after radiation of rectal cancer (Willett et al, 1995). Later, beneficial effects of radiotherapy for patients with various carcinoma with high Ki67 LIs were reported (Nakano et al, 1997; Raybaud-Diogene et al, 1997). However, in other reports, no relation was noted between Ki67 values in biopsy specimens before radiation and response rate in rectal cancers (Suzuki et al, 2004; Debucquoy et al, 2008). Suzuki et al (2004) performed preoperative radiotherapy only. Debucquoy et al (2008) combined preoperative radiotherapy and/or 5-FU/LV. Because we adopted CRT for all patients, the response may be more influenced by chemotherapy than radiation.

The second factor, Bax expression, was also reported by Chang et al (2005) to correlate well with chemoradiation therapeutic effects, and the authors considered that apoptosis may be important in rectal carcinoma response to CRT. Similarly, Bax overexpression has been found to correlate with anticancer drug sensitivity in a variety of human cancers, through enhanced induction of apoptosis (Krajewski et al, 1995; Guo et al, 2000; Teranishi et al, 2007). However, Gosens et al (2008) did not find any link between Bax expression and rectal cancer regression for neoadjuvant chemoradiation. They evaluated the regression grading system described by Rödel et al (2005): (1) no regression or <25% of tumour mass, (2) 25% to >50% tumour regression, and

**Table 3** Clinicopathological characteristics of the patients separated by Dworak grades 1, 2 vs 3, 4

| Variable             | Dworak grade 3, 4 (n = 29) | Dworak grade 1, 2 (n = 31) |
|----------------------|----------------------------|----------------------------|
| Age (year) (mean ± s.d.) | 63.5 ± 11.4 | 63.5 ± 9.8 |
| Sex                  | 21 | 24 |
| Tumor size (mm) (mean ± s.d.) | 46.7 ± 14.4 | 48.0 ± 19.7 |
| Histological type (biopsy) | Well | 17 |
| CEA (mg/100 ml) (mean ± s.d.) | 8.5 ± 12.7 | 9.4 ± 8.5 |
| CA19-9 (ng/ml) (mean ± s.d.) | 17 ± 25 | 22 ± 29 |

Well, well-differentiated adenocarcinoma; mod/por, moderately to poorly differentiated adenocarcinoma; s.d., standard deviation.

**Table 4** Results of multiple logistic regression analysis

| Variable | Regression coefficient | P-value | Odds ratio | 95% CI |
|----------|------------------------|---------|------------|--------|
| Ki67 LI  | 0.15                   | 0.002   | 1.17       | 1.06–1.29 |
| Bax score| 2.90                   | 0.001   | 18.1       | 3.11–105.7 |
| TS score | 0.60                   | 0.019   | 1.83       | 1.11–3.03 |

**Table 5** Criteria for Dworak grades 1, 2 vs 3, 4, and their validities tested among the 60 patients

| Category       | Definition (P) | Definition (II) | Pathological response | Validity |
|----------------|----------------|-----------------|-----------------------|----------|
| Responder      | 0.90 ≤ P       | 2.20 ≤ log₂ (P) | Responder (n = 29)    | Non-responder (n = 31) |
|                |                |                 | Se                    | Sp       |
| Probable responder | 0.50 ≤ P < 0.90 | 0 ≤ log₂ (P) < 2.20 | 13                    | 0        |
| Unknown        | 0.40 ≤ P < 0.50 | −0.41 < log₂ (P) < 0 | 1                     | 1        |
| Probable no-responder | 0.20 ≤ P < 0.40 | −1.39 < log₂ (P) ≤ −0.41 | 4                    | 7        |
| No-responder  | P ≤ 0.20       | log₂ (P) ≤ −1.39 | 0                     | 18       |

P: probability; DG, Dworak grade; Se, sensitivity; Sp, specificity; II = log₂ (P1/P).

**Figure 3** Receiver-operating characteristic curve with the logistic regression model. The area under the curve is 0.928 (95% confidence interval: 0.867–0.988).
complete regression. In addition, Bax immunohistochemical values were only intensity of cytoplasmic staining 0–3. Differences in grading systems and immunohistochemical expression scoring could clearly influence the results.

Rau et al. (2003) immunohistochemically investigated the expression of p53, Bax, p21, Ki67, hMSH2 in pre- and post-therapeutic rectal carcinoma with preoperative radiotherapy. Only low p21 expression in tumour samples was significant in no-response to neoadjuvant chemoradiation. They reported no relation with Bax expression but classified responders as CR or partial response, histopathologically defined with resected post-therapeutic rectum, again differing from our definition as Dworak grades 3 or 4.

The third factor, TS, is important in pyrimidine nucleotide synthesis and represents an important chemotherapeutic target for 5-FU chemotherapy. Immunohistochemically, high TS expression in pre-treatment locally advanced rectal cancer biopsies was earlier shown to be predictive of a higher pathological response in the fluorouracil/oxaliplatin-base chemotherapy (Negri et al., 2008). A trend toward a direct correlation between the level of TS expression and response of 5-FU/LV treatment in patients with metastatic colon cancer has been noted (Johnston et al., 2003). Similar results have also been reported by Edler et al. (2002) and Kornmann et al. (2003).

However, low TS expression was a predictor of response to 5-FU chemotherapy for colorectal cancer metastases (Aschele et al., 1999) and advanced colorectal cancer (Cascinu et al., 1999). Aschele et al. (1999) used a regimen of schedule-specific biochemical modulation of 5-FU plus methotrexate, and Cascinu et al. (1999) applied 5-FU/LV. In both studies, cases with metastasis and/or recurrence were included, and TS expression was evaluated as intensity 0 (undetectable staining) to 4 (very high intensity of staining), and then intensity levels 0–2 were considered as low, and 3 and 4 as high expression. We examined both cytoplasmic TS expression intensity and percentage of positive cells, as well as the Bax value. In another study, by Liersch et al. (2006), TS expression was examined in surgically resected rectal cancer. In the reports, high TS expression correlated with cancer relapse. The clinical meaning of evaluation of TS expression needs further clarification.

The multiple logistic regression analysis revealed Ki67 LI, Bax score, and TS score to be independent factors, with a sensitivity and specificity for prediction of responder cases of 82.8 and 85.9%, respectively. Although the logarithm model is difficult to calculate for daily use, it can be easily converted to a linear model. It is sufficient for users to know the values of $\log_e (P/1 – P)$ at the point of criteria. Practically, users can directly substitute the Ki67 LI, Bax score, and TS score into the formula:

$$\Pi = \log_e(P/1 – P) = -24 + 0.15 \times [\text{Ki67 LI}] + 2.90 \times [\text{Bax score}] + 0.60 \times [\text{TS score}]$$

If this value $\Pi$ ($\log_e (P/1 – P)$) is larger than 0.00, it indicates a responder case. If it is smaller than $-0.41$, it indicates a non-responder case (Table 5).

At present, CRT with subsequent surgical resection is performed without selection of cases. However, with our approach, likely responder cases can be chosen before therapy. In the future, our multivariate model should be revised using new factors to improve the sensitivity and specificity. The treatment strategy for locally advanced rectal cancer should be further developed toward so-called tailor-made therapy including such evaluation before preoperative therapy and/or surgical resection.

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