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Review

Morphological features and molecular markers in rectal cancer from 95 patients included in the European Organisation for Research and Treatment of Cancer 22921 trial: Prognostic value and effects of preoperative radio (chemo) therapy

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ABSTRACT

In this study, the prognostic and/or predictive value of different proteins (cyclo-oxygenase 2 (COX-2), Ki67 and cleaved cytokeratin (CK) 18) and fibro-inflammatory changes which might be of importance for the response to treatment were evaluated using tissue micro arrays. Samples were obtained from a subset of 95 patients included in the European Organisation for Research and Treatment of Cancer 22921 clinical trial, which randomised patients with rectal cancer to one of four arms treated with preoperative radiotherapy with or without pre- and/or postoperative chemotherapy. From our results, we can conclude that the addition of preoperative chemotherapy to radiotherapy led to significantly less COX-2 upregulation, less proliferation and more inflammation, as was seen in the resection specimen as well as less invasion and metastasis. For COX-2, Ki67 or cleaved CK18, no predictive or prognostic value could be identified. However, the fibro-inflammatory reaction after preoperative radiochemotherapy correlated with T-downstaging and seems to be an important factor for response.

1. Introduction

Standard treatment of an advanced rectal carcinoma is radical surgery with or without sphincter preservation. However, even with macroscopically complete resection of the tumour, 15–20% of patients will have a local pelvic relapse. The value of adding radiotherapy to surgery has been assessed in several trials using pre-or postoperative irradiation, either alone or in combination with chemotherapy and has been shown to induce tumour regression and downstaging and improve local control as well as to increase survival.[1] Interestingly, the response of clinically identical tumours to the (same) treatment

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may be different. This suggests that, although rather good results are obtained with the current treatment guidelines, which are based solely on clinical parameters, more 'patient-tailored' treatments might in the future give greater benefit. Molecular analyses of prognostic features for colorectal cancer have shown that response of the tumours might be dependent on certain tumour characteristics. Therefore, many studies have been initiated with the aim of identifying molecular correlates of response of colorectal cancer to chemoradiotherapy.

In colorectal cancer, it has been suggested that cyclo-oxygenase 2 (COX-2), which is often expressed constitutively and at high levels, indicates a poor prognosis. Expression of COX-2 also increases with progression from premalignancy (polyps) to carcinomas and correlates with poor response of rectal cancers to neoadjuvant radiochemotherapy as shown by Smith et al.[2] which makes it an attractive molecular target for therapeutic intervention. COX-2 is one of the three isoforms of COX, an enzyme that catalyses the conversion of arachidonic acid to biologically active prostanoids.[3] Unlike COX-1, which is constitutively expressed, COX-2 is normally induced in response to inflammatory stimuli, such as TNF-α, IL-1, IL-6 and IFN-γ or to mitogenic stimuli, such as EGF, VEGF and bFGF,[4] and is thus mainly expressed in inflammatory and tumoural tissue.

Increased proliferation is a well-known characteristic of tumours. Because of deregulation in the growth signalling pathways, tumours are much less dependent on growth signals and insensitive to anti-growth signals.[5] Therefore, proliferation might be important in the prediction of the response to treatment and the outcome of the patient. Ki67 is a nuclear protein involved in cell cycle regulation.[6] It is present in all cell cycles phases except for the G0 and early G1 phase, making it a good marker for proliferation. Ki67 labelling index has a prognostic value and/or predictive value in different tumour types. However, in colorectal cancer the results seem to be conflicting.[7]

The ability of tumour cell populations to expand in numbers is determined not only by the rate of cell proliferation but also by the rate of cell death. The ability to evade apoptosis is thought to be central in both tumourigenesis[5] and resistance to cytotoxic drugs and radiation,[8] which is why apoptosis might be an important prognostic or predictive factor. Apoptosis is characterised by specific changes in cell surface and nuclear morphologic features. One of the methods for the detection of apoptosis is the M30 antibody, which has been introduced by Leers et al.[9] This antibody defines an epitope on cytokeratin (CK) 18 that becomes available at an early caspase cleavage event during apoptosis and is not detectable in vital and necrotic cells. The use of this antibody permits the detection of early-phase apoptosis before other methods such as the TUNEL assay or the annexin V assay.[9] The cleaved cytokeratin staining seems to correlate very well with caspase-3 staining[10] and has been used before in paraffin embedded material from patients with rectal cancer.[11,12]

It is known that preoperative radio- and/or chemotherapy produces tumour regression by replacing neoplastic glands with fibrous or fibro-inflammatory tissue.[13–16] Some years ago, Shia et al. showed that certain morphological changes induced by preoperative radio- and/or chemotherapy are prognostic relevant in patients with rectal cancer. Patients whose post-treated rectal cancers show a stroma with abundant inflammatory cells or active surface ulceration have a better outcome.[17] Also Gallon et al. showed more recently that the presence of an adaptive immune reaction within the tumour seems to be a critical variable influencing the outcome of the patients with colorectal cancer after surgical treatment.[18]

In this study, the prognostic and/or predictive value of different proteins (COX-2, Ki67 and cleaved CK18) and fibro-inflammatory changes which might be of importance for the response to treatment will be evaluated. The expression of these proteins will be studied using tissue micro arrays on 95 patients included in the European Organisation for Research and Treatment of Cancer (EORTC) 22921 study.

2. Materials and methods

2.1. *Patient population*

The EORTC trial 22921 started with inclusion of patients in April 1993 and closed in March 2003 after 1011 patients were recruited. To minimise inter-centre variation, we studied a subset of 95 patients included in this trial from the 'Centre Hospitalier Universitaire de Besançon', Centre Paul Strass' in Strassbourg, 'Centre Hospitalier Universitaire de Charleroi' and 'Centre Georges-François Leclerc' in Dijon. This study was approved by the ethical committee of the different institutions and all patients gave informed consent.

Patients were randomised in four treatment arms: (1) preoperative radiotherapy; (2) preoperative radiotherapy with concurrent fluorouracil–leucovorin (5-FU–LV); (3) preoperative radiotherapy and postoperative 5-FU–LV; (4) preoperative radiotherapy with concurrent 5-FU–LV and postoperative 5-FU–LV. Radiotherapy was given in fractions of 1.8 Gy over five weeks to a total dose of 45 Gy. Concomitant chemotherapy was delivered in two 5-day courses during the first and fifth week of radiotherapy. 5-FU was given as a short intravenous infusion, 1 h before radiation at a dose of 350 mg/m^2/day. Leucovorin (LV) was delivered by i.v. push just before 5-FU at a dose of 20 mg/m^2/day. Surgery was performed 3–10 weeks after the end of the preoperative treatment. The postoperative chemotherapy dose was identical to the preoperative chemotherapy.[19]

2.2. *Immunohistochemistry*

2.2.1. *Material*

For the patients included in Besançon ($n = 62$), all tissues had been fixed in Bouins, whilst the material from the three other centres ($n = 33$) was fixed in formalin. For all markers no significant differences in expression were seen in the Bouin and formalin fixed material.

2.2.2. *Construction of tissue micro arrays*

After cutting 4 μm sections of the paraffin embedded material, the first section was stained for H&E to visualise the histology of the tumour. Based on the H&E stain, tumoural regions and regions of normal mucosa were delineated. This H&E stains could then be matched on the original paraffin embedded tissue of which small cores were taken in the different regions of interest and transferred to an empty paraffin block. For each patient, four cores were taken out of the

pre-treatment biopsy, six cores from the resection specimen and four from the normal mucosa. If metastasis material was available, another six cores were taken out of this paraffin block. After incubating the tissue micro array (TMA) blocks at 37 °C for 2–3 h, about 25 4 μm sections were cut. An H&E stain was performed on every fifth section. These sections were used for histological verification. The other sections were used for immunohistochemical stains for molecular markers (COX-2, Ki67 and cleaved CK18).

2.2.3. *Protocols for immunohistochemical stainings*

From each paraffin embedded block, one section was stained for COX-2, Ki67 and cleaved CK18. Standard immunoperoxidase procedures were used. All antibodies used for these stainings were monoclonal mouse antibodies: COX-2 (Cayman, 1/50), Ki67 (Labvision, Ready to use) and M30 (Roche, 1/10). For the COX-2 and cleaved CK18 stain, citrate buffer was used for antigen retrieval whilst Tris–EDTA buffer was used as antigen retrieval for Ki67 stains. All antibodies were visualised by diaminobenzidine.

2.2.4. *Scoring of the immunohistochemical stainings*

For COX-2 and Ki67, stained sections were analysed at a total magnification of 200× field-by-field. Each field was assigned a continuous score of percentage positivity, representative of the approximate area of immunostaining. For cleaved CK18, the number of positive tumour cells per punch (=1 mm^2) was counted on a 400× magnification.

2.2.5. *Scoring of H&E stains of the resection specimen*

Classical pathological variables, including blood vessel, lymph vessel and perineural invasion, the most prevalent grade of differentiation, ypT and ypN-stage and section margins were scored on whole sections of the resection specimen by an experienced pathologist. The differentiation grade in mucinous tumours ($n = 3$) was scored analogous to the non-mucinous tumours. Also, the Dworak regression grade was determined.[20] Stromal changes (fibrotic types versus fibro-inflammatory type) and tumour cell alterations (tumour cells with marked cytoplasmic eosinophilia and nuclear atypia, presence of mucin pools) induced by preoperative therapy were scored analogous to Shia et al.[17]

Biopsies taken before the start of preoperative treatment were available for 67 patients whilst tissue from the resection specimen was evaluable in 80 patients. Evaluation of the immunohistochemical stains and clinicopathological variables on the tissue was not possible in some patients due to complete remission or if only minimal residual tumour was left in the resection specimen.

2.2.6. *Statistical analysis*

For all statistical analysis, Statistica 7.0 was used. To compare pretreatment and post-treatment expression of proteins, the non-parametric Wilcoxon matched pair test was used. Correlation between the expression levels of different continuous markers was analysed with the Spearman Rank test. To compare the expression of several proteins with T-downstaging, we used the Mann–Whitney *U* test. Survival analysis was done by using Kaplan–Meier modelling for overall survival and disease-free survival. Since the number of patients with a local recurrence was small ($n = 8$), we did not study the effect of parameters on local control. Significant prognostic parameters were analysed afterwards in multivariate analyses using a COX regression model. For the survival analysis, the median was used as a cut-off for the continuous parameters (Ki67, COX-2 and cleaved CK18). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. *Patients characteristics*

Patients ($n = 95$) were included in the EORTC 22921 trial between April 1993 and November 2003. Median follow-up was 4.2 years with a minimum of 2.8 months and a maximum of 10.8 years. Characteristics of the patients and the tumours were well balanced amongst the four treatment arms (Table 1). The clinical data for the whole patient group ($n = 1011$) were published previously.[19]

3.2. *Immunohistochemical stains*

Cytoplasmic COX-2 expression was very limited in the normal mucosa. In 73% (49/67) of the preoperative biopsy specimens, COX-2 was detected in the cytoplasm with a median of 12% of the cells being positive (Fig. 1). This increased in the resection specimen to 96% (77/80) with a median expression of 55%. The Wilcoxon matched paired test showed a significant increase in COX-2 expression in the resection specimen ($p < 0.0001$).

Nuclear Ki67 staining was observed at the base of the proliferating crypts of the normal mucosa, as expected. Very strong Ki67 staining was seen in the tumour cells of the biopsy and resection specimens (Fig. 1). In 95% (64/67) of the biopsies, Ki67 was detected with a median of 37% positive cells. In the resection specimen, 93% (74/80) of the tumours were positive for Ki67 with a median value of 25%. No significant difference was found between the expression in the biopsies and the resection specimen.

Apoptotic cells were cytoplasmically stained by the cleaved CK18 antibody (Fig. 1). In the normal mucosa, almost no apoptosis was detected. In 31% (21/67) of the biopsies, apoptosis was detected which ranged between 1 and 16 positive cells/mm^2 (median = 0). After the preoperative treatment, positive cells were seen in 86% (69/80) of the tumours (range 0–63; median of 7 cells/mm^2). Apoptotic cells were spread heterogeneously throughout the tumour.

3.3. *Fibro-inflammatory and cytological alterations*

Tumour regression in post-treatment rectal carcinomas was marked by fibrosis or fibro-inflammatory changes replacing neoplastic glands. Forty-six percent of the evaluable tumours (37/80) had a marked inflammatory cell component, whilst the other 54% had a predominantly fibrotic type stromal response. Marked cytoplasmic eosinophilia was seen in 31% (25/80) of the tumours. In 16% (13/80) of the tumours, mucus lakes were present after preoperative treatment. Fifty percent of these cases were mucinous adenocarcinomas, since they were composed for at least 50% of mucin. In four of the 13 tumours with mucus lakes in the resection specimen, mucus

Table 1 – Characteristics of patients in the different treatment arms

	Arm 1 (preoperative radiotherapy – RT)	Arm 2 (preoperative radio (chemo) therapy – RCT)	Arm 3 (preoperative RT + postoperative chemotherapy – CT)	Arm 4 (preoperative RCT + postoperative CT)	Total
Number of patients	28	21	24	22	95
Age					
Median	60	59	66	64	64
Range	47–75	37–75	47–78	27–77	27–78
cT stage					
T3	27	20	23	22	92
T4	1	1	1	0	3
Histological differentiation					
Well	18	13	14	13	58
Moderate	8	5	8	5	26
Poor	0	0	2	1	3
Unknown	1	1	0	1	3
Complete resection	27	21	20	20	88
T-downstaging	10	8	7	6	31
Number of local recurrences	3	3	2	0	8
Number of metastasis	9	6	12	4	31
Number of deaths	8	8	10	5	31
Venous invasion	2	3	6	7	18
Lymphatic invasion	2	5	8	5	20
Perineural invasion	5	2	6	4	17
Fibro-inflammatory response[a]	12	8	6	11	37
Cytoplasmatic eosinophilia[a]	7	10	5	3	25

a Only evaluable in 80/95 patients due to complete remission or minimal residual tumour.

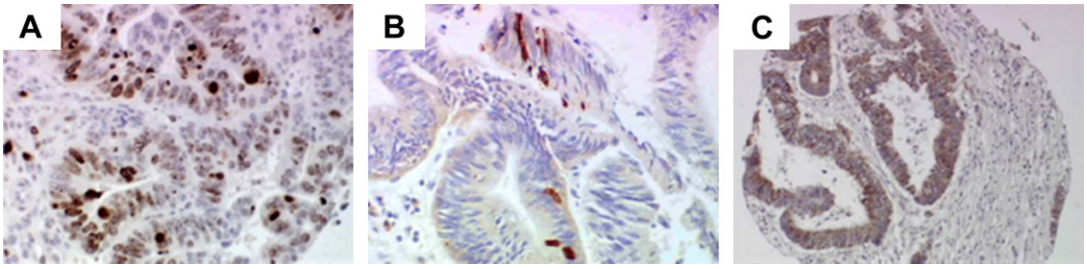

Fig. 1 – Immunohistochemical stains for (A) Ki67 (400×), (B) cleaved CK18 (400×), (C) COX-2 (200×).

lakes were induced during the preoperative therapy, whilst four other tumours already had mucus lakes before the start of therapy. For the remaining five tumours, this difference was not evaluable due to the absence of pretherapeutic biopsies.

3.4. *Differential effects of radiotherapy and chemoradiotherapy*

The addition of chemotherapy to the preoperative radiotherapy treatment significantly lowered expression of COX-2 ($p = 0.01$) (Fig. 2). Ki67 expression in the resection specimen was less in patients who were given preoperative radiochemotherapy compared to those given radiotherapy alone ($p = 0.04$) (Fig. 2). Specimens from patients receiving radiochemotherapy also had a more marked inflammatory cell component (63% versus 43%), although this effect was not significant ($p = 0.08$). No significant differences were seen in apoptosis (cleaved CK18) in the different treatment arms.

3.5. *Correlation between different markers*

To better understand the effect of preoperative (chemo) radiotherapy, we tried to correlate expression of different markers in the resection specimens to each other. The fibro-inflammatory changes induced by the preoperative treatment were present to a greater extent in patients with T-downstaging ($p = 0.002$) irrespective of the preoperative treatment. The presence of cytoplasmic eosinophilia correlated with the lower expression of Ki67 ($p = 0.02$) in the resection specimen. In tumours with cytoplasmic eosinophilia also, more fibro-inflammatory changes (64% versus 43%) and mucus lakes

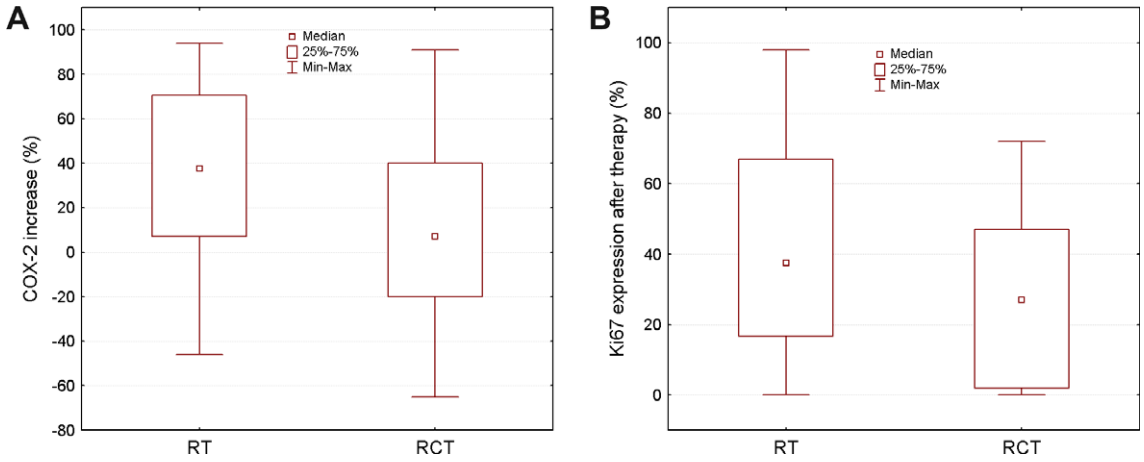

Fig. 2 – (A) Increase in COX-2 expression in the different preoperative treatment groups ($p = 0.01$). (B) Ki67 expression in the resection specimen in the different preoperative treatment groups ($p = 0.04$).

(24% versus 7%) were found than in tumours without cytoplasmic eosinophilia.

3.6. *Predictive and prognostic markers*

None of the proteins analysed with immunohistochemistry (COX-2, Ki67 and cleaved CK18) were predictive for T-down-staging, Dworak regression grade, ypT or ypN. Several clinico-pathological variables were analysed for their effect on disease-free survival (DFS) and overall survival (OS): treatment scheme, cT and ypT, T-down staging, Dworak regression grade, pN stage, differentiation grade, resection margins, blood vessel invasion, perineural invasion, lymph vessel invasion and the expression of different proteins (COX-2, Ki67 and cleaved CK18). Since the number of patients with a local recurrence was small ($n = 8$), we did not study the effect of parameters on local control. For Ki67 and COX-2, no prognostic value for DFS or OS was identified. A trend towards better DFS and OS was identified in patients with a predominantly inflammatory type stromal response ($p = 0.08$) (Fig. 3). The

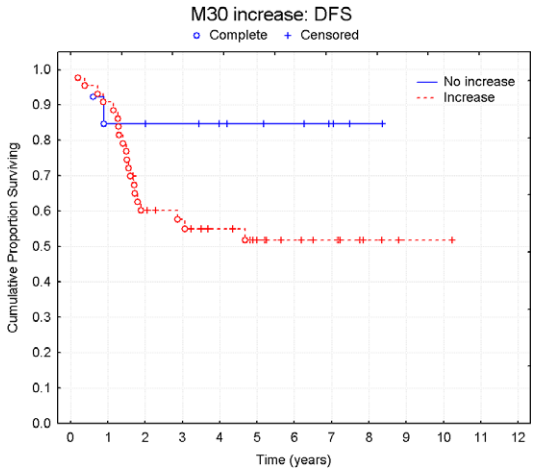

Fig. 4 – DFS for patients with and without an increase in apoptosis (cleaved CK18) during preoperative therapy.

number of apoptotic cells in the biopsy or the resection specimen did not correlate with survival. However, patients of

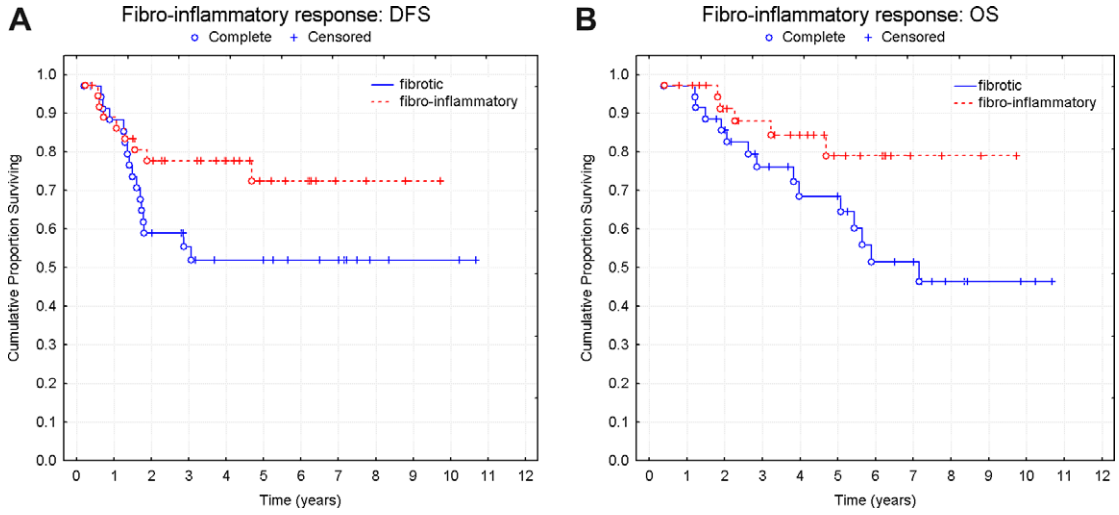

Fig. 3 – DFS (A) and OS (B) in patients with and without fibro-inflammatory response ($p = 0.08$).

Table 2 – Prognostic value for the different clinico-pathological parameters

	Univariate		Multivariate	
	DFS	OS	DFS	OS
T-downstaging	0.06	0.16	–	–
Dworak regression grade	**0.0008**	0.20	**0.01**	–
pN (0–1 versus 2)	**0.005**	**0.01**	**0.0002**	**0.03**
Complete resection	0.37	0.21	–	–
Venous invasion	**0.05**	0.17	**0.05**	0.48
Lymphatic invasion	**0.03**	**0.03**	0.523	0.50
Perineural invasion	**<0.0001**	**0.002**	**0.05**	0.81
Fibro-inflammatory response	0.08	0.08	–	–
Cytoplasmatic eosinophilia	0.64	0.31	–	–

Bold values are the significant values (below or equal to 0.05).

which the treatment induced apoptosis in the resection specimen was higher than the intrinsic apoptosis before treatment showed a better disease-free survival ($p = 0.05$) (Fig. 4). The same trend was seen for OS and LC, but this was not significant. Several established prognostic clinico-pathological parameters correlated with DFS and OS. In multivariate analysis, Dworak regression grade, pN, venous and perineural invasion correlated with DFS. For OS, only pN had a prognostic value (Table 2).

4. Discussion

Although good results are obtained with the current multimodal treatment of rectal cancer, 'patient-tailored' treatments are expected to give greater benefit. In this study, we compared the effect of preoperative radiotherapy with radio-(chemo) therapy on the expression of COX-2, proliferation (Ki67), apoptosis (cleaved CK18) and morphological alterations. Moreover, the potential value of these markers in predicting the response of the tumours to preoperative treatment was evaluated.

Overexpression of COX-2 has been reported in several human malignancies including rectal cancer.[21] In our study, a significant increase in COX-2 expression was noticed, shifting the median expression from 12% to 55%. This upregulation has also been shown earlier by our group and others.[22,23] The less pronounced increase of COX-2 expression in patients who were given the combination of chemo- and radiotherapy in comparison with patients, who were given radiotherapy alone, might be explained by an inhibiting effect of the chemotherapy on the COX-2 expression. Since COX-2 is known to play an important role in tumour growth and its expression has been correlated with outcome before,[21,23,24] this hypothesis supports the use of chemotherapy in the preoperative treatment.

In most patients, we observed a downregulation of Ki67 after preoperative treatment, which was, however, not significant. We did see that in patients that were given radiochemotherapy the proliferative capacity of the tumours was lower at moment of surgery in comparison with patients treated with radiotherapy alone, which again underlines the importance of adding chemotherapy.

Several studies have described the relevance of apoptosis for the clinical outcome of colorectal cancer patients. Whereas some studies reported a better prognosis for patients with high levels of intrinsic apoptosis, others could not find such a correlation or showed the opposite.[11,12] In our study, we did not find any predictive or prognostic value for apoptosis in the biopsy or in the resection specimen on its own. However, we did see that patients for whom the treatment induced apoptosis was higher than the intrinsic apoptosis had a better disease-free survival. This suggests that not only the intrinsic apoptosis might be an important prognostic factor, as has been shown before,[11,12] but also the changes in apoptosis should be taken into account.

One of the problems with immunohistochemical stains is the inter-laboratory difference caused by different fixation, age of the tissue and other variables such as different antibodies and protocols. These problems do not apply to the scoring of histopathological parameters on H&E stains. Stromal changes and tumour cell alterations were scored in this study in accordance with Shia et al.[17] In our study, the percentages scored for the stromal changes (fibrotic types versus fibro-inflammatory type) and tumour cell alterations (tumour cells with marked cytoplasmic eosinophilia and nuclear atypia, presence of mucin pools) induced by preoperative therapy were very similar to those reported by Shia et al.[17] Although the effect of fibro-inflammatory response on disease-free survival was less pronounced in our patient group, we saw the same trend as described by others[17,25]: patients with a marked inflammatory component in the stroma after preoperative treatment have a lower chance of recurrences. The fact that this inflammatory component was significantly more prominent in patients with T-downstaging underlines the importance of inflammation in response to treatment.

Cytoplasmic eosinophilia was found in 31% of the resection specimen and correlated with a higher presence of mucus lakes and fibro-inflammatory changes as well as with low proliferation. Several possible mechanisms have been suggested for the accumulation of tumour cells with a marked cytoplasmic eosinophilia, also known as oncocytes, after chemoradiation. This accumulation could be caused by differentiation of epithelial neoplastic cells towards the 'oncocyte-appearance' phenotype or by selective proliferation of oncocytes within tumour cells due to their resistance to radiochemotherapy.[26] Because of the association with low proliferation, which has also been shown before,[27] the first explanation seems most plausible.

To conclude, the combination of radio- and chemotherapy in the preoperative treatment seems beneficial to the patients on the molecular level, as shown by a lower Ki67 and COX-2 expression. Moreover, we confirmed the importance of the fibro-inflammatory reaction after preoperative radiochemotherapy, which correlated with T-downstaging and disease-free survival.

Conflict of interest statement

None declared.

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