Published in final edited form as:
Int J Colorectal Dis. 2009 February ; 24(2): 129–138. doi:10.1007/s00384-008-0608-8.

Molecular and clinico-pathological markers in rectal cancer: a tissue micro-array study

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Abstract

Aims—The aims of the study were to study the effect of pre-operative treatment on the expression of tumour-related proteins and to correlate the expression of these proteins with response and survival of patients with advanced rectal cancer.

Materials and methods—Tissue micro-arrays from pre- and post-treatment biopsies of 99 patients with rectal cancer treated with pre-operative (chemo)radiotherapy were stained for epidermal growth factor receptor (EGFR), carbonic anhydrase IX, Ki67, vascular endothelial growth factor, cyclo-oxygenase 2 (COX-2) and cleaved cytokeratin 18 (c-CK18). Also, fibro-inflammatory alterations after treatment were evaluated.

Results—Pre-operative (chemo)radiotherapy caused fibro-inflammatory changes, a downregulation of proliferation (Ki67) and EGFR and an upregulation of apoptosis (cleaved CK18). Patients with a good regression during pre-operative treatment showed less proliferating and apoptotic cells in the resection specimen. Multivariate analysis showed that T downstaging, fibro-

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inflammatory changes in the resection specimen and COX-2 expression in the biopsy correlated with overall survival.

Conclusions—Pre-operative treatment has an effect on proliferation, apoptosis, inflammation and EGFR expression. The classical clinical parameters as well as fibro-inflammatory changes and COX-2 expression seem most valuable as predictors for survival.

Keywords

Predictive; Prognostic; Colorectal tumours

Introduction

Colorectal cancer is a major cause of death in the Western Countries. The basic treatment is radical surgery with or without sphincter preservation. Despite the introduction of total mesorectal excision (TME) [1–3] the incidence of local recurrence remains between 5% and 20% depending on the expertise of the surgeon, the tumour location and stage at the time of diagnosis [4,5]. Adding radiotherapy to surgery has been shown to be of value in several trials using pre- or post-operative irradiation, either alone or in combination with chemotherapy [5–11]. The EORTC22921 trial concluded that chemotherapy, regardless of whether it is administered before or after surgery, confers a significant benefit with respect to local control. The addition of chemotherapy to the treatment scheme did not give rise to a difference in survival [12].

However, the response of individual tumours to adjuvant therapies is not uniform. In order to offer patients a patient tailored therapy, it would be of significant clinical relevance to identify predictive and/or prognostic markers of cancer response to radiotherapy or combined-modality therapy. We studied molecular pathways that are potentially responsible for resistance of some colorectal carcinomas to therapy. Three proteins that are known to play an important role in the growth and development of many tumours are cyclo-oxygenase 2 (COX-2), epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) [13,14]. These proteins are abundantly expressed in many tumour types and have been correlated with a bad prognosis, also in colorectal cancer [13,14]. By looking at the expression of these proteins, it might be possible to predict which patients will respond well to the standard (chemo) radiotherapy treatment, and the promising proteins might be useful targets for therapy in patients with rectal cancer.

Next to specific expression of these three important proteins, we also looked at some proteins which reflect more general factors important in the response to therapy such as proliferation, hypoxia and apoptosis. Ki67 is a nuclear protein involved in cell cycle regulation. It is present in all cell cycle phases except for the G0 and early G1 phase, making it a good marker for proliferation [15]. 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 [16].

The ability to evade apoptosis is thought to be central in both tumourigenesis [17] and resistance to cytotoxic drugs and radiation [18]. One of the methods for the detection of apoptosis is the cleaved CK1S (c-CK18) antibody, which has been introduced by Leers et al. [19]. 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 [19]. The cleaved cytokeratin staining seems to correlate very well with caspase-3 staining [20] and has been used before in paraffin-embedded material from patients with rectal cancer [21,22].

Since both the effect of radiotherapy and chemotherapy can be influenced by hypoxia, the presence of hypoxia is a problem for the treatment of both colon and rectal tumours. Various hypoxia-inducible proteins have been put forward as potentially useful endogenous hypoxia markers. Carbonic anhydrase IX (CA IX), which can be activated by HIF-1α, is over-expressed in many tumour types [23] and has been widely investigated. CA IX is a transmembrane protein that was initially characterised as a hypoxia-inducible gene due to its over-expression in von Hippel-Lindau-defective cell lines [24]. It is involved in glycolysis, catalysing the reversible hydration of carbon dioxide to carbonic acid and providing a mean of regulating pH in tumours. In most colorectal tumours, CA IX has an altered expression pattern [23,23], and it is suggested that this may be important in their pathogenesis [25].

In this study, the change in expression of different proteins (COX-2, VEGF, EGFR, Ki67, CAIX and c-CK18) and fibro-inflammatory alterations during pre-operative treatment and the correlation of these markers with response will be evaluated.

Materials and methods

Patient selection

Between 1996 and 2003, 99 patients with an adenocarcinoma of the rectum were neo-adjuvantly treated with radiotherapy alone or with a combination of chemotherapy and radiation. Of the 99 patients included in our study, 79 received a combination of radiotherapy (45 Gy in 25 fractions with or without a boost of 5.5 Gy to tumour and lymph nodes) with chemotherapy (325 mg/m^2 5-FU and 10 mg/m^2 LV as bolus injection five first and five last days of radiotherapy or 5-FU, 225 mg/m^2/day as continuous during the whole course of radiotherapy), and 20 were pre-operatively treated with radiation only (30 Gy in ten fractions with or without a boost of 9 Gy to the tumour). The tumours were located in the lower (47%), middle (36%) and upper parts (18%) of the rectum. All patients underwent surgery in the university hospital of Gasthuisberg, Leuven by three different abdominal surgeons. All patients received a TME procedure. The study was performed in accordance with the ethical standards of our institution. Due to the retrospective character of the study, no informed consent could be obtained from the patients.

Tissues and array construction

Paraffin-embedded tumour from both the biopsy before treatment (*n*=99) and the tumour tissue from the resection specimen (*n*=77) were used to make donor blocks. The missing resection specimens were due to the absence of tumour tissue after making the tissue arrays (*n*=6) or the absence of tumour after neo-adjuvant treatment (*n*=14). Morphologically defined regions of the tissue blocks were identified using a haematoxylin and eosin (H&E)-stained section from each.

The tissue micro-arrays (TMAs) were constructed using a fixed pattern. Tissue from the initial biopsy was punched (three to four cores per patient), followed by four to six core biopsies from different areas and from the surgical specimen (after the neo-adjuvant treatment).

The pathological characteristics were scored on the H&E sections of the whole tissue slices by an experienced pathologist. ‘Classical’ pathological variables, including the most prevalent grade of differentiation, section margins, ypT and ypN stage were noted. Since lymph vessel and venous invasion are often difficult to distinguish, this was scored as one entity (invasion). Regression of the tumour after pre-operative treatment was evaluated by T downstaging and Dworak regression grading. T downstaging was defined as a shift of the cT stage, evaluated by endoscopic ultrasound, towards a less advanced ypT stage, evaluated on H&E stains of the resected specimen. Dworak regression was scored on H&E stains of the resection specimen as

published before: grade 0, no regression; grade 1, minimal regression; grade 2, moderate regression; grade 3, good regression; and grade 4, total regression [26]. Patients with Dworak regression grade 0–2 were defined as non-responders, grade 3–4 as responders. Analogous to Shia et al. [27], stromal responses as were seen in the resection specimens after tumoural regression following the neo-adjuvant treatment, were scored as follows: fibrotic type = fibrosis/sclerosis with sparse inflammatory cell component, comprising <25% of the entire stromal tissue and fibro-inflammatory type = fibrosis/sclerosis with a prominent inflammatory component comprising <25% of the entire stromal tissue.

Immunohistochermstry (IHC)

IHC for each antigen was performed on 4-μm paraffin sections of each rectal cancer TMA section described above. From each block, one section was stained for COX-2, EGFR, CA IX, VEGF, c-CK18 and Ki67.

Standard immunoperoxidase procedures were used. EGFR staining was undertaken following the instructions of the DAKOCytomation EGFR kit. Antigen retrieval was necessary for the immunostaining with the COX-2 (mouse monoclonal, Cayman chemical, dilution 1/50), Ki67 (Lab Vision, mouse monoclonal antibody, ready to use), VEGF (rabbit polyclonal, Santa Cruz, dilution 1/100) and c-CK18 (M30, mouse monoclonal, Roche, 1/10) antigens. The primary antibody against CA IX was a mouse monoclonal antibody (M75, Bayer, USA, dilution 1/50) [28], which required no micro-wave antigen retrieval. Antibody binding was visualised by diaminobenzidine. All slides were counterstained with haematoxylin, dehydrated, cleared and mounted. As a negative control, we used PBS on sections that were proven to be positive for the different proteins in earlier experiments.

Scoring of the immunohistochemical staining

For COX-2, Ki67, VEGF, EGFR and CA IX, stained sections were analysed at a total magnification of ×200. Each core was assigned a continuous score of percentage positivity, representative of the approximate area of immunostaining. For VEGF and COX-2, the percentage of cells with positivity in the cytoplasm were counted, while for CA IX, only cells with expression in the membrane were considered positive. For EGFR, both cytoplasmic and membranous positive cells were taken into account. For Ki67, all stained nuclei were scored. For C-CK18, the number of positive tumour cells per punch (=1 mm^2) were counted on a ×400 magnification. All samples were evaluated and scored without knowledge of the patients' histories.

Statistics

The statistical software Statistica 7.0 was used for analysis. Differences for the expression of a protein between the classes for clinical variables were evaluated using a Mann–Whitney *U* or Kruskal Wallis test. The difference in expression in the biopsies and the resection specimen was assessed with a Mann–Whitney *U* test.

Survival rates (local control, disease free survival and overall survival) were calculated by Kaplan–Meier modelling. The influence of risk factors on survival was evaluated by the log-rank test looking at the expression patterns of the studied proteins and classical prognostic factors such as pre-treatment clinical stage, pathological stage, involvement of the resection margins, degree of differentiation and changes in T stage. For the immunohistochemical stains, the median was used as a cut-off for the analysis. A multivariate Cox proportional hazard regression model was used to explore the relative risk of death and (local) recurrence for the above mentioned prognostic factors. Differences were considered statistically significant at $p<0.05$.

Results

Patient characteristics

Clinical characteristics of the patients such as sex, T stage, N stage and histopathological grades of tumours at the time of the biopsy are presented in Table 1. Forty-two out of 99 tumours (42.4%) showed T downstaging. The Dworak regression grades were distributed as follows: grade 0 (3%), grade 1 (13%), grade 2 (55%), grade 3 (12%), grade 4 (17%). The median follow-up time was 42.4 months (2.3–97.3), with a median local control (LC), disease free survival (DFS) and overall survival (OS) of 42.7 months (4.1–92.7), 39.2 months (4.1–92.7) and 44.2 months (4.1–98.7), respectively. The percentages for the development of local recurrences and metastases were 9% and 24%, respectively, and 17% of the patients died. No significant differences were found in response (T downstaging, Dworak), local control and survival (DPS, OS) for patients receiving pre-operative radiotherapy or radiochemotherapy.

Fibro-inflammatory alterations after pre-operative treatment

Tumour regression in rectal carcinomas after pre-operative treatment was in most cases associated with fibrosis or fibro-inflammatory changes replacing neo-plastic glands. In all of the examined resection specimens (n=77), a form of fibrosis was present. Fibrosis with sparse inflammatory infiltrates was more commonly seen (n=44) than fibrosis with a marked inflammatory cell component (n=33). No differences in fibro-inflammatory response were seen in patients treated with either radiotherapy or radiochemotherapy.

Effect of pre-operative treatment

The results of the immunostainings of all markers are represented in Table 2 and Fig. 1. The pre-operative treatment seems to have the most effect on Ki67, EGFR and c-CK18 expression with a significant downregulation of Ki67 and EGFR (p<0.0001) and an upregulation of c-CK18 (p<0.0001; Fig. 2). For COX-2, also a significant upregulation (p=0.02) was noticed but to a lesser extent. For VEGF and CA IX, no significant differences were seen before and after therapy. No significant different effect was seen in the patients treated with pre-operative radiotherapy and those treated with pre-operative radiochemotherapy.

Correlations between pre-treatment factors and regression

Clinical pre-treatment factors and expression of proteins in the biopsies were correlated with the regression grade T downstaging (0–1) and Dworak regression grade (0–1–2 versus 3–4) of the tumours. No correlation was found between the clinical factors cT and differentiation grade in the biopsies with T downstaging or Dworak regression.

The expression of VEGF correlated with response in patients with a low Dworak regression grade having a higher VEGF expression before treatment (p=0.01). This could however not be confirmed for T downstaging. For the other proteins, no correlation between expression in the biopsy and regression was found.

Correlations between post-treatment factors and regression

Dworak regression (0–1–2 versus 3–4) correlated significantly with T downstaging (0–1; p<0.0001). Both parameters correlated significantly with ypT, showing more regression (p=0.006) and T downstaging (p=0.0001) in ypT1 and ypT2 tumours compared to ypT3 and ypT4 tumours, as expected. No correlation was found between Dworak regression and ypN or positive resection margins. No correlations were found between Dworak regression or T downstaging with differentiation grade or fibro-inflammatory alterations in the resection specimen.

In patients with a high Dworak grade, thus good regression, a lower expression of Ki67 (p=0.07) and c-CK18 (p=0.0007) was noticed in the resection specimen (Fig. 3). The expression of Ki67 in the resection specimen was also higher in those patients with inflammatory fibrosis in the resection specimen (p=0.002).

Univariate analysis of clinico-pathological variables and immunostaining data

Several of the clinico-pathological variables that were tested correlated with either DFS, local control (LC) or OS (Table 3). For DFS, high cT stage, cN stage, ypT stage and ypN stage were negative prognostic factors, whereas T downstaging and fibrosis in the resection specimen were positive prognostic factors, OS was associated with ypT, T downstaging, differentiation grade, fibrosis in the resection specimen and positive section margins. Positive resection margins, invasion, a high cN stage, ypT and ypN stage correlated with local recurrence.

A set of six biomarkers was tested by immunohistochemistry on the TMAs (Fig. 1). For DPS, no prognostic value could be identified for the six biomarkers, OS showed a negative correlation with the amount of COX-2 expression and high COX-2 expression in the resection specimen correlated with local recurrences (Table 3, Fig. 4).

Multivariate analysis of clinico-pathological variables and biomarkers

In the multivariate Cox analysis, fibrosis in the resection specimen (p=0.01) and COX-2 expression in the biopsy (p=0.04) were significant predictors for OS. There was no significant correlation between COX-2 expression and inflammation in the resection specimen (p=0.42).

Hierarchical cluster analysis

We performed a hierarchical cluster analysis to find expression patterns of responders and non-responders to define patients with ‘good response’. However, no significant patterns associated with response were found, possibly due to the small patient group.

Discussion

Anticancer therapies aim for the complete eradication of tumour cells. Nevertheless, it is well known that although some tumours respond to cytotoxic therapies, such as irradiation and chemotherapy, other tumours persist or relapse early after therapy. Until now, little is known about the mechanisms responsible for persistence or recurrence of tumour cells after cytotoxic therapies. One difficulty is the rare availability of longitudinal tumour material. Here, we present a study of 99 locally advanced colorectal tumours treated pre-operatively with (chemo-) radiotherapy, from which tumour material was available before and after pre-operative treatment. The aim of the present study was twofold; on the one hand, we wanted to get insight into the changes in protein expression caused by neo-adjuvant treatment. Moreover, we aimed to correlate the expression of several biological markers with response in a patient population with locally advanced rectal tumours.

Our patients were treated with either pre-operative radiotherapy (30 Gy) or pre-operative chemoradiotherapy (45 Gy + 5-FU). Although 5-FU is interfering with DNA synthesis, leading to less efficient repair of DNA stranded breaks, which could enhance the effect of radiation, no significant differences were found in response (T downstaging, Dworak, LC, DFS, OS) or the effect on expression of proteins in our patient group. This is in agreement with the randomised trial in rectal cancer published by Bosset et al. where also no significant effect of pre-operative radiotherapy or radiochemotherapy was seen on OS and DFS [12]. Although the different doses of radiotherapy used in both treatment groups might seem quite different (30 versus 45 Gy), the biological effective dose of both the 30-Gy treatment, given in ten fractions of 3 Gy, and the 45-Gy treatment given in 25 fractions of 1.8 Gy, is very comparable. The

biological effective dose on the tumour equals 36 Gy for the 30-Gy treatment and 37.5 Gy for the 45-Gy treatment. Because of the negligible effect of the different radiation doses and the similar response found in the different treatment groups combined with the small number of patients available in our study, the different groups were regarded as one to identify the predictive and prognostic markers.

By comparing expression of proteins before and after therapy, we get an idea of which pathways are influenced by the pre-operative (chemo)radiotherapy. We can conclude that proliferation, as measured by Ki67 expression, was clearly diminished after pre-operative therapy, while the number of apoptotic cells (c-CK18) was significantly increased. Rau et al. [29] also found a downregulation of Ki67 after neo-adjuvant treatment, which could indicate that the most proliferative tumour cells are the most sensitive for the neo-adjuvant treatment. In agreement with this, our data also show that patients with a good regression have a lower percentage of Ki67 positive cells left after treatment (Fig. 3).

Also, the expression of EGFR and to a lesser extent CAIX and COX-2 was influenced by the chemoradiation: EGFR expression was downregulated, and CAIX and COX-2 expression increased. Although radiotherapy is known to upregulate EGFR [30–32], we believe the delay between the end of the radiotherapy and the surgery is the main reason for the downregulation of EGFR in our study. The upregulation of COX-2 after radiotherapy is a phenomenon described in vitro [33]. None of these parameters correlated with T downstaging or Dworak regression.

In agreement with Gosens et al. [34], we found a significant correlation between regression (T downstaging and Dworak regression) and ypT stage. We did however not see a correlation with positive resection margins, possibly due to the low number of patients with positive resection margins (n=6). The good correlation between regression and the ypT stage confirms the idea by Gosens et al. suggesting that pre-operative treatment results in tumour shrinkage rather than tumour fragmentation [34].

Most clinico-pathological prognosticators found in our study (Table 3) are variables proven to have prognostic value in many studies, indicating the representativeness of our patient population [27,35]. The observation that patients who have an extensive inflammatory response at the tumour bed have a better outcome (DFS, p=0.007; OS, p=0.003) fits the hypothesis that the tumour-associated inflammatory infiltrate is a type of host response and is an important factor in tumour progression [36]. This may therefore be supportive for further studies on therapeutic regimens that aim at intensifying host immune reaction in this patient population.

Next to the clinico-pathological markers, a set of six biomarkers was tested by immunohistochemistry on the TMAs (Fig. 1), where we identified a prognostic value for COX-2. Whilst the intrinsic COX-2 expression in the biopsies seems to be important for DFS, the chance of local recurrences seems more dependent on the COX-2 expression in the resection specimen. In literature, conflicting results concerning the role of COX-2 as prognostic marker in colorectal tumours are reported. Recently, a large study by Fux et al. [37] found no prognostic significance for COX-2 expression in pooled colon and rectal carcinomas on survival. A major difference with our study is that we only looked at rectal adenocarcinomas. They claimed that the definite answer on that issue was given due to the size of their study. But, the literature shows as many published studies with prognostic value for COX-2 [38–42] as there are with none [37,43–46].

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 [21,22]. Ki67 is the most popular protein studied as a prognosticator in colorectal cancer, although, so

far, only one study reported an independent prognostic role for Ki67 in colorectal tumours when determined at the invasive margin [47]. Chen et al. [48] failed to demonstrate any prognostic role for high Ki67, and in several large series of colorectal patients, Ki67 did not correlate with survival [49–53]. In our study, no prognostic role for Ki67 or c-CK18 was found. However, we did see that patients with a good response during pre-operative radiochemotherapy showed a lower expression of Ki67 and c-CK18 in the resection specimen, which suggests that the effect of the treatment on proliferation and apoptosis is important for a good response.

To conclude, pre-operative chemoradiation gives rise to fibro-inflammatory changes, more apoptosis, less proliferation and less EGFR expression. The effect on proliferation and apoptosis seems to be important for tumour regression. Moreover, the fibro-inflammatory changes induced during chemoradiation and COX-2 expression seem to be prognostic for the outcome of the patients.

Acknowledgements

This work was supported by a FWO grant (G.0434.04) and Varian Biosynergy. Karin Haustermans is supported by a fundamental clinical mandate of the FWO.

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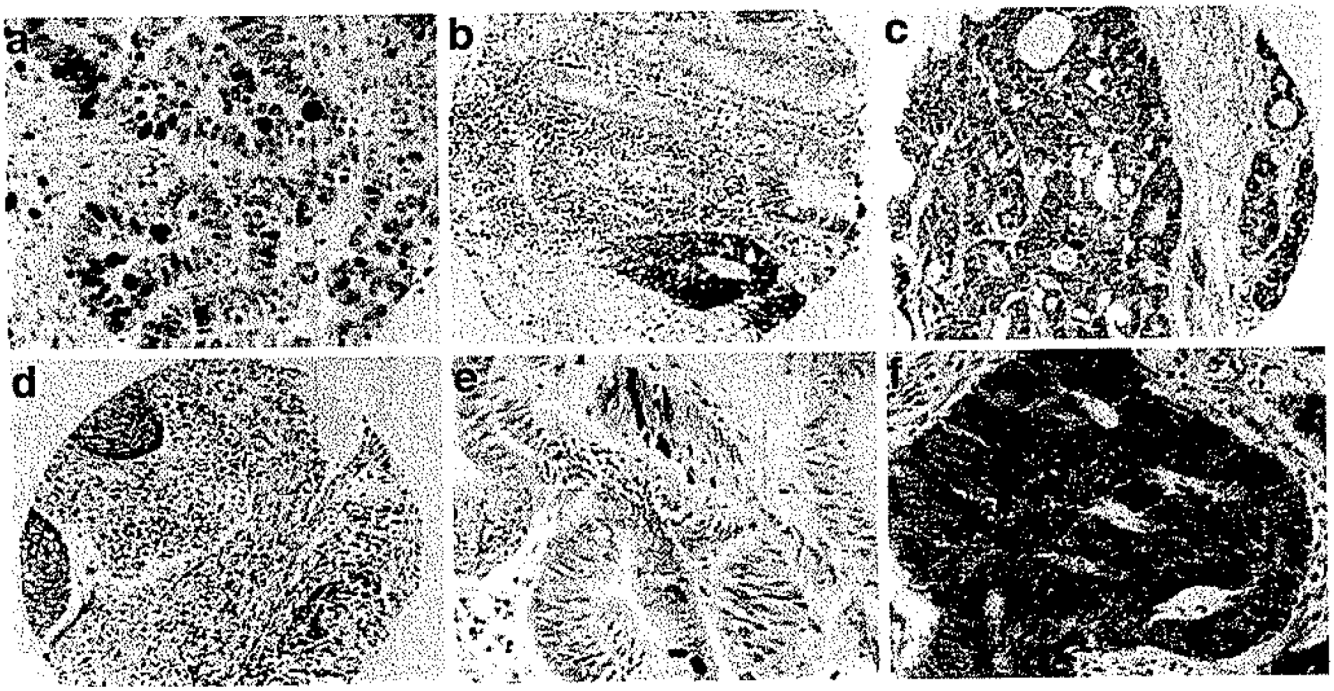

Fig. 1.
Examples of six core biopsies stained for Ki67 (**a**), CA IX (**b**), VEGF (**c**), EGFR (**d**), c-CK18 (**e**) and COX-2 (**f**), **a, e** and **f** ×200 magnification; **b, c** and **d** ×100 magnification

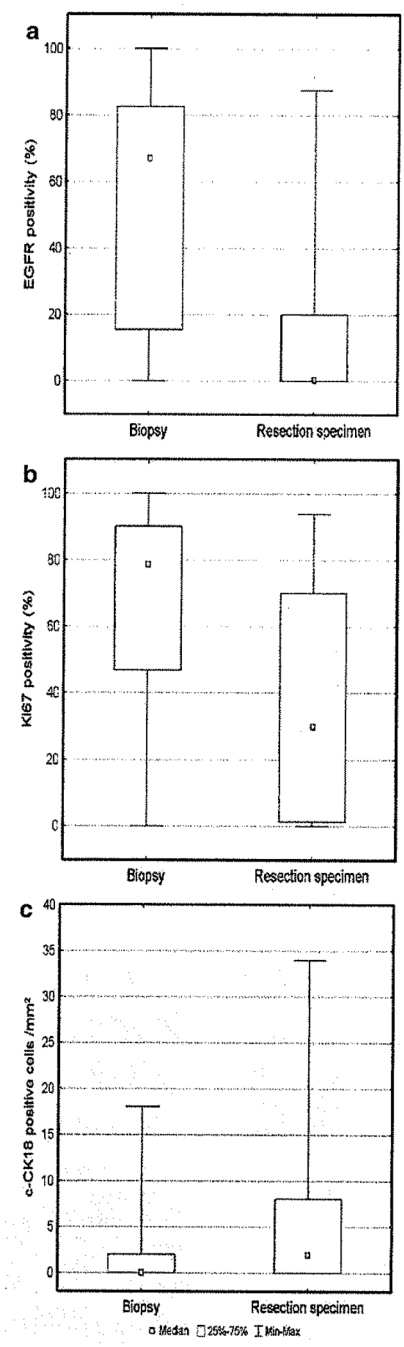

Fig. 2.
Expression of EGFR (**a**), Ki67 (**b**) and c-CK18 (**c**) in the biopsy and the resection specimen

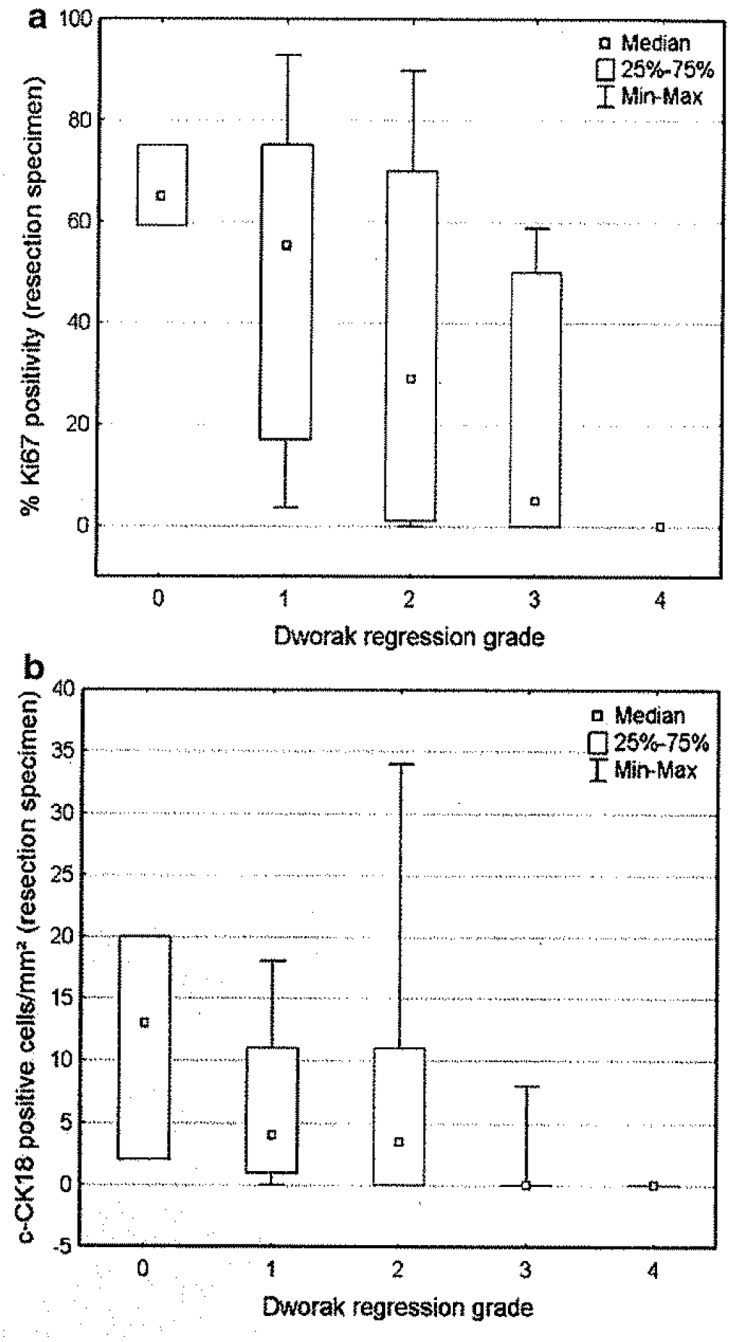

Fig. 3.

Expression of Ki67 (**a**) and c-CK18 (**b**) in the resection specimen of patients with different regression grades. Patients with a good regression (=high Dworak grade) have a lower expression of Ki67 and c-CK18

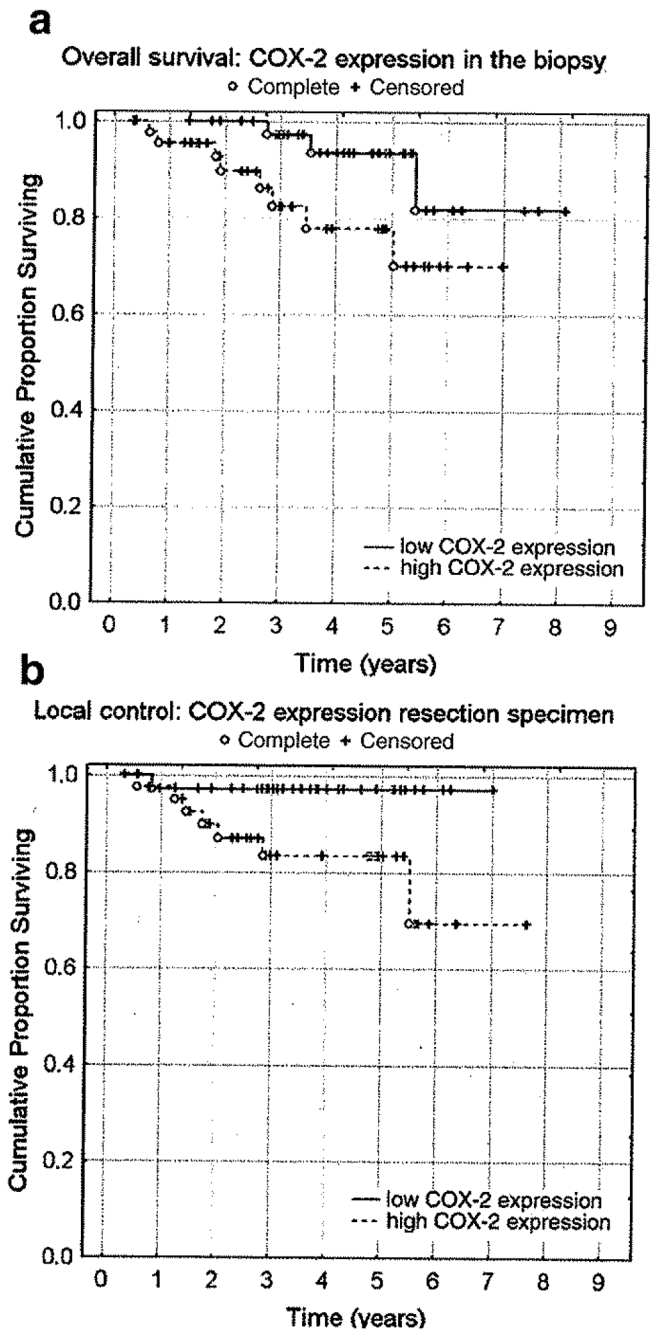

Fig. 4.

Overall survival curve for COX-2 expression (**a**) in the biopsy. Local recurrence curve for COX-2 expression in the resection specimen (**b**)

Table 1

Patient characteristics

	Number	Percentage
Number of patients	99	
Age (years)	63 (39–84)	
Gender (male/female)	64/35	
Tumour stage		
cT2	10	10.1
cT3	75	75.8
cT4	12	12.1
cTx	2	2.0
Nodal stage		
cN0	24	24.2
cN1-2	68	68.7
cNx	7	7.1
Histopathologic grade		
G1	10	10.1
G2	39	39.4
G3	37	37.4
Unknown	13	13.1

Table 2

Characteristics of expression patterns of studied proteins

		Median percentage positivity	Range of percentage positivity	% of tumours showing expression
Ki67	Biopsy	78.8	(0–100)	95
	Res. spec.	32	(0–87.5)	63
CA IX	Biopsy	31.7	(0–100)	87
	Res. spec.	10	(0–89.1)	61
COX-2	Biopsy	65	(0–100)	81
	Res. spec.	87.8	(0–100)	80
VEGF	Biopsy	95	(0–100)	94
	Res. spec.	90	(7–100)	85
EGFR	Biopsy	67	(0–100)	77
	Res. spec.	0	(0–100)	40
c-CK18	Biopsy	0[a]	(0–17)[a]	44
	Res. spec.	2[a]	(0–34)[a]	59

Res. spec. resection specimen

[a]Number of positive tumour cells per punch (=1 mm^2)

Table 3

Prognostic markers, clinical variables and cluster groups in univariate analysis of disease free survival, local control and overall survival

Marker or factor (cut-off)	Significance (*p* value) DFS	Significance (*p* value) LC	Significance (*p* value) OS
cT (1, 2–3, 4)	0.03, (−)	0.21	0.13
cN (0, 1–2)	0.007, (−)	0.0004, (−)	0.06
ypT (is, 0, 1–2, 3, 4)	0.005, (−)	0.009, (−)	0.0007, (−)
ypN (0–+)	0.001, (−)	0.002, (−)	0.38
T downstaging (no- yes)	0.04, (+)	0.16	0.003, (+)
Dworak regression grade (0–1–2 versus 3–4)	0.69	0.99	0.17
Fibrosis res. spec. (without–with inflammation)	0.007, (+)		0.003, (+)
Differentiation res. spec. (I–II–III)	0.08		=0.04[a]
Invasion (no–yes)	0.15	0.04, (−)	0.16
Section margins (neg–pos)	0.15	0.0004, (−)	0.04, (−)
COX-2 biopsy (median)	0.11	0.19	0.05, (−)
CA IX biopsy (median)	0.76	0.54	0.29
EGFR biopsy (median)	0.72	0.98	0.98
VEGF biopsy (median)	0.59	0.90	0.25
Ki67 biopsy (median)	0.23	0.36	0.42
c-CK18 biopsy (median)	0.96	0.06	0.64
COX-2 res. spec. (median)	0.23	0.05, (−)	0.42
CA IX res. spec. (median)	0.33	0.97	0.94
EGFR res. spec. (median)	0.93	0.55	0.76
VEGF res. spec. (median)	0.48	0.66	0.83
Ki67 res. spec. (median)	0.12	0.48	0.84
c-CK18 res. spec. (median)	0.57	0.77	0.26

(−) negative factor, (+) positive factor, *Res. spec.* resection specimen, *DFS* disease free survival, *LC* local control, *OS* overall survival

[a]Confounding data, grade II is better than I and III