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Title: Histological grading based on poorly differentiated clusters (PDC) is predictive of tumor response and clinical outcome in rectal carcinoma treated with neo-adjuvant chemo-radiotherapy

Running title: PDC in rectal cancer treated with neo-adjuvant CRT

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ABSTRACT

Aims

The clinical outcome of patients with locally advanced rectal cancer submitted to neo-adjuvant chemo-radiotherapy (CRT) is influenced by tumour response to treatment, which is reflected by tumour regression grade (TRG) and post-treatment (y) TNM stage. Little is known on the prognostic value of pre-treatment histopathological features of the tumour which may be useful to discriminate potential non-responders and to design tailored therapeutic strategies.

In this study, we aimed to investigate the prognostic role of poorly differentiated clusters (PDC) of neoplastic cells in pre-treatment biopsies of rectal cancer submitted to neo-adjuvant CRT.

Methods and results

Grading based on PDC counting was retrospectively applied to 204 pre-treatment endoscopic biopsies of rectal carcinomas treated with neo-adjuvant CRT and surgery. Inter-observer agreement in the assessment of PDC grade was good. High PDC grade was significantly associated with high yT stage (P= 0.044), yM+ status (P= 0.0004) and unchanged TNM stage or TNM upstaging (P= 0.032). In addition, high PDC grade was a significant and independent prognostic factor for cancer specific survival (CSS).

Conclusions

PDC grade may be assessed in pre-operative biopsy of rectal cancer with good reproducibility. High PDC grade in pre-treatment tumour is significantly associated with low response to therapy. Hence, we suggest that PDC grading might be used as a significant predictive and prognostic factor in patients with locally advanced rectal cancer submitted to neo-adjuvant CRT and to identify high-risk patients who need surgery and adjuvant chemotherapy.

Key words: rectal cancer; poorly differentiated clusters; grading; prognosis; Dworak; neo-adjuvant chemo-radiotherapy

INTRODUCTION

Neo-adjuvant chemo-radiotherapy (CRT) is currently applied to patients with locally advanced (clinical T stage 3/4 or N+) rectal cancer[1,2] in order to improve tumour resectability and sphincter preservation and to decrease the probability of local recurrence after surgery[3,4]. The efficacy of neo-adjuvant CRT is highly heterogeneous and it can be measured in the surgical specimen through the assessment of post-treatment Tumour Node Metastasis stage (yTNM) and so-called Tumour Regression Grade (TRG). Interestingly, while some rectal carcinomas have total histological regression (absence of neoplastic cells in the surgical specimen and yT0N0M0), others have no change in TNM stage or even TNM upstaging after neo-adjuvant CRT[5]. Identification of clinical and histopathological factors able to predict tumour response to CRT is a main research focus. Indeed, in the aim to avoid side-effects of surgery, "watch and wait" approach could be applied to patients with high probability of having complete tumour regression after CRT[6]. In a recent study, our group demonstrated that lower/extensive involvement of the rectum (i.e. involvement of the lower third of the rectum or involvement of the whole rectum), the presence of nodal metastases (i.e. clinical (c) N+ status) and mucinous histotype are significantly associated with poor tumour regression after neo-adjuvant therapy[5]. This suggests that "watch and wait" policy should be avoided in patients having a rectal cancer with those features due to significantly higher probability of residual disease.

In the last years, a novel grading system based on the counting of poorly differentiated clusters (PDC) of neoplastic cells was proposed for colorectal cancer (CRC)[7]. According to the original definition, PDC are aggregates of at least five neoplastic cells which do not form

a glandular structure (Ueno et al., 2012). By counting the number of PDC in a x20 microscopic field, CRC in surgical specimens can be graded into G1, G2 or G3, in the presence of <5, 5-9, or ≥ 10 PDC, respectively[8]. Interestingly, several studies have shown that PDC grading is more reproducible and prognostically informative than conventional (based on the percentage of glandular differentiation) histological grading in CRC[7-17]. In addition, our group demonstrated that PDC grading may also be applied to pre-operative endoscopic biopsies[9]. In detail, after adjusting the scale with cut-off values (Barresi et al., 2014a) different from those used in surgical specimens, we showed that PDC grade in the pre-operative biopsy is highly predictive of nodal status[9]. Indeed, CRC graded as G3 had significantly higher probability to have nodal metastases compared to G1 and G2 carcinomas[9]. Although endoscopic biopsies of rectal carcinomas were also included in that study, none of the patients had been submitted to neo-adjuvant CRT. Hence, whether pre-surgical PDC grade has prognostic relevance in patients with rectal carcinoma submitted to neo-adjuvant CRT is still to be investigated. For this reason, the aim of this study was to assess PDC grading in the endoscopic biopsies of rectal cancer submitted to neo-adjuvant CRT and surgery, and to analyze its correlation with the other clinical and histopathological parameters, with tumour response and with clinical outcome as well.

MATERIALS AND METHODS

All relevant ethical issues were discussed with the Local Ethics Committee. Since this was a retrospective study, based on revision of histological slides, no formal approval was required.

A total of 249 rectal adenocarcinomas treated through neo-adjuvant CRT and surgical resection with mesorectal excision were identified in the Tumour Registry of Colorectal Cancer of the University of Modena and Reggio Emilia, Italy, in the period between 2001 and 2013.

23 patients were excluded from this study because they had clinical TNM (cTNM) stage IV. Then, after revision of the histological slides of the endoscopic biopsies, we excluded 12 more cases, since the excessive fragmentation of the specimen did not allow assessing PDC grading.

Thus the final cohort in this analysis was composed of 204 patients (138 males; 66 females; mean age: 66 years; age range: 30-85 years) with cT3/T4 or cN+ rectal cancer. Those cases were already included in a previous study carried out to establish the prognostic relevance of TRG in rectal cancer[5].

In all cases, TNM staging workup had been performed by using digital rectal examination, chest radiography, total-body computed tomography (CT), magnetic resonance imaging (MRI), endorectal ultrasonography and coloscopy with biopsy. For each case, we reviewed the clinical records to obtain information on the localization in the rectum (upper, medium, lower or extensive), circumferential involvement of rectal wall (one third, middle or complete), distance from the anal verge (more or less than 1 cm) and craniocaudal extension (more or less than 3 cms) of the tumour.

After the histological diagnosis on endoscopic biopsy, all patients had received a total dose of 50 Gy radiotherapy, administered in 28 fractions of 1,8 Gy each for five consecutive days per week, and daily continuous infusion of 5-fluorouracil (5-FU). Then, they had been submitted to surgical resection. After surgery, the patients were monitored for disease progression with total-body CT scan, coloscopy and blood tests (including measurement of CEA and CA 19-9). This allowed achieving data on cancer specific survival (CSS) and disease free survival (DFS). Patients who died of diseases independent from rectal cancer were censored. Both local and distant recurrences were considered in the assessment of DFS. Information on

adjuvant therapy was available for 127 patients. Decision to give neo-adjuvant treatment was based on the age of the patients, on the presence of comorbidities and on cTNM stage and yTNM stage as well.

Pathological examination

For each case we reviewed the histological slides of pre-operative biopsy and established the histological grade and histotype of the tumour according to the World Health Organization criteria[18,19]. In addition, we assessed PDC grade as previously described[9]. In brief, we scanned the whole tumour at low power magnification (using a 4x objective lens) to identify the area with the highest number of PDC. Then we counted the clusters under the microscopic field of objective lens ×20 (i.e., a microscopic field with a major axis of 1 mm) using a Zeiss microscope (Oberkochen, Germany). Cases with 0, 1 to 2 and more than 2 PDC were graded as grade 1 (G1), grade 2 (G2), and grade 3 (G3), respectively. Assessment was carried out by two independent observers and blinded to the other clinico-pathological variables. In case of discordance, consensus was reached using a double-headed microscope. A main cause of discordance was the distinction between PDC and fragmented glands.

For each case, the corresponding surgical specimen had been fixed in formalin for 24 hours at room temperature and grossly examined for obvious or presumable remains of tumour as a mass, ulcer or fibrotic lesion. At least 3 samples had been taken for paraffin embedding from specimens showing obvious tumour mass. On the other hand, specimens with questionable residual tumour- i.e. those showing a fibrotic area or a scar- had been completely embedded, and if no tumour cells were detected on first paraffin sections, three additional levelled sections had been examined from each paraffin block. In each case, at least 12 lymphnodes had been retrieved from perirectal fat.

TRG and yTNM staging were assessed in each case[5]. In particular, TRG was established by using Dworak system and a two-tiered Dworak scale as well[5,20]. In two-tiered Dworak grading scale, cases with no regression or those with easy-to-find neoplastic cells (Dworak 0/1/2) were classified as absent/partial regression, while those with evidence of very few neoplastic cells (difficult to find) and those with no tumour cells (Dworak 3/4) were classified as total/subtotal regression[5]. Cases with acellular mucin pools and complete absence of tumour cells in the stroma were classified as total regression[5].

yTNM staging was performed on the criteria of Union for International Cancer Control (UICC) (TNM 7th edition)[21].

Radial (circumferential resection) margin was defined as positive if the distal edge of the tumour was located 1 mm or less from the nonperitonealized surface [22].

Finally, in each case we compared cTNM and yTNM stage to verify changes in T, N and TNM staging after neo-adjuvant CRT; by doing so, we subdivided cases into three groups: 1) rectal cancer with no change in TNM staging; 2) rectal cancers with downstaging after therapy; 3) rectal carcinomas with upstaging after therapy.

Statistical analyses

Fleiss-Cohen weighted k statistics were used to establish interobserver variability in the assessment of PDC grade.

The Chi-squared test was applied to analyze the statistical correlations between PDC grade and the various clinico-pathological parameters. DFS and CSS were assessed by the Kaplan-Meier method, with the date of primary surgery as entry date. The end point for the DFS analysis was the length of survival to disease progression (either local or distant). CSS was characterized as the length of survival to death from rectal cancer or to the last follow-up date. The Mantel-Cox log-rank test was applied to assess the strength of association between

DFS or CSS and each of the parameters as a single variable. Correlation between adjuvant chemotherapy and CSS or DFS were not investigated since this information was available in only a part of cases.

Subsequently, a stepwise multivariate analysis (Cox regression model) was utilized to determine the independent effect of each variable on survival. Only variables with significant prognostic significance in univariate analyses were considered in multivariate analyses for CSS and DFS.

A probability (*P*) value less than 0.05 was considered statistically significant. Statistical analysis was done using MedCalc 12.1.4.0 statistical software (MedCalc Software, Mariakerke, Belgium).

RESULTS

PDC were identified in a total of 46 biopsies of rectal carcinoma. PDC counting ranged between 0 and 6 (median number: 0). When we applied PDC grading, PDC grade was G1 in 158 (77%), G2 in 37 (18%) and G3 in 9 (5%) carcinomas (Figure 1). Inter-observer agreement in the assessment of PDC grade was good (K: 0.79). Disagreement mainly concerned the distinction between PDC and fragmented glands (Figure 2).

Cases with PDC G3 were considered to be high-grade, while those with PDC G1/G2 were considered to be low-grade.

High PDC grade (G3) was significantly more frequent among carcinomas of conventional histotype (*P*= 0.034) (Table 1). On the other hand, no statistically significant association was found between PDC grade and the other pre-treatment clinico-pathological parameters (localization in the rectum, circumferential spread, craniocaudal extension, distance from the anal verge, cT and cN status) (Table 1). When we analyzed the correlations between PDC grade and the histopathological characteristics of the surgical specimen obtained after CRT,

we found that cases with high PDC grade in the pre-operative biopsy had high yT (yT3/T4) stage (P= 0.044), yM+ status (P= 0.0004), unchanged T status or T upstaging (P = 0,022) and unchanged TNM stage or TNM upstaging (P= 0.032) with significantly higher frequency than cases with low PDC grade (Table 1). However, PDC grade was not associated with TRG (Table 1).

Median follow-up of the patients was 68 months (range: 3-183). During the follow-up 71 (35%) patients developed recurrences and 57 (28%) died of disease. In detail, 5 patients had local recurrence, while 66 had distant recurrences. Distant recurrence were diffuse in 34 patients, hepatic in 14, cutaneous in 2, pulmonary in 13, pelvic in 2 and osseous in 1 case.

Univariate analyses showed that high PDC grade (P= 0.015; P= 0.013) (Figures 3 and 4), craniocaudal extension > 3cms (P= 0.01; P=0.006), high yT (P < 0.0001; P < 0.0001), yN+ (P< 0.0001; P< 0.0001), yM+ (P= 0.0002; P=0.01), high y stage (P< 0.0001; P< 0.0001), unchanged TNM stage or TNM upstaging (P< 0.0001; P <0.0001), absent/partial tumour regression (P= 0.0002; P= 0.007) and positive radial margin (P= 0.007; P= 0.01) were significant prognostic factors associated with shorter CSS and DFS, respectively (Tables 2 and 3). Craniocaudal extension > 3 cms (P= 0.012), unchanged TNM stage (P < 0.0001), TNM upstaging (P = 0.0005) and PDC G3 (P = 0.0486) were independent prognostic factors for CSS (Table 2). Craniocaudal extension (P= 0.014), TNM stage variation (P = 0.0001) and y stage (P = 0.0045) were independent variables for DFS (Table 3).

DISCUSSION

There is evidence that DFS and CSS to rectal cancer are significantly associated with post-treatment TRG and TNM variation in patients submitted to neo-adjuvant CRT[5,23-26]. For this reason, “watch and wait” approach was suggested to prevent side-effects of surgery in patients with rectal cancer with complete regression after CRT[6]. Tumour regression can be

evaluated through the assessment of post-treatment cTNM staging. However, recognition of clinico-pathological factors associated with complete tumour response may contribute to discriminate patients in whom watch and wait approach could be safely applied from those in whom it should be avoided. Over the years, many studies analyzed the prognostic significance of clinico-pathological variables in rectal cancer submitted to neo-adjuvant CRT and surgery[26-32]. Nonetheless, only few studies investigated the prognostic value of histopathological features in pre-treatment biopsy of rectal cancer[5,33,34]. According to the study performed by Shia and coll.[33] and to our recent findings[5], WHO histological grade in pre-therapy biopsy shows no statistically significant correlation with the CSS or DFS of patients with locally advanced rectal cancer treated with neo-adjuvant CRT. On the other hand, Rogers et al.[34] demonstrated that intra-tumoral budding in pre-treatment biopsy is significantly associated with poor tumour response to neo-adjuvant CRT, and with shorter DFS and CSS as well. Intra-tumoral budding was defined as a single cancer cell or a group of less than 5 detached tumour cells in the stroma of the biopsy specimen[34]. As known, tumour budding is a histological hallmark of epithelial-mesenchymal transition (EMT)[35], a process by which neoplastic cells lose their epithelial properties and acquire the potential of mesenchymal cells to detach, migrate and give rise to metastatic disease[36]. In accordance with the findings reported by Rogers et al.[34], rectal cancer with lower response to CRT has higher expression of biomarkers of EMT and higher incidence of tumour budding in the post-treatment surgical specimen[27]. However, tumour budding in the post-treatment specimen may be confused with tumour fragmentation due to neo-adjuvant treatment and this may bias the results. PDC represent another morphological signature of EMT in CRC[37]. Similarly to tumour budding, PDC are associated with higher incidence of nodal metastases, lymphovascular invasion and bad prognosis in CRC[7-16]. However, as they are composed of at least five neoplastic cells, their identification in haematoxylin and eosin stained slides is

easier, which may account for the higher inter-observer reproducibility in their assessment compared to that of tumour budding[16].

In this study we investigated for the first time the prognostic role of PDC grading in pre-treatment biopsy of rectal cancer submitted to neo-adjuvant CRT. The inter-observer reproducibility in the assessment of PDC grade was good. In addition, we demonstrated that PDC grade in pre-treatment biopsy is significantly associated with high yT stage and yM+ and with unchanged TNM or with TNM upstaging after neo-adjuvant CRT. Indeed, the majority of tumours with PDC G1 had TNM downstaging after therapy, while tumours with PDC G2 and G3 in pre-therapy biopsy more frequently showed unchanged TNM or even TNM upstaging. Although we did not find statistical correlation between TRG and PDC grade, none of the cases graded as PDC G3 had complete pathological regression in the primitive tumour. In addition, none of PDC G3 cases with cN+ status had N downstaging after treatment, and one of them even developed nodal metastases during the treatment. These findings, together with the correlation between PDC grade and TNM variation, suggests that cases with a high number of PDC are more refractory to neo-adjuvant CRT. Besides, the correlation between tumour response to CRT and EMT had already been demonstrated by other authors[27]. Interestingly, PDC grade was not only correlated with tumour response to CRT, but also with CSS and DFS. What's more, it was an independent and significant predictor of CSS in our cohort of patients. In a recent study, PDC grade was applied to surgical specimens obtained from patients with rectal cancer treated with neo-adjuvant CRT and surgery[38]. High PDC grade (G3) was significantly associated with lower tumour regression and with the presence of tumour budding[38]. However, no correlation between PDC grade and recurrence or death from rectal cancer was evidenced[38]. In our opinion, PDC grade in post-CRT specimens may not be reliable because PDC may be difficultly discriminated from glands morphologically altered by post-radiation fibrosis and chemotherapy as well.

This may account for the lack of correlation between PDC grade and clinical outcome in that study[38]. Besides, although the authors used immunohistochemistry against MUC1 to distinguish PDC from glands altered by CRT, this analysis was performed in only 5/72 cases[38].

In conclusion, this study is the first to show that PDC grade in pre-treatment biopsy of rectal cancer submitted to neo-adjuvant CRT is associated with response to therapy and with DSF and CSS as well. Hence its assessment may be a useful tool to identify non-responders to neo-adjuvant CRT and in whom wait and see approach should be avoided. Our findings suggest that PDC grade may be added in the histopathological report of rectal biopsies as a prognostic parameter. Nonetheless, it should be mentioned that, although reproducibility in PDC grading assessment was good, both the observers in this study have experience in PDC recognition and have been working together for several years. Therefore multicentric studies, aimed at further investigation of PDC grading in biopsies of rectal cancer are warranted to definitely establish whether this histological parameter may be used in routine practice.

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Authors contribution to the study

L.R.B. designed the study, revised the histological slides for the assessment of PDC grading, interpreted the results, wrote the paper.

S.L. performed the statistical analysis, interpreted the data, revised the manuscript draft.

F.D. retrieved the clinical data, interpreted the results, revised the manuscript draft.

G.P. retrieved the histological data, interpreted the results, revised the maknuscript draft.

E.M. retrieved the histological data, interpreted the results, revised the manuscript draft.

V.B. designed the study, revised the histological slides for the assessment of PDC grading, performed the statistical analyses, revised the manuscript draft.

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TABLES

Table 1. Statistical correlations between PDC grade in pre-treatment biopsy and other clinico-pathological variables of 204 rectal carcinomas submitted to neo-adjuvant chemo-radiotherapy and surgery.

		PDC grade			*P*
		G1	**G2**	**G3**	
Localization	Upper	70	20	1	
	Medium	24	5	2	
	Lower	54	12	6	
Circumferential spread	Extensive	10	0	0	0.177
	one third	71	15	4	
	middle	45	10	3	
	complete	42	12	2	0.951
Craniocaudal extension	< 3 cms	37	10	1	
	≥ 3 cms	121	27	8	0.599
distance from the anal verge	≥ 1 cm	149	37	8	
	< 1 cm	9	0	1	0.238
WHO Histological grade	1	3	1	0	
	2	132	32	6	
	3	23	4	3	0.535
Histotype	conventional	148	37	7	
	mucinous	10	0	2	**0.0349**
cT	cT2	9	2	0	
	cT3	126	31	6	

	cT4	23	4	3	0.51
cN	cN0	70	18	5	
	cN+	88	19	4	0.738
	yT0	29	6	0	
	yT1	15	3	1	
yT	yT2	49	8	1	
	yT3	58	17	4	
	yT4	7	3	3	**0.0449**
yN	yN0	111	23	3	
	yN+	47	14	6	0.0558
yM	yM0	156	36	7	
	yM+	2	1	2	**0.0004**
	unchanged	49	19	5	
TNM variation	downstaging	96	15	2	
	upstaging	13	3	2	**0.0329**
	unchanged	58	19	5	
T variation	downstaging	99	17	3	
	upstaging	1	1	1	**0.0222**
	unchanged	36	12	5	
N variation	downstaging	51	7	0	
	upstaging	11	2	1	0.071
Radial Margin	negative	147	35	8	
	positive	11	2	1	0.379
	0	17	9	1	
	1	53	14	4	
Dworak Regression grade	2	44	5	2	
	3	15	4	2	
	4	29	5	0	0.235
Regression	absent	114	28	7	
	Total/subtotal	44	9	2	0.862
Status	alive	120	24	3	
	dead	38	13	6	**0.012**
Recurrence	not	112	19	2	
	yes	46	18	7	**0.0017**

Table 2. Statistical correlations between the various clinico-pathological parameters and cancer-specific survival (CSS) of 204 patients with rectal cancer treated through neo-adjuvant chemo-radiotherapy and surgery.

Variables	**Category**	**Univariate**		**Multivariate***	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Localization	Upper	1			
	Medium	1.2 (0.5-2.6)			
	Lower	1.1 (0.6-2)			
	Extensive	1.1 (0.3-4)	0.935		
Circumferential spread	one third	1			
	middle	1 (0-5-1.9)			
	complete	1 (0.5-1.9)	0.988		
Craniocaudal extension	< 3 cms	1		0.3 (0.1-0.9)	
	≥ 3 cms	2.8 (1.5-5.2)	0.01	1	0.028
distance from the anal verge	≥ 1 cm	1			
	< 1 cm	1.2 (0.3-4.4)	0.788		
WHO Histological grade	1	/			
	2	1			
	3	1.7 (0.7-3.6)	0.13		
Histotype	conventional	1			
	mucinous	1.3 (0.4-4.4)	0.534		
PDC grade	G1	1			
	G2	1.2 (0.6-2.4)			
	G3	3.2 (0.8-13.2)	**0.0157**		
cT	uT2	1			
	uT3	3.1 (1-9.6)			
	uT4	5.4 (1.4-19.6)	0.097		
cN	uN0	1			
	uN+	1.4 (0.8-2.3)	0.19		
yT	yT0	1			
	yT1	0.6 (0.2-1.7)			
	yT2	2.6 (1.2-5.5)			
	yT3	6.9 (3.3-14.4)			
	yT4	11.3 (3-42)	<0.0001		
yN	yN0	1		1	
	yN+	2.9 (1.6-5.3)	<0.0001	2.1 (1.2-3.8)	0.006
yM	yM0	1		1	
	yM+	5.4 (0.5-51.7)	0.0002	3.1 (1.1-8.9)	0.03
TNM variation	unchanged	3.6 (2-6.4)			
	downstaging	1			
	upstaging	4.6 (1.5-13.6)	< 0.0001		
T variation	unchanged	2.9 (1.6-5)			

	downstaging	1			
	upstaging	8.9 (0.3-211.6)	<0.0001		
N variation	unchanged	3.3 (1.7-6.5)			
	downstaging	1			
	upstaging	2.8 (0.9-8.8)	0.0018		
Radial Margin	negative	1		1	
	positive	2.8 (0.8-9.5)	0.007	2.3 (1-5.2)	0.04
Dworak Regression grade	Dworak 0	7.4 (2.8-19.3)			
	Dworak 1	4.2 (2-8.8)			
	Dworak 2	5.2 (2.3-11.5)			
	Dworak 3	1.4 (0.5-3.7)			
	Dworak 4	1	0.0015		
Regression	no regression	4.2 (2.4-7.4)		1	
	Total/subtotal regression	1	0.0002	0.3 (0.1-0.8)	0.028

*Only significant variables are indicated in multivariate analysis.

Table 3. Statistical correlations between the various clinico-pathological parameters and disease-free survival (DFS) of 204 patients with rectal cancer treated through neo-adjuvant chemo-radiotherapy and surgery.

Variables	**Category**	**Univariate**		**Multivariate***	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Localization	Upper	1			
	Medium	1 (0.5-2.1)			
	Lower	1 (0.5-1.7)			
Circumferential spread	Extensive	0.9 (0.2-2.8)	0.992		
	one third	1			
	middle	1.1 (0.6-1.9)			
	complete	1 (0.5-1.7)	0.926		
Craniocaudal extension	< 3 cms	1		0.3 (0.1-0.7)	
	≥ 3 cms	2.6 (1.5-4.5)	**0.0067**	1	**0.004**
distance from the anal verge	≥ 1 cm	1			
	< 1 cm	1.5 (0.4-4.8)	0.556		
WHO Histological grade	1	/			
	2	1			
	3	1.3 (0.3-2.6)	0.276		
Histotype	conventional	1			
	mucinous	0.8 (0.3-2.1)	0.774		
PDC grade	G1	1			
	G2	1.6 (0.8-2.9)			

	G3	2.7 (0.8-8.6)	**0.013**		
cT	uT2	1			
	uT3	2.8 (1.2-6.7)			
	uT4	4.3 (1.5-12.3)	0.061		
cN	uN0	1			
	uN+	1.3 (0.8-2.1)	0.241		
yT	yT0	1			
	yT1	0.2 (0.09-0.5)		0.1 (0.01-0.8)	**0.03**
	yT2	1 (0.5-2)		0.5 (0.2-0.9)	**0.03**
	yT3	2.7 (1.4-5.3)		1	
	yT4	5.8 (1.7-19.5)	**<0.0001**	2.3 (1.1-4.4)	**0.02**
yN	yN0	1		1	
	yN+	2.7 (1.6-4.6)	**<0.0001**	2.1 (1.3-3.5)	**0.028**
yM	yM0	1			
	yM+	2.8 (0.6-12.4)	**0.0107**		
TNM variation	unchanged	2.9 (1.7-4.9)			
	downstaging	1			
	upstaging	3 (1.2-7.7)	**<0.0001**		
T variation	unchanged	2.4 (1.5-3.9)			
	downstaging	1			
	upstaging	21.8 (0.3-1404)	**< 0.0001**		
N variation	unchanged	1.3 (0.4-3.9)			
	downstaging	1			
	upstaging	2.4 (0.8-6.8)	**0.0007**		
Radial Margin	negative	1			
	positive	2.3 (0.8-6.7)	**0.018**		
Dworak Regression grade	Dworak 0	3.2 (1.3-7.6)			
	Dworak 1	1.7 (0.9-3.5)			
	Dworak 2	1.8 (0.8-3.7)			
	Dworak 3	0.7 (0.3-1.7)			
	Dworak 4	1	**0.0155**		
Regression	no regression	2.2 (1.3-3.7)			
	Total/subtotal regression	1	**0.007**		

*Only significant variables are indicated in multivariate analysis.

FIGURE LEGENDS

Figure 1. Pre-treatment endoscopic biopsies of rectal cancer showing PDC G2 (A) and (B) PDC G3 (Haematoxylin and eosin stain; original magnification. x200). C. PDC surrounded by extracellular mucin in PDC G3 mucinous carcinoma. Green circles highlight PDC (Haematoxylin and eosin stain; original magnification. x200).

Figure 2. a. Pre-treatment endoscopic biopsy of rectal cancer. showing PDC (green circles) and fragmented glands (red circles). In some cases it was impossible to distinguish between PDC and fragmented glands (black circles) (Haematoxylin and eosin stain; original magnification, x100) b. Higher magnification of PDC (green circles) and clusters difficult to classify as PDC or rather as fragmented glands (Haematoxylin and eosin stain; original magnification, x200). c. Higher magnification of fragmented glands, showing milder nuclear atypia and architectural distorsion compared to PDC (Haematoxylin and eosin stain; original magnification, x200)

Figure 3. Kaplan-Meier curves showed that cancer specific survival (CSS) was significantly shorter in patients with rectal carcinoma classified as PDC G3 in pre-treatment biopsy.

Figure 4. Kaplan-Meier curves showed that disease-free survival (DFS) was significantly shorter in patients with rectal carcinoma classified as PDC G3 in pre-treatment biopsy.