Carbonic Anhydrase IX, Hypoxia-inducible Factor-1α, Ezrin and Glucose Transporter-1 as Predictors of Disease Outcome in Rectal Cancer: Multivariate Cox Survival Models following Data Reduction by Principal Component Analysis of the Clinicopathological Predictors

EIJA A. KORKEILA[1], JARI SUNDSTRÖM[2], SEPPO PYRHÖNEN[1] and KARI SYRJÄNEN[1]

[1]*Department of Oncology and Radiotherapy, University of Turku and Turku University Hospital, Turku, Finland;*
[2]*Department of Pathology, University of Turku and Turku University Hospital, Turku, Finland*

Abstract. *Strong expression of carbonic anhydrase IX (CAIX), hypoxia-inducible factor-1α (HIF-1α), ezrin and glucose transporter-1 (GLUT-1) was previously shown to be related to adverse disease outcome in rectal cancer. In this study, operative samples of 178 rectal cancer patients 77 treated with short-course and 47 with long-course preoperative radiotherapy (RT), and 54 with no preoperative treatment, as well as 80 preoperative biopsies from the RT group patients were analyzed for these markers. For data reduction, principal component analysis (PCA) was used to extract a set of factors from the original clinicopathological variables that would explain as much as possible of their variance. After extraction and promax rotation, this set of five first-order factors (F1-F5) was used in multivariate (Cox) modeling together with the four biomarkers. In model 1 (biomarkers in operative samples), F1 was the only independent determinant of disease-free (DFS) (p=0.043) and disease-specific survival (DSS) (p=0.029). In model 2 (biomarkers in preoperative biopsies), none of the five factors or biomarkers were significantly associated with DFS. However, HIF-1α (p=0.024), ezrin (p=0.034), F1 (p=0.011), and F3 (p=0.001) were significant independent predictors of DSS. Similarly, in model 3 (ezrin in preoperative biopsies and others in operative samples), none of the factors or biomarkers were significant predictors of DFS. However, CAIX (p=0.028), and F1 (p= 0.017) were significantly associated with DSS. Preoperative RT markedly modifies the expression of these four biomarkers and also interferes with the original clinicopathological prognosticators (loaded to F1-F5), emphasizing the complexity of prognostication in rectal cancer.*

Correspondence to: Eija Korkeila, Department of Oncology and Radiotherapy, Turku University Hospital, Hämeentie 11, PB 52, FIN-20521 Turku, Finland. Tel: +35 823152803, Fax: +35 823132850, e-mail: eija.korkeila@tyks.fi or eijakorkeila@gmail.com

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The most important prognostic factors of rectal cancer include the number of metastatic and examined lymph nodes, the depth of tumor invasion and the involvement of the circumferential margin (CRM) (1-3). Local disease recurrences have a detrimental effect on the patient's quality of life and survival (4, 5). Consequently, macroscopical and microscopical radical resection is a cornerstone in the management of rectal cancer (6).

Removal of the mesorectum intact by total mesorectal excision technique (TME) reduces the amount of local recurrences (7). Preoperative treatment is shown to further improve local disease outcome even after TME is utilized (8). Preoperative short-course RT is generally used in the treatment of T3 (stage IIA) rectal cancer, whereas long-course RT with concomitant chemotherapy is recommended for locally advanced T4 (stage IIB) tumors or tumors with predicted CRM involvement (9).

Tumor hypoxia is associated with resistance to radio- and chemotherapy (10, 11). In addition, overexpression of markers linked with hypoxia is coupled with poor disease outcome (12, 13). Hypoxia starts a cascade of events, leading to the stabilization of hypoxia-inducible factor-1α (HIF-1α) and the activation of several genes involved in angiogenesis, glycolysis, invasion, metastasis, apoptosis, pH regulation and growth factor signaling (14,15). The down-stream targets of HIF-1α include carbonic anhydrase IX (CAIX) and glucose transporter-1 (GLUT-1) (14, 15). In several types of cancers, mammalian target of rapamycin (mTOR) is a key regulator of HIF-1α protein synthesis (16). As a cross-linker between the cell membrane and the cytoskeleton (17, 18) ezrin is a crucial

protein for metastatic behavior in cancer (19). Ezrin-related metastatic behavior is in turn found to be associated with mTOR pathway (17). Blocking mTOR is shown to inhibit experimental lung metastasis (20). Thereby, HIF-1α, mTOR and ezrin are interrelated.

In rectal cancer, strong expression of HIF-1α is linked to shorter overall survival and inferior local disease control (13). We have previously reported the effect of CAIX, HIF-1α, ezrin and GLUT-1 expression on disease outcome in rectal cancer treated with preoperative RT or chemoradiotherapy (21, 24). Since these biomarkers are interrelated, their correlations with common prognostic markers were analyzed here in multivariate (Cox) analyses for disease-free (DFS) and disease-specific survival (DSS).

Patients and Methods

Patients. The study population consisted of 178 consecutive patients with rectal cancer treated according to the standard treatment protocols at Turku University Hospital in 2000-2008. Patients with upper rectal cancer, superficial tumors operated by excision and those with metastatic disease were excluded from the study. Patients were treated either with short-course (5×5 Gy) (n=77) or long-course RT (50.40 Gy) (n=47), or no treatment preoperatively (n=54). Long-course RT was given with (n=37) or without chemotherapy (n=10). Treatment was based on the stage and localization of the tumor, as decided by the multidisciplinary team. In addition, 80 preoperative biopsies from the patients who had received preoperative short- or long-course RT were studied. Postoperative adjuvant chemotherapy was given to patients with lymph node-positive disease or high-risk lymph node-negative disease, as well as postoperative adjuvant chemotherapy or chemoradiotherapy to eligible control group patients. After completion of the treatment protocols, patients were scheduled for follow-up visits at the Department of Surgery. The median and mean follow-up times of the patients were 35 months and 40 months (range 2-114 months, respectively). The patient population is described in detail in our earlier publications of the same cohort (21-24).

The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland and the collection and use of archival tissue material by the National Authority for Medico-Legal affairs. The study was conducted in accordance with the Declaration of Helsinki.

Methods. Immunohistochemistry of the samples was evaluated by two observers (EK and JS), blinded to all clinical data and radiological findings. The immunohistochemical staining procedures and the analyses of CAIX, HIF-1α, ezrin and GLUT-1 are described in detail in our earlier publications of our group (21-24).

Statistical analysis. Statistical analyses were carried out using two statistical software packages: IBM SPSS 18.03 for Windows (IBM, NY, USA) and STATA/SE 11.1 software (STATA Corp., TX, USA). A wide variety of clinical variables were first tested in factor analysis to reduce the number of potential predictors to be used in the subsequent multivariate models together with the four markers (Table I). Firstly, principal component analysis (PCA) was used for the extraction of the initial factors (SPSS). Only factors with eigen values ≥1.0 were retained in analysis. The initial factors were then rotated. The analyses were performed using both a varimax and a promax rotation to explore the underlying pathogenetic relationships. By definition, the varimax rotation generates uncorrelated factors, which might simplify the interpretation of the factors, but may not be biologically feasible. Therefore, we also carried out a promax rotation, which generates correlated factors, which are biologically meaningful in the multivariate models (25). In the next step, the extracted factors were interpreted for their loadings of the original variables, using the recommended cut-offs for factor loadings (coefficients) as follows: variables with loadings ≥0.60 were considered to be heavily loaded by a particular clinical variable, those with loadings 0.40-0.59 were considered moderately loaded, and those with co-efficients 0.3-0.39 were modestly loaded (Tables II and III).

Table I. *Clinical variables entered in the principal component analysis*.

Preoperative tumor extent (T)
Postoperative T
Postoperative stage
Postoperative tumor differentiation grade (G)
Postoperative lymph node status (N)
Number of metastatic lymph nodes (LN)
Number of examined lymph nodes (LN)
Preoperative carcinoembryonic antigen (CEA)
Circumferential margin (CRM), <1 mm/≥1 mm
Vessel invasion, yes/no
Necrosis, yes/no
Vital cancer cells, yes/no
Type of operation, anterior resection/abdominoperineal resection
Operation before/after year 2005
Postoperative CEA

In PCA analysis, the scores of the rotated factors were saved (separately after varimax and promax rotations) into the SPSS file and used as variables factors 1-5 (F1-F5) in the multivariate models run in STATA. To adjust for all covariates (four biomarkers and F1-F5), Cox proportional hazards regression models were constructed separately for promax and varimax-rotated factors 1-5, by using three different approaches (models) for the four biomarkers. The expression of ezrin in the preoperative biopsies and the expression of all the other markers in the operative samples were significantly associated with disease outcome, as reported in the original publications (21-24). For this reason, the three models in the current analysis were: i) all four biomarkers (CAIX, HIF-1α, GLUT-1, ezrin) analyzed in the operative samples; ii) all marker data analyzed in the preoperative biopsy samples; and iii) ezrin analyzed in the preoperative samples and the others in the operative samples. All Cox proportional hazards models were run in STATA using robust variance estimator to calculate the 95% confidence intervals (95% CI) for the hazard ratios (HR). The validity of the proportional hazards assumption was tested graphically using log−minus−log plots for all binary covariates (independent variables). All statistical tests were two-sided and declared significant at a *p*-value of <0.05 level.

Results

Factor analysis. To derive uncorrelated factors, we first carried out factor analysis of all the clinical variables listed in Table I, by extracting only the factors with an eigen value ≥1 and using a varimax rotation. The results are shown in Table II. Five

Table II. *Correlation coefficients of the original clinical variables with the 5 factors derived from extraction and varimax rotation.*

Original clinical factor	Varimax-rotated factor				
	1 (F1)	2 (F2)	3 (F3)	4 (F4)	5 (F5)
Preoperative T	–0.077	0.140	0.247	**0.755*****	–0.137
Postoperative T	0.530**	0.558**	0.139	0.172	0.167
Postoperative G	–0.026	**0.740*****	–0.057	–0.105	0.083
Number of LN involved (4 cut-off)	–0.420**	–0.519**	0.024	0.504**	0.055
Number of LN examined (12 cut-off)	–0.066	0.061	**–0.691*****	0.021	0.013
Postoperative LN status (N0/ *vs*. N1/N2)	**0.927*****	0.056	–0.055	–0.084	–0.040
Postoperative stage	**0.944*****	0.112	0.065	0.045	0.100
Tumor necrosis	–0.213	0.091	0.432**	–0.674***	–0.012
Vital cancer tissue left	0.046	**0.736*****	–0.332*	0.153	–0.071
CRM (1 m cut-off)	–0.395*	–0.558**	–0.221	–0.015	–0.030
Vascular invasion	–0.260	–0.217	0.054	–0.004	**–0.648*****
Year of diagnosis/therapy (2005 cut-off)	0.010	–0.049	0.825***	0.075	–0.022
Type of operation	–0.135	–0.107	0.011	–0.104	0.870***

Factor loadings: *0.3-0.39 modest factor loading; **0.4-0.59 moderate loading; ***>0.6 heavy loading (bold); [1]with Kaiser normalization. T, tumor extent; G, tumor differentiation grade; LN lymph nodes; CRM, circumferential margin.

Table III. *Correlation coefficients of the original clinical variables with the 5 factors derived from extraction and promax rotation.*

Original clinical factor	Promax-rotated factor				
	1 (F1)	2 (F2)	3 (F3)	4 (F4)	5 (F5)
Preoperative T	–0.142	0.174	0.283	0.771***	–0.112
Postoperative T	0.403**	0.485**	0.147	0.192	0.135
Postoperative G	–0.237	**0.819*****	–0.060	–0.082	0.068
Number of LN involved (4 cut-off)	–0.325*	–0.462**	0.049	0.494**	0.103
Number of LN examined (12 cut-off)	–0.066	0.110	**–0.693*****	0.002	0.010
Post-operative LN status (N0 vs. N1/N2)	**0.999*****	–0.157	–0.063	–0.093	–0.094
Postoperative stage	**0.988*****	–0.102	0.064	0.043	0.049
Tumor necrosis	–0.258	0.124	0.403**	–0.657***	–0.018
Vital cancer tissue left	–0.146	**0.811*****	–0.326*	0.167	–0.084
Circumferential margin (1 mm cut-off)	–0.262	–0.511**	–0.221	–0.038	–0.002
Vascular invasion	–0.193	–0.183	0.053	–0.011	**–0.632*****
Year of diagnosis/therapy (2005 cut-off)	–0.003	–0.087	**0.832*****	0.100	–0.013
Type of operation	–0.155	–0.082	0.009	–0.101	**0.879*****

Factor loadings: *0.3-0.39 modest factor loading; **0.4-0.59 moderate loading; ***>0.6 heavy loading (bold); [1]with Kaiser normalization. T, tumor extent; G, tumor differentiation grade; LN lymph nodes; CRM, circumferential margin.

factors were obtained. Altogether, these five factors explained 67.7% of the total variance. The factor (F1) explaining the greatest variance, 24.3%, was most heavily loaded by two variables: postoperative stage (0.944) and postoperative lymph node status (0.927). The second factor (F2) which explained 12.9% of the variance, showed the highest loading of postoperative tumor grade (0.740) and the remaining vital cancer tissue (0.736). The third factor (F3) explaining 11.5% of the variance, had the highest factor loadings by the year of therapy (2005 cut-off) (0.825) and number of lymph nodes examined (–0.691; negative loading). F4 explained 10.2% of the variance and was significantly loaded by preoperative tumor extent (0.755) and a negative loading by tumor necrosis (–0.674). The final factor (F5) explained 8.6% of the variance and had the highest loading by the type of surgical operation (0.870), and a negative loading (–0.648) by vascular invasion. As evident in Table II, several additional variables showed at least moderate or modest loadings to F1-F5, both positive and negative. By definition, these factors were completely uncorrelated (r=1.000 for all correlations).

Table IV. *Predictors of disease-free survival (DFS) in multivariate (Cox) survival analysis.*

Covariate	Hazard ratio	Significance	95% Confidence interval
Model 1:			
CAIX	1.20	0.80	0.27-5.26
HIF-1α	0.83	0.78	0.22-3.11
GLUT-1	0.87	0.83	0.24-3.09
Ezrin	0.97	0.96	0.23-4.00
Factor 1	1.81	0.04	1.02-3.20
Factor 2	1.05	0.78	0.73-1.53
Factor 3	1.25	0.51	0.64-2.45
Factor 4	1.25	0.42	0.72-2.16
Factor 5	0.93	0.81	0.50-1.73
Model 2:			
CAIX	1.18	0.80	0.33-4.19
HIF-1α	3.42	0.27	0.39-30.29
GLUT-1	2.47	0.45	0.23-26.51
Ezrin	0.62	0.41	0.20-1.91
Factor 1)	1.51	0.36	0.62-3.68
Factor 2	0.81	0.54	0.41-1.60
Factor 3	1.85	0.35	0.51-6.66
Factor 4	1.40	0.41	0.63-3.10
Factor 5	0.46	0.23	0.13-1.63
Model 3:			
CAIX	1.57	0.63	0.26-9.52
HIF-1α	0.37	0.45	0.03-4.75
GLUT-1	0.99	0.99	0.58-16.71
Ezrin	0.67	0.77	0.44-10.19
Factor 1	1.22	0.51	0.68-2.20
Factor 2	1.04	0.86	0.69-1.55
Factor 3	1.81	0.20	0.74-4.45
Factor 4	1.68	0.44	0.46-6.15
Factor 5	0.53	0.32	0.16-1.82

Model 1: All biomarkers analyzed in operative samples; Model 2: biomarkers analyzed in pre-operative biopsies; Model 3: ezrin in preoperative, all others in operative specimens; all 5 factors were generated by promax rotation (*i.e.* correlated factors).

Table V. *Predictors of disease-specific survival (DSS) in multivariate (Cox) survival analysis.*

Covariate	Hazard ratio	Significance	95% Confidence interval
Model 1			
CAIX	3.56	0.15	0.63-20.1
HIF-1α	0.36	0.32	0.96-2.16
Ezrin	0.91	0.91	0.17-4.76
GLUT-1	0.54	0.51	0.83-3.46
Factor 1	2.23	0.029	1.08-4.59
Factor 2	1.09	0.67	0.73-1.63
Factor 3	1.72	0.11	0.88-3.35
Factor 4	0.81	0.41	0.49-1.33
Factor 5	0.69	0.36	0.31-1.4
Model 2			
CAIX	3.25	0.08	0.86-12.30
HIF-1α	4287.04	0.024	3.051-6022850
Ezrin	4051.83	0.034	1.88-8729454
GLUT-1	0.45	0.43	0.061-3.29
Factor 1	13.63	0.011	1.80-103
Factor 2	0.75	0.62	0.24-2.36
Factor 3	8.89	0.001	2.39-33,08
Factor 4	0.0027	0.064	0.00000054-1.41
Factor 5	0.48	0.62	0.028-8.47
Model 3			
CAIX	5.37	0.028	1.19-24.13
HIF-1α	0.36	0.58	0.010-24.13
Ezrin	8.23	0.17	0.41-166.61
GLUT-1	0.28	0.45	0.011-7.35
Factor 1	2.15	0.017	1.15-4.01
Factor 2	1.44	0.063	0.98-2.10
Factor 3	2.08	0.13	0.81-5.33
Factor 4	0.21	0.055	0.043-1.03
Factor 5	0.55	0.16	0.24-1.27

Model 1: All biomarkers analyzed in operative samples; Model 2: biomarkers analyzed in preoperative biopsies; Model 3: ezrin in preoperative, all others in operative specimens; all 5 factors were generated by promax rotation (*i.e.* correlated factors).

Because uncorrelated factors may not be biologically meaningful (25), we repeated the same analyses using the promax rotation, as shown in Table III. The same five factors with identical variances were obtained, and the loadings of the individual variables on the factors differed only slightly from those generated by the varimax rotation, the correlation coefficients being invariably somewhat higher with the promax rotation. Of these promax-rotated factors, F1 and F2 were significantly (p=0.0001) correlated (r=0.451), while the others were not (r ranged from –0.005 to 0.1, p>0.05).

A second-order factor analysis of the factors derived using the promax rotation was carried out. Two factors explaining 51.2% of the variance were derived. Out of these second-order factors, factor 1 was heavily loaded by the first-order F1 (0.850), and F2 (0.828), whereas the second-order factor 2 had the highest loading by first-order F4 (0.767), and a negative loading by first-order F3 (–0.696). These two second-order factors had modest to high (positive or negative) loadings by most of the original clinical variables (data not shown). The two second-order factors were no longer correlated to each other anymore (r=–0.31, p=0.791).

Multivariate Cox analysis. The results of multivariate Cox survival analysis for DFS and DSS are shown in Tables IV and V, separately for the three models and using only promax-rotated factors. In model 1 (biomarkers in operative samples), F1 is the only independent determinant of DFS (p=0.043) and also DSS (p=0.029). In model 2 (markers in preoperative biopsies), none of the five factors or biomarkers were significantly associated with DFS. However, HIF-1α (p=0.024), ezrin (p=0.034), F1 (p=0.011), and F3 (p=0.001) were significant independent predictors of DSS. Similarly, in

model 3 (ezrin in preoperative biopsies and other markers in operative samples), none of the factors or biomarkers were significant predictors of DFS. However, CAIX (p=0.028), and F1 (p=0.017) were significantly associated with DSS.

Discussion

In the current study we analyzed four hypoxia-related biomarkers: CAIX, HIF-1α, ezrin and GLUT-1 in a cohort of 178 rectal cancer patients treated by preoperative RT or chemoradio-therapy. CAIX intensity was the only independent predictor of DSS (21). In the long-course RT group, negative HIF-1α and negative/weak ezrin expression were associated with favorable DSS (22). Negative/weak ezrin expression in the biopsy was also linked to longer DSS as compared with moderate/strong expression (23). Likewise, a trend for longer DFS in favor of negative/weak GLUT-1 staining was observed among patients in the long-course RT group (24).

HIF-1α, CAIX, ezrin and GLUT-1 are, however, biologically interrelated. CAIX and GLUT-1 are downstream targets of HIF-1α (14, 15, 26). Activation of receptor tyrosine kinases including protein kinase B (AKT), increases mTOR activity (16, 17), which in turn induces HIF-1α (16, 28, 29). Rapamycin, a protein kinase that inhibits mTOR activity, is found to regulate HIF-1α in hypoxia (30). Thus, mTOR is a key regulator of HIF-1α protein synthesis in many cancers (16). Ezrin-related metastatic behavior is shown to be linked to the mTOR pathway (19). When the mTOR pathway is blocked by rapamycin, experimental lung metastasis is inhibited *in vivo* (19). Thereby, the regulation of mTOR, HIF-1α, CAIX and GLUT-1 are interrelated and univariate analysis of the individual markers is of limited power to disclose these interrelations.

In addition to analyzing individual biomarkers separately, other approaches are needed to explore their interactions and particularly their role as independent predictors of disease outcome together with the classical prognostic factors. The latter include: postoperative tumor stage, tumor size and differentiation, lymphovascular invasion, positive circumferential margin, examination of less than 12 lymph nodes, and type of operation (1, 2, 31-33). In the current cohort, several other clinicopathological variables (*e.g.* number of metastatic lymph nodes, and disease recurrence) were also recorded and originally analyzed as potential predictors of disease outcome (21, 24). Together with the four biomarkers, however, the number of all these covariates is too large to rend direct multivariate modeling feasible. A useful option is to attempt data reduction using PCA to generate a set of factors that explain as much as possible of the variance by the original covariates (=clinicopathological variables). This set of factors (*i.e.* their saved scores) can then be directly used in multivariate modeling together with the four markers of interest.

PCA was used in the present study to elucidate the role of these four biomarkers as independent predictors of disease outcome in rectal cancer. The five factors generated by PCA explained almost 70% of the total variance of the original variables subjected to extraction. These five factors also located in the steep slope of the plot, and the rest of the factors located on the shallow slope did not contribute much to the solution. The extracted factors were subjected to varimax and promax rotation to generate uncorrelated and correlated factors, respectively. Although the generation of these five factors by PCA was relatively straightforward, the complexity of the interplay between the original clinicopathological variables is well illustrated by the fact that all these five factors were heavily loaded by more than one original variable. For this reason, these five factors were not given any specific names in order to avoid confusion with the original variables.

By definition, varimax-rotated factors showed no correlation (r=0.000, p=1.000), while promax-rotated factors should be correlated. In our case, this linear correlation (Pearson r) was observed only between F1 and F2 (r=0.451, p=0.0001), implicating a close interrelationship between the original variables loaded into these two first-order factors. In all subsequent analyses, only the five promax-rotated factors were used in order to be able to exploit their complex biological interrelationships. In further analysis, we also generated second-order factors by performing promax rotation for the promax-rotated first-order factors F1-5 (data not shown). Using the same eigen value cut-off (≥1), two second-order factors were generated, showing heavy loadings by the first-order factors F1, F2, F4 and F3, but much less by F5. Not unexpectedly, the two second-order factors were no longer linearly correlated (r=–0.31, p=0.791), reflecting the fact that out of the first-order factors F1-5, only F1 and F2 were significantly correlated.

In multivariate Cox analysis, on the operative samples, F1 was the only independent predictor of DFS and DSS. On the preoperative biopsies, no factor or biomarker predicted DFS, whereas F1 and F3 as well as HIF-1α and ezrin were significantly linked with DSS. When ezrin in the preoperative biopsies and the other biomarkers in the operative samples were analyzed, none of the factors or biomarkers were significantly associated with DFS. However, CAIX and F1 predicted DSS. Deviating results in Cox analyses for the three models would most feasibly be explained by given preoperative treatment. RT may shrink the tumor (34), delivering complete remission in 0-29% of tumors (35-37). Decreased tumor size may alleviate hypoxia and thereby alter the expression of hypoxia-associated biomarkers. RT can also affect the tumor microenvironment (38) or cause direct damage in DNA replication (39). To circumvent this problem, preoperative biopsies as well as tissue samples from patients who had not received preoperative RT were used as controls. However, pre-treatment biopsies may represent only a small portion of the

tumor, not enabling the determination of the differentiation grade. Furthermore, preoperative tumor assessment may only be directional due to the limitations of imaging methods (40). Postoperative tumor staging can in turn be affected by RT, which may also influence the results. It would have been advisable to analyze separately the operative samples from the control group of patients (who had not received any treatment prior to surgery). However, the control group was too small to be analyzed alone, and thereby we had to settle for analyzing the pre-RT biopsies.

In the original studies, we were unable to find any significant correlations between the biomarkers and common clinicopathological factors, which may partly be due to the impact of RT. Postoperative tumor stage is known to be the most important clinical prognostic factor of disease outcome (2, 41). In the current PCA analyses, F1 was most heavily loaded by postoperative stage and lymph node status. Thus, it is not unexpected that F1 was singled out as the only significant prognosticator of disease outcome in all three models. F3, most heavily loaded by the number of examined lymph nodes and the year of therapy was significantly associated with DSS only when analyzed in the preoperative biopsies. In our institution, a multidisciplinary team has been active since 2005, in parallel with the introduction of a structured pathology report, bringing about improved quality control and feedback to the radiologists, surgeons, pathologists and oncologists. In fact, at least twelve lymph nodes were examined in 69% of the patients after and including year 2005, whereas this was the case in only 31% operated on before 2005 (unpublished data). The retrieval of lymph nodes after long-course RT may be complex, especially after excellent tumor response (42). Not unexpectedly, the number of examined lymph nodes and the year of therapy seem to be clearly interconnected in this study, reflecting the importance of the acuity in lymph node retrieval.

The present study showed that HIF-1α and ezrin as well as CAIX, but not GLUT-1, significantly predicted DSS in multivariate Cox models, adjusted for the first-order factors 1-5. This is in line with the original studies analyzing each of these markers separately, showing strong expression of these biomarkers to be associated with adverse disease outcome (21-24). The current data implicate their predictive value should not be confounded with the classical prognosticators. Further analyses are ongoing in order to examine the interactions between HIF-1α, CAIX, ezrin and GLUT-1 in full factorial models. It remains to be seen whether any panels can be established and be proven useful as independent predictors of disease outcome.

Taken together, factor analysis using PCA of a multitude of clinicopathological prognosticators generated five first-order factors which proved to be variably loaded by most of the original variables. F1 was the only significant predictor of DFS and DSS in model 1. In model 2, HIF-1α, ezrin, F1 and F3 were significantly associated with DSS. In model 3, DSS was significantly predicted by CAIX and F1. Preoperative RT appears to markedly modify the expression of the studied biomarkers and also interfere with the original clinicopathological prognosticators, emphasizing the complexity of prognostication in rectal cancer.

Conflicts of Interest

The Authors declare no conflicts of interest.

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References

1 Dukes C and Bussey H: The spread of rectal cancer and its effect on prognosis. Br J Cancer *12*: 309-320, 1958.
2 Swanson R, Compton C, Stewart A and Bland K: The prognosis of T3N0 colon cancer is dependent on the number of lymph nodes examined. Ann Surg Oncol *10*: 65-71, 2003.
3 Nagtegaal I and Quirke P: What is the role for the circumferential margin in the modern treatment of rectal cancer? J Clin Oncol *26*: 303-312, 2008.
4 Kapiteijn E, Marijnen C, Nagtegaal I, Putter H, Steup W, Wiggers T, Rutten H, Pahlman L, Glimelius B, van Krieken J, Leer J and van de Velde C: Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N Engl J Med *345*: 638-646, 2001.
5 Marijnen C, Nagtegaal I, Kapiteijn E, Kranenbarg E, Noordijk E, van Krieken J, van de Velde C and Leer J: Radiotherapy does not compensate for positive resection margins in rectal cancer patients: report of a multicenter randomized trial. Int J Radiat Oncol Biol Phys *55*: 1311-1320, 2003.
6 Meredith K, Hoffe S and Shibata D: The multidisciplinary management of rectal cancer. Surg Clin North Am *89*: 177-215, 2009.
7 Heald R, Husband E and Ryall R: The mesorectum in rectal cancer surgery – The clue to pelvic recurrence? Br J Surg *69*: 613-616, 1982.
8 Peeters K, Marijnen C, Nagtegaal I, Kranenbarg E, Putter H, Wiggers T, Rutten H, Pahlman L, Glimelius B, Leer J and van de Velde C: The TME trial after a median follow-up of 6 years: increased local control but no survival benefit in irradiated patients with resectable rectal carcinoma. Ann Surg *246*: 693-701, 2007.
9 Glimelius B, Påhlman L and Cervantes A, Group EGW. Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol *21*: 82-86, 2010.
10 Höckel M, Schlenger K, Mitze M, Schäffer U and Vaupel P: Hypoxia and radiation response in human tumors. Semin Radiat Oncol *6*: 3-9, 1996.
11 Brennan D, Jirstrom K, Kronblad A, Millikan R, Landberg G, Duffy M, Rydén L, Gallagher W and O'Brien S: CAIX is an independent prognostic marker in premenopausal breast cancer patients with one to three positive lymph nodes and a putative marker of radiation resistance. Clin Cancer Res *12*: 6421-6431, 2006.

12 Hoskin P, Sibtain A, Daley F and Wilson G: GLUT1 and CAIX as intrinsic markers of hypoxia in bladder cancer: relationship with vascularity and proliferation as predictors of outcome of ARCON. Br J Cancer *89*: 1290-1297, 2003.
13 Lu X, Xing C, Feng Y, Chen J and Deng C: Clinical significance of immunohistochemical expression of hypoxia-inducible factor-1alpha as a prognostic marker in rectal adenocarcinoma. Clin Colorectal Cancer *5*: 350-353, 2006.
14 Harris A: Hypoxia – a key regulatory factor in tumour growth. Nat Rev Cancer *2*: 38-47, 2002.
15 Semenza G: Targeting HIF-1 for cancer therapy. Nat Rev Cancer *3*: 721-732, 2003.
16 Semenza G: Evaluation of HIF-1 inhibitors as anticancer agents. Drug Discov Today *12*: 853-859, 2007.
17 Bretscher A, Reczek D and Berryman M: Ezrin: a protein requiring conformational activation to link microfilaments to the plasma membrane in the assembly of cell surface structures. J Cell Sci *110*: 3011-3018, 1997.
18 Algrain M, Turunen O, Vaheri A, Louvard D and Arpin M: Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. J Cell Biol *120*: 129-139, 1993.
19 Wan X, Mendoza A, Khanna C and Helman L: Rapamycin inhibits ezrin-mediated metastatic behavior in a murine model of osteosarcoma. Cancer Res *65*: 2406-2411, 2005.
20 Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A, Yeung C, Gorlick R, Hewitt S and Helman L: The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. Nat Med *10*: 182-186, 2004.
21 Korkeila E, Talvinen K, Jaakkola P, Minn H, Syrjänen K, Sundström J and Pyrhönen S: Expression of carbonic anhydrase IX suggests poor outcome in rectal cancer. Br J Cancer *100*: 874-880, 2009.
22 Korkeila E, Jaakkola P, Syrjänen K, Sundström J and Pyrhönen S: Preoperative radiotherapy down-regulates the nuclear expression of hypoxia-inducible factor-1alpha in rectal cancer. Scand J Gastroenterol *45*: 340-348, 2010.
23 Korkeila EA, Syrjänen K, Bendardaf R, Laulajainen M, Carpén O, Pyrhönen S and Sundström J: Preoperative radiotherapy modulates ezrin expression and its value as a predictive marker in patients with rectal cancer. Hum Pathol *42*: 384-392, 2011.
24 Korkeila E, Jaakkola PM, Syrjänen K, Pyrhönen S and Sundström J: Pronounced tumour regression after radiotherapy is associated with negative/weak glucose transporter-1 expression in rectal cancer. Anticancer Res *31*: 311-315, 2011.
25 Laaksonen DE, Niskanen L, Nyyssönen K, Lakka TA, Laukkanen JA and Salonen JT: Dyslipidaemia as a predictor of hypertension in middle-aged men. Eur Heart J *29*: 2561-2568, 2008.
26 Yeh C, Wang J, Chung F, Lee S, Huang M, Kuo C, Yang M and Lin S: Significance of the glycolytic pathway and glycolysis related-genes in tumorigenesis of human colorectal cancers. Oncol Rep *19*: 81-91, 2008.
27 Sekulić A, Hudson C, Homme J, Yin P, Otterness D, Karnitz L and Abraham R: A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Res *60*: 3504-3513, 2000.
28 Semenza G: Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Crit Rev Biochem Mol Biol *35*: 71-103, 2000.
29 Laughner E, Taghavi P, Chiles K, Mahon P and Semenza G: HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol *21*: 3995-4004, 2001.
30 Hudson C, Liu M, Chiang G, Otterness D, Loomis D, Kaper F, Giaccia A and Abraham R: Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. Mol Cell Biol *22*: 7004-7014, 2002.
31 Nagtegaal I, Marijnen C, Kranenbarg E, van de Velde C and van Krieken J: Circumferential margin involvement is still an important predictor of local recurrence in rectal carcinoma: not one millimeter but two millimeters is the limit. Am J Surg Pathol *26*: 350-357, 2002.
32 Compton C, Fenoglio-Preiser C, Pettigrew N and Fielding L: American Joint Committee on cancer prognostic factors consensus conference: Colorectal Working Group. Cancer *88*: 1739-1757, 2000.
33 Haward RA, Morris E, Monson JR, Johnston C and Forman D: The long term survival of rectal cancer patients following abdominoperineal and anterior resection: results of a population-based observational study. Eur J Surg Oncol *31*: 22-28, 2005.
34 Ruo L, Tickoo S, Klimstra D, Minsky B, Saltz L, Mazumdar M, Paty P, Wong W, Larson S, Cohen A and Guillem J: Long-term prognostic significance of extent of rectal cancer response to preoperative radiation and chemotherapy. Ann Surg *236*: 75-81, 2002.
35 Moore H, Gittleman A, Minsky B, Wong D, Paty P, Weiser M, Temple L, Saltz L, Shia J and Guillem J: Rate of pathologic complete response with increased interval between preoperative combined modality therapy and rectal cancer resection. Dis Colon Rectum *47*: 279-286, 2004.
36 Vironen J, Juhola M, Kairaluoma M, Jantunen I and Kellokumpu I: Tumour regression grading in the evaluation of tumour response after different preoperative radiotherapy treatments for rectal carcinoma. Int J Colorectal Dis *20*: 440-445, 2005.
37 Rödel C, Martus P, Papadoupolos T, Füzesi L, Klimpfinger M, Fietkau R, Liersch T, Hohenberger W, Raab R, Sauer R and Wittekind C: Prognostic significance of tumor regression after preoperative chemoradiotherapy for rectal cancer. J Clin Oncol *23*: 8688-8866, 2005.
38 Lorimore S, Coates P, Scobie G, Milne G and Wright E: Inflammatory-type responses after exposure to ionizing radiation *in vivo*: a mechanism for radiation-induced bystander effects? Oncogene *20*: 7085-7095, 2001.
39 Riley P: Free radicals in biology: oxidative stress and the effects of ionizing radiation. Int J Radiat Biol *65*: 27-33, 1994.
40 Bipat S, Glas A, Slors F, Zwinderman A, Bossuyt P and Stoker J: Rectal cancer: local staging and assessment of lymph node involvement with endoluminal US, CT, and MR imaging –a meta-analysis. Radiology *232*: 773-783, 2004.
41 Liersch T, Langer C, Ghadimi B, Kulle B, Aust D, Baretton G, Schwabe W, Häusler P, Becker H and Jakob C: Lymph node status and TS gene expression are prognostic markers in stage II/III rectal cancer after neoadjuvant fluorouracil-based chemoradiotherapy. J Clin Oncol *24*: 4062-4068, 2006.
42 Baxter N, Morris A, Rothenberger D and Tepper J: Impact of preoperative radiation for rectal cancer on subsequent lymph node evaluation: a population-based analysis. Int J Radiat Oncol Biol Phys *61*: 426-431, 2005.

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