Main Effects and Interactions of Carbonic Anhydrase IX, Hypoxia-inducible Factor-1α, Ezrin and Glucose Transporter-1 in Multivariate Analysis for Disease Outcome in Rectal Cancer

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Abstract. *Strong expression of carbonic anhydrase IX (CA IX), hypoxia-inducible factor-1α (HIF-1α), ezrin and glucose transporter-1 (GLUT-1) were previously shown to be interrelated and to affect clinicopathological prognostic factors. In the current study, operative samples from 178 rectal cancer patients, 77 treated with short-course, 47 with long-course preoperative radiotherapy (RT), and 54 with no preoperative treatment, as well as 80 preoperative biopsies from the RT group were analysed using multivariate modelling in order to assess the role of these markers as predictors of disease outcome. Multivariate survival analysis revealed several sets of panels with the potential ability to identify patients at increased or decreased risk of dying from their disease or disease recurrence. The most remarkable panel, consisting of moderate/strong expression of CA IX, positive HIF-1α expression and negative/weak GLUT-1 expression in operative samples and negative/weak ezrin expression in preoperative biopsies was associated with 47.5-fold risk of death from this disease. These results should, however, be interpreted with caution due to the heterogeneity of the patient population in this retrospective study.*

Radical surgery, completed with adequate resection margins is the cornerstone in the treatment of rectal cancer (1). The number of metastatic and examined lymph nodes, the circumferential margin and the depth of tumour invasion are among the most important prognostic factors in rectal cancer (2, 3). Treatment decisions are based on pre- and postoperative tumour staging, which also affect disease outcome (2, 4). Although patients with node-negative rectal cancer have more favourable disease outcome than their node-positive counterparts, some 10-50% of patients are eventually diagnosed with recurrent disease (5). Additional predictive and prognostic tools could help identify those high-risk patients who would benefit from more intense treatment.

In non-cancerous tissues, cellular energy is produced *via* oxidative phosphorylation in normoxic conditions and by aerobic glycolysis in an oxygen-deprived environment (6), whereas malignant tumours have a tendency to turn to aerobic glycolysis even in the presence of oxygen (7). The end product of aerobic glycolysis is lactate, which promotes an acidic environment and thereby, cellular proliferation in cancer (8).

Hypoxia-inducible factor-1α (HIF-1α) is the key protein in tissue response in an oxygen-deprived environment (9). HIF-1α can also be activated by oncogenes or defects in its degradation, irrespective of the tissue oxygenation level (10). HIF-1α is involved in the activation of several mechanisms associated with tumour progression and resistance to treatment, including angiogenesis, glycolysis, invasion, metastasis, apoptosis, pH regulation and growth factor signal transduction (11, 12). Carbonic anhydrase IX (CA IX) and glucose transporter-1 (GLUT-1) are downstream targets of HIF-1α (11-13). CA IX is involved in maintaining the acidic pH and GLUT-1 in the glucose transport in cancer cells (11, 13).

Ezrin belongs to the ezrin-radixin-moesin family of proteins. It acts as a cross-linker between the cytoskeleton and the cell membrane (14). Ezrin-related metastatic behaviour is associated with the mammalian target of rapamycin (mTOR), which is also involved in the control of HIF-1α (9). Thereby, the four biomarkers in question are provided with a common crossroad linking.

We have previously studied the individual effects of CA IX, HIF-1α, ezrin and GLUT-1 expression on disease outcome in rectal cancer treated by preoperative RT or chemoradiotherapy (5, 15-18) individually and adjusted for the classical clinical prognostic factors (19). In this study, we

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focus on mutual interactions of these four markers, analysed in multivariate (Cox) models, to disclose marker panels (four-way interactions) with eventual significance as predictors of disease-specific survival (DSS) and disease-free survival (DFS).

Patients and Methods

Patients. Patients (n=178) in this study were treated at the Turku University Hospital in 2000-2009, according to the standard treatment protocols. Patients received either 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 localisation of the tumour (on clinical examination), histological biopsies and magnetic resonance imaging or computerised tomography of the rectum. In addition to operative samples, 80 preoperative biopsies from the patients who had received preoperative short- or long-course RT were studied. Patients who had upper rectal cancer, superficial tumours removed by excision and those with metastatic disease were excluded from the study. Postoperative adjuvant chemotherapy and postoperative chemoradiotherapy were given, as indicated, to eligible patients according to standard practice protocols. The median follow-up time was 35 months, and the mean follow-up time 114 months (range=2-114 months). The characteristics of the patient population are described in detail in our previous reports on this same cohort (5, 15-18). The Ethics Committee of the Hospital District of Southwest Finland approved the study protocol and the National Authority for Medico-Legal affairs granted permission for the collection and use of archival tissue material. The study was conducted in accordance with the Declaration of Helsinki.

Immunohistochemical (IHC) staining procedure. CA IX, HIF-1α, ezrin and GLUT-1 expression was analysed in all preoperative diagnostic biopsies available for study and in all tumour samples obtained at operation (5, 15-18). The most representative blocks were selected and cut into 5-μm sections. After pre-treatment in a microwave oven, the specimens were subjected to IHC staining with polyclonal rabbit anti-human CA IX (antibody 15086, Abcam, Cambridge, UK), monoclonal mouse anti-human HIF-1α (CAT# 610958; BD Transduction Laboratories San Jose, CA, USA), mouse monoclonal IgG antibody to human ezrin (clone 3 C12) (20), provided by Antti Vaheri, and polyclonal rabbit anti-human GLUT-1 (Alpha Diagnostic International, Cat # GT12 Rabbit Anti-Human GLUT-1 IgG, affinity pure; San Antonio, TX).

Evaluation of immunohistochemical staining. Two observers (EK and JS), blinded to all clinical data and radiology reports, evaluated the IHC stainings. The IHC staining procedures and the analyses of CA IX, HIF-1α, ezrin and GLUT-1 are described in detail in our earlier publications (5, 15-18).

Statistical analyses. Statistical analyses were performed using two statistical software packages: the IBM SPSS 19.0.1 for Windows (IBM, New York, NY, USA) and the STATA/SE 11.2 software (STATA Corp., College Station, TX, USA). In these analyses, only the four biomarkers were analysed using multivariate modelling to assess their role as predictors of disease outcome: DSS and DFS. The correlations of clinicopathological variables were not analysed in these models. DSS was calculated based on the time from diagnosis to death (due to disease) or to the latest follow-up visit, and DFS based on the time from diagnosis to the appearance of metastatic disease, respectively. Cox proportional hazards regression models were constructed using three different approaches (models), the four biomarkers as the only covariates (19). These three models were: i) all four biomarkers (CA IX, HIF-1α, GLUT-1, ezrin) analysed in the operative samples (n=175); ii) all marker data analysed in the preoperative biopsy samples (n=175); and iii) ezrin analysed in the preoperative samples and all 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). Both the main effects of individual biomarkers and all their four-way interactions were tested as independent variables in the Cox model. The validity of the proportional hazards assumption was tested graphically using the log minus-log plots for the main effect covariates. All statistical tests were two-sided and considered significant at a p-value of <0.05.

Results

The main results of multivariate Cox survival analysis for DFS and DSS are shown in Tables I and II, separately for the two models, including the main effects of the four markers and their (four-way) interactions. A panel of 15 possible combinations of the four markers was analysed. Panels containing one or more empty cells could not be computed, and were excluded from the tables.

In model 1 (biomarkers in operative samples), the HR for dying from cancer was 9.26 when moderate/strong CA IX intensity was present (95% CI=1.23-69.99). None of the 15 panel combinations was significantly associated with DSS (Table I). Similarly, none of the markers (main effects) or their interactions proved to be significantly associated with DFS in this model (data not shown).

In model 2 (biomarkers in preoperative biopsies), the HR for dying from disease was 2.25-fold with moderate/strong ezrin expression (95% CI=0.40;12.62). Due to the limited number of biopsies, some of the marker combinations were present only in single cases or entirely absent, precluding the calculation of the risk estimates for DSS, and even for DFS (data not shown in tables).

In model 3 (ezrin in preoperative biopsies and the remaining markers in operative samples), the HR for dying of disease was 5.13-fold (95% CI=1.58-16.58) when moderate/strong CA IX intensity was present, while the others were not significant or not computable. Of the marker combinations, negative/weak CA IX, ezrin and GLUT, together with positive HIF-1α staining was associated with a 5.87-fold risk for dying of cancer (95% CI=0.29-116.65). As shown in Table II, the single most powerful predictor of DSS, was the marker combination of moderate/strong CA IX, negative/weak ezrin, negative/weak GLUT-1 and positive HIF-1α expression, increasing the risk of dying from disease

Table I. *Multivariate (Cox) analysis for disease-specific survival (DSS), in Model 1, studying of the biomarkers in the operative samples. Combinations of markers that could not be computed are excluded.*

				Main effects	Four-wag interactions
Variable				Hazard ratio	95% Confidence interval
Moderate/Strong CA IX intensity				9.26	[1.23; 69.99]
Positive HIF-1α				1.66	[0.48; 5.74]
Moderate/Strong ezrin				0.62	[0.26; 1.48]
				Four-way interactions	
Variable				Hazard ratio	95% Confidence interval
CA IX	HIF-1α	Ezrin	GLUT-1		
Negative/Weak	Positive	Negative/Weak	Moderate/Strong	1.56	[0.07; 33.87]
Negative/Weak	Negative	Moderate/Strong	Moderate/Strong	5.37	[0.36; 79.2]
Negative/Weak	Negative	Negative/Weak	Moderate/Strong	1.28	[0.14; 11.89]
Moderate/Strong	Positive	Negative/Weak	Moderate/Strong	0.45	[0.04; 5.09]

Table II. *Multivariate (Cox) analysis for disease-specific survival (DSS) in Model 3, analysing ezrin in the preoperative samples, and CA IX, HIF-1α, and GLUT-1 in the operative samples. Combinations of markers that could not be computed are excluded.*

				Main effects	
Variable				Hazard ratio	95% Confidence interval
Moderate/Strong CA IX intensity				5.13	[1.58; 16.58]
Positive HIF-1α				1.22	[0.34; 4.36]
				Four-way interactions	
Variable				Hazard Ratio	95% Confidence interval
CA IX	HIF-1α	Ezrin	GLUT-1		
Negative/Weak	Positive	Negative/Weak	Negative/Weak	5.87	[0.29; 116.65]
Negative/Weak	Negative	Negative/Weak	Negative/Weak	1.70	[0.19; 14.85]
Moderate/Strong	Positive	Negative/Weak	Negative/Weak	47.46	[4.97; 453.14]

with HR=47.46 (95% CI=4.97-453.14). None of the markers (main effects) or their different combinations were significantly associated with DFS (data not shown in tables).

Discussion

Multivariate survival analysis was used to test the four-way interactions of HIF-1α, CA IX, ezrin and GLUT-1, creating 15 marker panels with potential predictive value of DFS and DSS. Importantly, one of these panels was shown to be associated with a greater than 47-fold risk of dying from the disease. This panel included moderate/strong expression of CA IX and positive HIF-1α together with negative/weak expression of GLUT-1 in the operative samples and negative/weak expression of ezrin in the preoperative biopsies.

We have previously separately analysed the prognostic and predictive value of CA IX, HIF-1α, ezrin and GLUT-1 analyses in a cohort of 178 rectal cancer patients treated by preoperative radiotherapy (or chemoradiotherapy) or with no treatment prior to operation (5, 15-18). In multivariate models, the single most intriguing observation was that moderate/strong CA IX intensity was a strong predictor of DSS, conferring a greater than 9-fold HR for dying from the disease. In our previous study, these four markers were evaluated as independent predictors adjusted for the classical

clinical prognostic factors (after data reduction by principal component analysis), further emphasising the important role of CA IX as such a predictor (19).

To determine the four-way interactions of the biomarkers, a set of 15 panels was created, each used as a covariate (together with the four markers) in the Cox model, to identify individuals with unfavourable or favourable prognosis. The rationale was to assess whether any such marker panel might be used as an additional tool to separate patients who need additional treatment from those with favourable prognosis, *i.e.* while considering postoperative adjuvant treatment in rectal cancer (21, 22). In this respect, markers targeting the molecular pathways linked to tumour hypoxia are of interest, because tumours frequently have poorly oxygenated areas, irrespective of their size (23).

Hypoxia renders a tumour resistant to both chemo- and radiotherapy (24) and predicts unfavourable prognosis (12). Therefore, it is not unexpected that several panels of markers were associated with 5-6-fold increased risk. Furthermore, a panel predicting a favourable prognosis was identified in the present analyses. Negative/weak expression of all four markers in preoperative biopsies was associated with a lower risk for dying from the disease. Risk of disease relapse was also lower when all markers were negative/weak.

Out of all different settings analysed, there is one panel that stands out, identifying a group of patients with an over 47-fold risk of dying from disease, consisting of moderate/strong CA IX, negative/weak GLUT-1 and positive HIF-1α expression in the operative samples, together with negative/weak ezrin expression in the preoperative biopsies. To the best of our knowledge, there are no earlier reports concerning the interactions of these four biomarkers. The results of these analyses suggest that HIF-1α, GLUT-1, CA IX and ezrin are indeed biologically related. The data also support the theoretical background linking the four biomarkers. Furthermore, these findings are particularly interesting since the idea of inhibiting these molecules is already being exploited in treatment of some types of cancer (25). The results of the current analyses are predominantly in line with our previous publications with some differences. In this multivariate analysis we were not able to fully assess the biomarkers in different treatment groups due to the limited number of patients in each category. Preoperative treatment is chosen based on disease stage, with advanced stages possibly reflecting more aggressive tumour behaviour. Hence, it would be important to have samples of a larger patient population available for analyses. However, keeping in mind the small number of biopsies and the heterogeneity of the patient population (the preoperative treatment groups were analysed together), as well as the relatively broad confidence interval, the results of this study should be interpreted with caution. Preferably a randomised prospective study evaluating these panels together with clinical prognosticators would clarify the feasibility of the use of these panels in clinical practice.

Taken together, multivariate survival analysis of four hypoxia-associated biomarkers revealed several sets of panels with the potential to identify patients at increased or decreased risk of dying from disease or disease recurrence. The most remarkable panel, consisting of moderate/strong CA IX, negative/weak ezrin (in preoperative biopsy), negative/weak GLUT-1, and positive HIF-1α expression was associated with a 47.5-fold risk of dying from the disease. However, this was a retrospective, non-randomised study, with a limited number of heterogeneous patients. Thus, the results should be considered preliminary and should be further validated in prospective clinical trials. If confirmed in a larger prospective cohort, this panel could provide an extremely useful tool in tailoring postoperative treatment for those patients with the most unfavourable prognosis.

Conflicts of Interest

None to declare.

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