ORIGINAL CONTRIBUTION

Hypoxia-Related Proteins in Patients With Rectal Cancer Undergoing Neoadjuvant Combined Modality Therapy

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BACKGROUND: We have previously demonstrated the prognostic significance of rectal cancer pathologic response to neoadjuvant chemoradiation. Recent studies in other cancers have reported that hypoxia influences response to neoadjuvant chemoradiation.

OBJECTIVE: This study aimed to 1) characterize hypoxia-related protein expression in locally advanced rectal cancer before neoadjuvant chemoradiation, 2) determine the comodulation of hypoxia-related protein expression, and 3) evaluate the relationship between hypoxia-related protein expression and overall survival, time to recurrence, and tumor regression grade.

DESIGN: Immunohistochemical analysis of 4 hypoxia-related proteins (HIF-1α, CA-IX, VEGF, and GLUT-1) was performed on archival pretreatment rectal cancer biopsies.

PATIENTS: Eighty-five patients with locally advanced rectal cancer treated with neoadjuvant radiation and 5-fluorouracil-based chemotherapy were included.

MAIN OUTCOME MEASURES: The impact of hypoxia-related protein expression on outcome was evaluated by use of Cox proportional hazards model. Hypoxia-related protein expression was correlated with tumor regression grade by use of Spearman correlation coefficients.

RESULTS: Median follow-up was 54 months. CA-IX expression was associated with overall survival ($p = 0.01$). HIF-1α expression was weakly correlated with VEGF ($r = 0.26$, $p = 0.02$) and GLUT-1 ($r = 0.35$, $p = 0.001$). Hypoxia-related protein expression was not associated with time to recurrence or Mandard tumor regression grade.

CONCLUSIONS: Elevated CA-IX expression may be associated with poorer overall survival in locally advanced rectal cancer treated by neoadjuvant chemoradiation and resection. The expression of the hypoxia-related proteins HIF-1α, VEGF, and GLUT-1 may be comodulated in locally advanced rectal cancer. Further studies are needed to evaluate the mechanisms governing hypoxia regulation and the role of hypoxia in rectal cancer response to neoadjuvant chemoradiation.

KEY WORDS: Rectal cancer; Hypoxia; Chemotherapy; Radiation; CA-IX; Survival.

Hypoxia has been reported across a multitude of cancers in humans and has been linked with increased rates of metastatic disease and increased mortality.[1–4] Hypoxic tumors may not only have a more aggressive phenotype, but data from cervical cancer and head and neck cancer have demonstrated that tumor hypoxia increases resistance to radiation therapy.[5–8] The effect of tumor hypoxia on response to radiation therapy is

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relevant to the management of rectal cancer, as the current standard of care for locally advanced rectal cancer (LARC) consists of neoadjuvant combined modality therapy (CMT) followed by rectal resection.[9]

Rectal cancer hypoxia has been evaluated directly through measurements with polarographic needle electrodes[10–12] and indirectly through immunohistochemical (IHC) analysis of exogenous[13] and endogenous[14–25] hypoxia-related proteins, as well as through PET imaging with radiolabeled hypoxia markers.[26,27] Direct measurement with polarographic needle electrodes is the standard for measuring tissue oxygenation; however, this approach is invasive and not widely practiced. The disadvantages of PET imaging include high cost and limited availability of radiolabeled hypoxia markers. In contrast, IHC analysis uses readily available antibodies to endogenous hypoxia-related proteins and may prove to be an affordable, convenient method of measuring tumor hypoxia.

The 4 hypoxia-related proteins chosen for this study include hypoxia-inducible factor 1-alpha (HIF-1α), carbonic anhydrase-9 (CA-IX), vascular endothelial growth factor (VEGF), and glucose transporter-1 (GLUT-1). HIF-1α is a protein centrally involved in the cellular response to hypoxia; it activates a variety of downstream genes involved in anaerobic metabolism, cell cycle arrest, differentiation, stress adaptation, and angiogenesis.[28–31] These downstream genes' protein products, which include CA-IX, VEGF, and GLUT-1, promote cell survival under hypoxic conditions. The availability of antibodies directed against these 4 hypoxia-related proteins allows for IHC analysis of their expression in tumors. Among patients with colorectal cancer, expression of these 4 hypoxia-related proteins has been shown to correlate with rates of lymph node metastasis (VEGF and GLUT-1),[32–35] liver metastasis (HIF-1α and VEGF),[32,33,36] disease-free survival (HIF-1α, VEGF, and GLUT-1),[35–37] and overall survival (HIF-1α, CA-IX, VEGF, and GLUT-1).[32,35,36,38]

The aims of this exploratory study were to 1) characterize hypoxia-related protein expression in LARC before neoadjuvant CMT, 2) determine the comodulation of hypoxia-related protein expression, and 3) evaluate the relationship between hypoxia-related protein expression and tumor regression grade (TRG), time to recurrence, and overall survival.

METHODS

The study design was approved by the Institutional Review Board at Memorial Sloan-Kettering Cancer Center and the Ethics Committee at the University of Padova, Italy. At these 2 institutions, 101 consecutive patients with LARC treated between 1994 and 2004 were identified through prospectively maintained databases. At both institutions, patients received neoadjuvant 5-fluorouracil (5-FU)-based chemotherapy and long-course pelvic radiation. Patients were scheduled to undergo rectal resection 6 to 8 weeks after completion of neoadjuvant chemoradiation. Patients subsequently received adjuvant chemotherapy, per institutional protocols. Surgical specimens were evaluated for standard histopathologic staging as well as for pathologic response to neoadjuvant CMT (as described by Mandard TRG, TRG 1 to 5[39]). Pretreatment tumor biopsies and corresponding surgical specimens were available for analysis in 85 patients; these patients constitute the study population.

TABLE 1. Overview of antibodies used for immunohistochemical analysis

Antibody	*Dilution*	*Source*
HIF-1α	1:1600	Novus Biological, Littleton, CO
CA-IX	1:500	Private source
VEGF	1:500	Santa Cruz Biotechnology, Santa Cruz, CA
GLUT-1	1:400	Chemicon (now Millipore), Temecula, CA

Immunohistochemistry

Immunohistochemical analysis was performed on pretreatment tumor biopsies. The primary antibodies, dilutions, and commercial sources for HIF-1α, CA-IX, VEGF, and GLUT-1 are listed in Table 1. For CA-IX, VEGF and GLUT-1, the stains were performed using an automated IHC strainer (Discovery XT, Ventana Medical Systems, Tucson, AZ). The retrieval buffer (CC1 reagent) was used for pretreatment (heat-induced epitope retrieval), followed by a 60-minute primary antibody incubation. Omi-Map DAB Kit was used for visualization. For HIF-1α, slides were pretreated and stained according to DAKO catalyzed signal amplification System Kit, primary antibody dilution: 1:1600. Avidin biotin block was applied before the primary antibody. Diaminobenzidine was used as the chromogen.

Scoring of the IHC staining of all 4 tested markers was based on the staining percentage, defined by the proportion of tumor showing positive staining relative to the total amount of tumor present in the section. For the staining intensity, given that the intensity was relatively uniform for all 4 markers, a binary system was used, ie, a stain was scored as either negative or positive. Examples of positive staining for the 4 tested markers are illustrated in Figure 1.

Statistical Analysis

Protein expression, based on the staining percentage, was evaluated as a continuous variable; no cutoff expression level was used. The Cox proportional hazards model was used to evaluate the relationship between hypoxia-related protein expression, time to recurrence, and overall survival. The Spearman rank correlation coefficient (r) was used to look for associations between hypoxia-related protein expression and pathologic response to neoadjuvant CMT

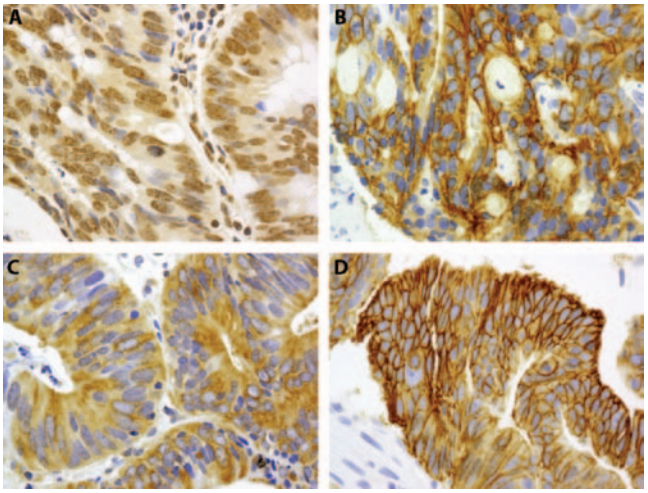

FIGURE 1. Representative pretreatment tumor biopsies staining positive on IHC for protein expression of HIF-1α (A), CA-IX (B), VEGF (C), and GLUT-1 (D). The staining percentage is the proportion of tumor showing positive staining relative to the total amount of tumor present in the section. IHC = immunohistochemical; CA-IX = carbonic anhydrase-9; VEGF = vascular endothelial growth factor; GLUT-1 = glucose transporter-1; HIF-1α = hypoxia-inducible factor 1-α.

(TRG), and for associations of individual hypoxia-related proteins with one another, as well.

RESULTS

Pretreatment tumor biopsies from 85 patients (69% male, mean age 61 years) underwent IHC analysis of HIF-1α, CA-IX, VEGF, and GLUT-1. The number of pretreatment tumor biopsies with a staining percentage of ≥10% was 37 of 82 (45%) for HIF-1α, 41 of 85 (48%) for CA-IX, 34 of 84 (40%) for VEGF, and 49 of 85 (58%) for GLUT-1. Representative pretreatment tumor biopsies staining positive for HIF-1α, CA-IX, VEGF, and GLUT-1 on IHC analysis are shown in Figure 1.

Neoadjuvant CMT consisted of 5-FU continuous infusion in 44 patients, including 10 patients who also received oxaliplatin and 4 patients who also received carboplatin, and bolus 5-FU/leucovorin in 41 patients, including 2 patients who also received irinotecan; and long-course external beam radiation therapy of 5040 cGy (83 patients), 4500 cGy (1 patient), and 3600 cGy (1 patient). The median time between completion of neoadjuvant CMT and surgery was 46 days (25–75% quartile: 42–51 days).

The pathologic complete response (TRG1, no residual cancer cells) rate of the primary tumor was 15% (n = 12). The remainder of pathologic responses to neoadjuvant CMT among the posttreatment surgical specimens included 14 (17%) TRG2, 28 (34%) TRG 3, 23 (28%) TRG 4, and 5 (6%) TRG 5. Median follow-up was 54 months.

CA-IX expression was found to be associated with overall survival, with a higher staining percentage indicating poorer overall survival ($p = 0.01$). No association was identified between hypoxia-related protein expression and time to recurrence (Table 2). HIF-1α expression was weakly correlated with VEGF ($r = 0.26$, $p = 0.02$) and GLUT-1 ($r = 0.35$, $p = 0.001$) expression (Fig. 2). CA-IX expression was not correlated with expression of any of the other hypoxia-related proteins. No association was found between hypoxia-related protein expression and pathologic response to neoadjuvant CMT, as measured by Mandard TRG (Table 3).

TABLE 2. Hypoxia-related protein expression and time to recurrence, overall survival

	Cox proportional hazards model HR[a] (p value)	
Protein	*Time to recurrence*	*Overall survival*
CA-IX	1.13 (0.10)	1.27 (0.01)
VEGF	1.00 (0.98)	0.90 (0.35)
GLUT-1	1.08 (0.40)	0.92 (0.51)
HIF-1α	1.22 (0.06)	1.16 (0.21)

[a]HR per 10% increase in protein expression

DISCUSSION

The results of our exploratory study demonstrate that increased CA-IX expression predicts decreased overall survival. This is of interest because our results support a previous study that showed an association between moderate/strong CA-IX expression and decreased disease-free and disease-specific survival in patients with rectal cancer treated by neoadjuvant CMT.[20] Although another study found no association between CA-IX expression and disease-specific survival, that study consisted of a distinct patient population, because it excluded patients who received neoadjuvant CMT.[19] Similar to its effects in patients with rectal cancer, the presence of stromal CA-IX expression has been shown to be associated with poorer overall survival in patients with colorectal cancer.[38]

CA-IX is a transmembrane glycoprotein that participates in pH regulation by catalyzing the reversible hydration of carbon dioxide to carbonic acid. CA-IX expression is induced by prolonged hypoxia, when anaerobic metabolism predominates and tissue pH decreases. Within other cancer types (with the exception of renal cancer),[40,41] increased CA-IX expression has been shown to be associated with decreased response to radiation therapy[7,8] and poorer survival.[42–48] If CA-IX expression was conclusively found to be an independent predictor of prognosis in patients with rectal cancer treated by neoadjuvant CMT, multiple theoretical clinical implications exist. CA-IX expression could potentially: influence the administration of neoadjuvant radiation therapy, contribute information to a rectal cancer nomogram for prediction of survival, and serve as an immunotherapy target for adjuvant treatment.

We did not find HIF-1α, VEGF, or GLUT-1 to have an association with overall survival or time to recurrence.

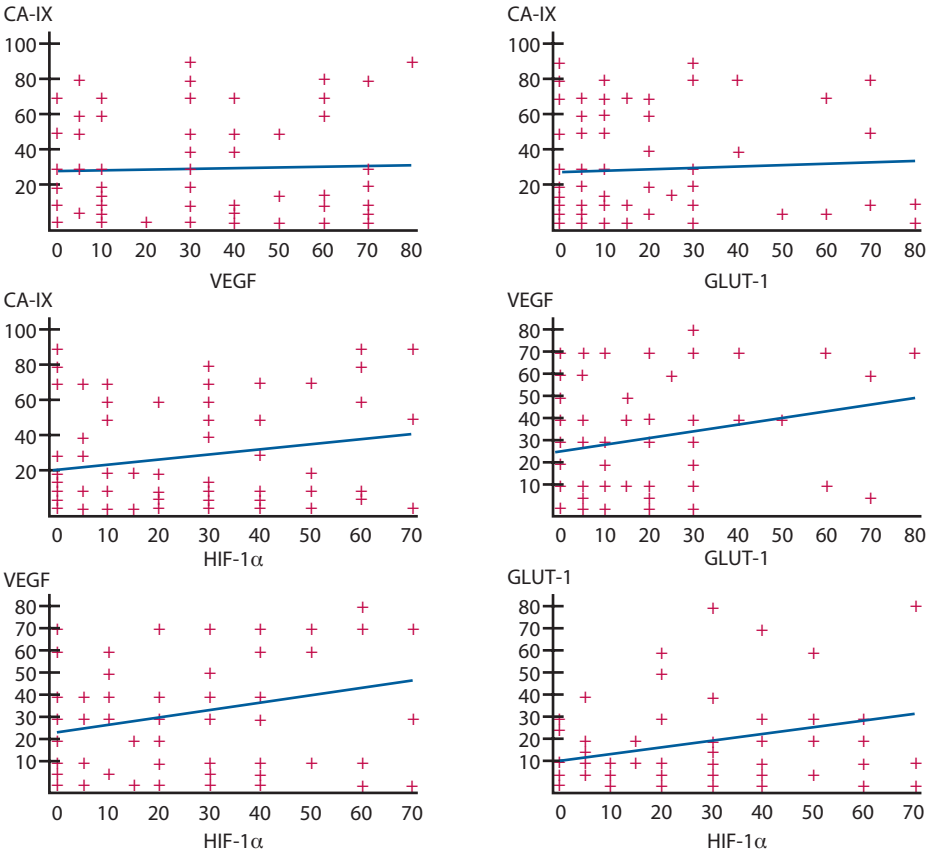

FIGURE 2. Evaluation of cosegregation of hypoxia-related protein expression with the use of Spearman correlation coefficients. HIF-1α expression was weakly correlated with VEGF ($r = 0.26$, $p = 0.02$) and GLUT-1 ($r = 0.35$, $p = 0.001$) expression. No other pairs demonstrated statistically significant cosegregation of protein expression. IHC = immunohistochemical; CA-IX = carbonic anhydrase-9; VEGF = vascular endothelial growth factor; GLUT-1 = glucose transporter-1; HIF-1α = hypoxia-inducible factor 1-α.

However, an association between HIF-1α and prognosis has been described among patients with rectal cancer who have *not* received neoadjuvant CMT.[15,16,18] The lack of association with time to recurrence supports a study that showed no association between GLUT-1 and recurrence-free survival among patients with rectal cancer of whom the majority (86%) did not receive neoadjuvant CMT, although that study did find an association between GLUT-1 expression and overall survival.[14] These discrepancies may be due to differences in neoadjuvant CMT administration. We did not observe a relationship between expression of hypoxia-related proteins and pathologic response to neoadjuvant CMT. The limited sample size and the heterogeneity of intratumoral oxygenation may be responsible for this negative finding.

TABLE 3. Hypoxia-related protein expression and Mandard tumor regression grade

Hypoxia-related protein	*Spearman correlation coefficient* (p *value*)
HIF-1α	−0.07 (0.53)
CA-IX	−0.04 (0.70)
VEGF	−0.01 (0.90)
GLUT-1	−0.02 (0.84)

We found that HIF-1α expression is weakly comodulated with VEGF ($p = 0.02$) and GLUT-1 ($p = 0.001$) expression in patients with LARC treated with long-course neoadjuvant CMT and rectal resection. The significance of HIF-1α comodulation with other hypoxia-related proteins is still being defined. Although HIF-1α expression is known to drive expression of downstream proteins, differences in individual protein half-lives may not allow for a direct relationship between HIF-1α and other proteins. In regard to the weak comodulation of HIF-1α expression with VEGF and GLUT-1 expression, the downstream proteins may have been influenced by other stimulatory or inhibitory signaling pathways independent from HIF-1α, making their expression somewhat variable in relation to HIF-1α. An association between HIF-1α expression and VEGF expression has been reported previously in patients with rectal cancer, with higher HIF-1α expression shown to be associated with higher VEGF expression.[15,16,23] The correlation between HIF-1α and GLUT-1 has not yet been shown in patients with rectal cancer, and the colorectal cancer literature contains contradictory findings.[34,38] It is curious that CA-IX expression was not shown to be comodulated with HIF-1α expression, given that HIF-1α is known to regulate CA-IX expression.[49]

Early studies evaluating hypoxia in rectal cancer performed direct p_{O_2} measurements in a limited number of patients with rectal cancer by use of polarographic needle electrodes, and demonstrated lower oxygen tension in tumors than in surrounding normal mucosa.[10–12] Another early study measured mean hemoglobin saturation of red blood cells within tumor microvessels and showed that rectal cancers are heterogeneous in their oxygenation distribution.[50] Although these results helped set the stage for the study of hypoxia in rectal cancer, they did not address the potential impact of hypoxia in terms of prognosis or pathologic response to neoadjuvant CMT.

Noninvasive imaging via PET has recently emerged as an attractive alternative modality to detect hypoxia in a number of tumor types, and we have begun to use this technology in the study of patients with rectal cancer. A theoretical benefit of hypoxia-PET imaging is that global assessment of the entire tumor is possible, as opposed to regional assessments using IHC or direct p_{O_2} measurements. ^{18}F-Fluoro-Misonidazole is currently considered the reference standard PET hypoxia tracer. One small study has demonstrated radiotracer uptake in recurrent rectal cancers and metastases. [26] In addition, a pilot study utilizing the novel hypoxia tracer ^{60}Cu-diacetyl-bis N^4-methylthiosemicarbazone in 17 patients with rectal cancer proposed that ^{60}Cu-diacetyl-bis N^4-methylthiosemicarbazone PET may predict survival and pathologic response to neoadjuvant CMT.[27] Although PET assessment of tumor hypoxia is an attractive modality to assess global tumor hypoxia, the lack of readily available and reliable tracers precludes its routine clinical use at this time.

Research in a variety of solid tumors has demonstrated an important association between oxygenation status and pathologic response to neoadjuvant CMT, likelihood of metastasis, and survival. With the commercial availability of antibodies against hypoxia-related proteins, IHC analysis has been increasingly used in the study of hypoxia. This exploratory study is unique in that we have characterized hypoxia based on IHC-quantified expression of 4 hypoxia-related proteins in rectal cancer biopsies obtained before neoadjuvant CMT. We found that HIF-1α expression is weakly comodulated with VEGF and GLUT-1 expression and that CA-IX expression may predict long-term outcome in patients with rectal cancer who undergo neoadjuvant CMT. Additional studies are needed to help further clarify the relationship between hypoxia, pathologic response to neoadjuvant CMT, and prognosis in patients with LARC.

REFERENCES

1. Terris DJ. Head and neck cancer: the importance of oxygen. *Laryngoscope*. 2000;110(5 Pt 1):697–707.
2. Knocke TH, Weitmann HD, Feldmann HJ, Selzer E, Pötter R. Intratumoral pO2-measurements as predictive assay in the treatment of carcinoma of the uterine cervix. *Radiother Oncol*. 1999;53:99–104.
3. Brizel DM, Scully SP, Harrelson JM, et al. Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. *Cancer Res*. 1996;56:941–943.
4. Nordsmark M, Alsner J, Keller J, et al. Hypoxia in human soft tissue sarcomas: adverse impact on survival and no association with p53 mutations. *Br J Cancer*. 2001;84:1070–1075.
5. Fyles AW, Milosevic M, Wong R, et al. Oxygenation predicts radiation response and survival in patients with cervix cancer. *Radiother Oncol*. 1998;48:149–156.
6. Nordsmark M, Overgaard J. A confirmatory prognostic study on oxygenation status and loco-regional control in advanced head and neck squamous cell carcinoma treated by radiation therapy. *Radiother Oncol*. 2000;57:39–43.
7. Koukourakis MI, Giatromanolaki A, Danielidis V, Sivridis E. Hypoxia inducible factor (HIf1alpha and HIF2alpha) and carbonic anhydrase 9 (CA9) expression and response of head-neck cancer to hypofractionated and accelerated radiotherapy. *Int J Radiat Biol*. 2008;84:47–52.
8. Koukourakis MI, Bentzen SM, Giatromanolaki A, et al. Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited in the CHART randomized trial. *J Clin Oncol*. 2006;24:727–735.
9. Sauer R, Becker H, Hohenberger W, et al.; German Rectal Cancer Study Group. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med*. 2004;351:1731–1740.
10. Feldmann HJ, Molls M, Füller J, Stüben G, Sack H. Changes in oxygenation patterns of locally advanced recurrent tumors under thermoradiotherapy. *Adv Exp Med Biol*. 1994;345:479–483.
11. Molls M, Feldmann HJ, Füller J. Oxygenation of locally advanced recurrent rectal cancer, soft tissue sarcoma and breast cancer. *Adv Exp Med Biol*. 1994;345:459–463.
12. Mattern J, Kallinowski F, Herfarth C, Volm M. Association of resistance-related protein expression with poor vascularization and low levels of oxygen in human rectal cancer. *Int J Cancer*. 1996;67:20–23.
13. Goethals L, Debucquoy A, Perneel C, et al. Hypoxia in human colorectal adenocarcinoma: comparison between extrinsic and potential intrinsic hypoxia markers. *Int J Radiat Oncol Biol Phys*. 2006;65:246–254.
14. Cooper R, Sarioglu S, Sökmen S, et al. Glucose transporter-1 (GLUT-1): a potential marker of prognosis in rectal carcinoma? *Br J Cancer*. 2003;89:870–876.
15. Lu XG, Xing CG, Feng YZ, Chen J, Deng C. Clinical significance of immunohistochemical expression of hypoxia-inducible factor-1alpha as a prognostic marker in rectal adenocarcinoma. *Clin Colorectal Cancer*. 2006;5:350–353.
16. Theodoropoulos GE, Lazaris AC, Theodoropoulos VE, et al. Hypoxia, angiogenesis and apoptosis markers in locally advanced rectal cancer. *Int J Colorectal Dis*. 2006;21:248–257.
17. Korkeila E, Jaakkola PM, Syrjänen K, Sundström J, Pyrhönen S. Preoperative radiotherapy downregulates the nuclear expression of hypoxia-inducible factor-1alpha in rectal cancer. *Scand J Gastroenterol*. 2010;45:340–348.
18. Rasheed S, Harris AL, Tekkis PP, et al. Hypoxia-inducible factor-1alpha and -2alpha are expressed in most rectal cancers but only hypoxia-inducible factor-1alpha is associated with prognosis. *Br J Cancer*. 2009;100:1666–1673.

19. Rasheed S, Harris AL, Tekkis PP, et al. Assessment of microvessel density and carbonic anhydrase-9 (CA-9) expression in rectal cancer. *Pathol Res Pract.* 2009;205:1–9.
20. Korkeila E, Talvinen K, Jaakkola PM, et al. Expression of carbonic anhydrase IX suggests poor outcome in rectal cancer. *Br J Cancer.* 2009;100:874–880.
21. Brophy S, Sheehan KM, McNamara DA, Deasy J, Bouchier-Hayes DJ, Kay EW. GLUT-1 expression and response to chemoradiotherapy in rectal cancer. *Int J Cancer.* 2009;125:2778–2782.
22. Saigusa S, Tanaka K, Toiyama Y, et al. Clinical significance of CD133 and hypoxia inducible factor-1a gene expression in rectal cancer after preoperative chemoradiotherapy. *Clin Oncol (R Coll Radiol).* 2011;23:323–332.
23. Toiyama Y, Inoue Y, Saigusa S, et al. Gene expression profiles of epidermal growth factor receptor, vascular endothelial growth factor and hypoxia-inducible factor-1 with special reference to local responsiveness to neoadjuvant chemoradiotherapy and disease recurrence after rectal cancer surgery. *Clin Oncol (R Coll Radiol).* 2010;22:272–280.
24. Korkeila E, Jaakkola PM, Syrjänen K, Pyrhönen S, Sundström J. Pronounced tumour regression after radiotherapy is associated with negative/weak glucose transporter-1 expression in rectal cancer. *Anticancer Res.* 2011;31:311–315.
25. Havelund BM, Sørensen FB, Lindebjerg J, Spindler KL, Jakobsen A. Pretreatment HIF-1a and GLUT-1 expressions do not correlate with outcome after preoperative chemoradiotherapy in rectal cancer. *Anticancer Res.* 2011;31:1559–1565.
26. Loi S, Ngan SY, Hicks RJ, et al. Oxaliplatin combined with infusional 5-fluorouracil and concomitant radiotherapy in inoperable and metastatic rectal cancer: a phase I trial. *Br J Cancer.* 2005;92:655–661.
27. Dietz DW, Dehdashti F, Grigsby PW, et al. Tumor hypoxia detected by positron emission tomography with 60Cu-ATSM as a predictor of response and survival in patients undergoing Neoadjuvant chemoradiotherapy for rectal carcinoma: a pilot study. *Dis Colon Rectum.* 2008;51:1641–1648.
28. Lal A, Peters H, St Croix B, et al. Transcriptional response to hypoxia in human tumors. *J Natl Cancer Inst.* 2001;93:1337–1343.
29. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, Harris AL. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. *Cancer Res.* 2001;61:6669–6673.
30. Zhong H, De Marzo AM, Laughner E, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res.* 1999;59:5830–5835.
31. Mabjeesh NJ, Amir S. Hypoxia-inducible factor (HIF) in human tumorigenesis. *Histol Histopathol.* 2007;22:559–572.
32. Ishigami SI, Arii S, Furutani M, et al. Predictive value of vascular endothelial growth factor (VEGF) in metastasis and prognosis of human colorectal cancer. *Br J Cancer.* 1998;78:1379–1384.
33. Konno H, Abe J, Kaneko T, et al. Urokinase receptor and vascular endothelial growth factor are synergistically associated with the liver metastasis of colorectal cancer. *Jpn J Cancer Res.* 2001;92:516–523.
34. Wincewicz A, Sulkowska M, Koda M, Sulkowski S. Clinicopathological significance and linkage of the distribution of HIF-1alpha and GLUT-1 in human primary colorectal cancer. *Pathol Oncol Res.* 2007;13:15–20.
35. White JD, Hewett PW, Kosuge D, et al. Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. *Cancer Res.* 2002;62:1669–1675.
36. Rajaganeshan R, Prasad R, Guillou PJ, Poston G, Scott N, Jayne DG. The role of hypoxia in recurrence following resection of Dukes' B colorectal cancer. *Int J Colorectal Dis.* 2008;23:1049–1055.
37. Haber RS, Rathan A, Weiser KR, et al. GLUT1 glucose transporter expression in colorectal carcinoma: a marker for poor prognosis. *Cancer.* 1998;83:34–40.
38. Cleven AH, van Engeland M, Wouters BG, de Bruïne AP. Stromal expression of hypoxia regulated proteins is an adverse prognostic factor in colorectal carcinomas. *Cell Oncol.* 2007;29:229–240.
39. Mandard AM, Dalibard F, Mandard JC, et al. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. *Cancer.* 1994;73:2680–2686.
40. Bui MH, Seligson D, Han KR, et al. Carbonic anhydrase IX is an independent predictor of survival in advanced renal clear cell carcinoma: implications for prognosis and therapy. *Clin Cancer Res.* 2003;9:802–811.
41. Leibovich BC, Sheinin Y, Lohse CM, et al. Carbonic anhydrase IX is not an independent predictor of outcome for patients with clear cell renal cell carcinoma. *J Clin Oncol.* 2007;25:4757–4764.
42. Beasley NJ, Wykoff CC, Watson PH, et al. Carbonic anhydrase IX, an endogenous hypoxia marker, expression in head and neck squamous cell carcinoma and its relationship to hypoxia, necrosis, and microvessel density. *Cancer Res.* 2001;61:5262–5267.
43. Chia SK, Wykoff CC, Watson PH, et al. Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. *J Clin Oncol.* 2001;19:3660–3668.
44. Loncaster JA, Harris AL, Davidson SE, et al. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. *Cancer Res.* 2001;61:6394–6399.
45. Hoskin PJ, Sibtain A, Daley FM, Wilson GD. 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.* 2003;89:1290–1297.
46. Swinson DE, Jones JL, Richardson D, et al. Carbonic anhydrase IX expression, a novel surrogate marker of tumor hypoxia, is associated with a poor prognosis in non-small-cell lung cancer. *J Clin Oncol.* 2003;21:473–482.
47. Driessen A, Landuyt W, Pastorekova S, et al. Expression of carbonic anhydrase IX (CA IX), a hypoxia-related protein, rather than vascular-endothelial growth factor (VEGF), a pro-angiogenic factor, correlates with an extremely poor prognosis in esophageal and gastric adenocarcinomas. *Ann Surg.* 2006;243:334–340.
48. Sathornsumetee S, Cao Y, Marcello JE, et al. Tumor angiogenic and hypoxic profiles predict radiographic response and survival in malignant astrocytoma patients treated with bevacizumab and irinotecan. *J Clin Oncol.* 2008;26:271–278.
49. Wykoff CC, Beasley NJ, Watson PH, et al. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Res.* 2000;60:7075–7083.
50. Wendling P, Manz R, Thews G, Vaupel P. Heterogeneous oxygenation of rectal carcinomas in humans: a critical parameter for preoperative irradiation? *Adv Exp Med Biol.* 1984;180:293–300.