Published OnlineFirst April 11, 2017; DOI: 10.1158/1078-0432.CCR-17-0038

Personalized Medicine and Imaging

Clinical Cancer Research

A Gene Signature for Selecting Benefit from Hypoxia Modification of Radiotherapy for High-Risk Bladder Cancer Patients

Lingjian Yang[1], Janet Taylor[1,2,3], Amanda Eustace[1], Joely J. Irlam[1], Helen Denley[4], Peter J. Hoskin[5], Jan Alsner[6], Francesca M. Buffa[7], Adrian L. Harris[7], Ananya Choudhury[1], and Catharine M.L. West[1]

Abstract

Purpose: Hypoxia modification improves overall survival in muscle-invasive bladder cancer patients who undergo radiotherapy. There is evidence that hypoxic tumors benefit most from hypoxia modification. The study aimed to identify or derive a hypoxia gene signature that predicts benefit from hypoxia-modifying treatment in bladder cancer.

Experimental Design: Published hypoxia signatures were tested and a new one derived by analyzing bladder cancer transcriptomic data from public databases. Tumor samples were available from the BCON phase III randomized trial of radiotherapy alone or with carbogen and nicotinamide (CON). Gene expression data were generated for 151 tumors using Affymetrix Human 1.0 Exon ST arrays and used for independent validation.

Results: A 24-gene signature was derived, which was prognostic in four of six independent surgical cohorts ($n = 679$; meta HR, 2.32; 95% CI, 1.73–3.12; $P < 0.0001$). The signature was also prognostic in BCON patients receiving radiotherapy alone ($n = 75$; HR for local relapse-free survival, 2.37; 95% CI, 1.26–4.47; $P = 0.0076$). The signature predicted benefit from CON ($n = 76$; HR, 0.47; 95% CI, 0.26–0.86; $P = 0.015$). Prognostic significance ($P = 0.017$) and predictive significance ($P = 0.058$) remained after adjusting for clinicopathologic variables. A test for interaction between hypoxia status and treatment arms was significant ($P = 0.0094$).

Conclusions: A 24-gene hypoxia signature has strong and independent prognostic and predictive value for muscle-invasive bladder cancer patients. The signature can aid identification of patients likely to benefit from the addition of carbogen and nicotinamide to radiotherapy. *Clin Cancer Res; 23(16); 4761–8.* *©2017 AACR.*

Introduction

Muscle-invasive carcinoma is a high-risk bladder cancer subtype (1). Surgery is often the preferred treatment choice while radical radiotherapy, with the advantage of preserving the normal bladder function, is also an option (2). Hypoxia is a common micro-environmental component in most solid tumors and is associated with a poor prognosis in multiple cancer types (3–7). Adding concurrent hypoxia modification to radiotherapy can improve treatment outcomes with good evidence that more hypoxic tumors benefit most from the hypoxia-modifying therapy (4, 6, 8).

Gene expression signatures show promise for clinical application (7). To date, hypoxia gene signatures were successfully developed for head and neck, breast, and lung cancers, and demonstrated to be not only prognostic in multiple tumor types, but also predictive of benefit from hypoxia-modifying therapy in head and neck cancers (4, 6, 7, 9). However, current evidence suggests a need to tailor signatures for different types of tumors (3). Specifically, we (6) showed that a hypoxia gene signature developed using samples from head and neck cancer biopsies failed to predict the benefit of adding hypoxia-modifying treatment to radiotherapy for bladder cancer. The aim here, therefore, was to identify a hypoxia gene signature for muscle-invasive bladder cancer patients, which predicted benefit from hypoxia-modifying therapy. In order to achieve this, published signatures were tested and a bladder-specific signature derived using an approach previously proven effective by us (6, 9) and others (10). The BCON phase III trial which randomized bladder cancer patients to radiotherapy alone or with hypoxia modification was used as a cohort to validate the predictive performance of the signatures (11).

Materials and Methods

BCON cohort

The study was conducted in accordance with European GCP and approved by local research ethics committee (LREC 09/H1013/24). Written informed consent was obtained.

[1]Translational Radiobiology Group, Division of Cancer Sciences, University of Manchester, Manchester Academic Health Science Centre, Christie Hospital, Manchester, United Kingdom. [2]Applied Computational Biology and Bioinformatics Group, CRUK-MI, Manchester, United Kingdom. [3]HMDS, Leeds Cancer Centre, St James University Hospital, Leeds, United Kingdom. [4]Department of Cellular Pathology, Manchester Royal Infirmary, Manchester, United Kingdom. [5]Cancer Centre, Mount Vernon Hospital, Rickmansworth Road, Northwood, Middlesex, United Kingdom. [6]Department of Clinical Medicine, Aarhus University, Aarhus, Denmark. [7]Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Catharine M.L. West, University of Manchester, Christie Hospital, Wilmslow Road, Manchester, M20 4BX, UK. Phone: 44-161-446-8275; Fax: 44-161-446-8111; E-mail: catharine.west@manchester.ac.uk

doi: 10.1158/1078-0432.CCR-17-0038

Published OnlineFirst April 11, 2017; DOI: 10.1158/1078-0432.CCR-17-0038

Translational Relevance

Hypoxia is a common component in the microenvironment of most solid tumors, including bladder cancers. Adding concurrent hypoxia modification into radiotherapy improves the survival of patients, with good evidence that more hypoxic tumors benefit the most from hypoxia-modifying therapy. However, there are no clinically validated biomarkers that can select the muscle-invasive tumors that would benefit from adding hypoxia-modifying therapy to radical radiotherapy. A signature was derived from public databases and independently validated in a *de novo* cohort from the BCON trial, which randomly assigned patients to radiotherapy alone or with carbogen and nicotinamide. The signature was both prognostic and predictive of benefit from adding concurrent hypoxic modification to radiotherapy. The signature has translational relevance, as tumors from the BCON trial were FFPE samples, a routine technique for preserving tissues in hospitals. The signature can help identify patients likely to benefit from the addition of carbogen and nicotinamide to radiotherapy.

Pretreatment formalin-fixed, paraffin-embedded (FFPE) samples were available from the prospective BCON multicenter phase III clinical trial of radiotherapy alone or with carbogen and nicotinamide (CON). Patients had T1-T4a urothelial (transitional) cell bladder carcinoma and were randomized between November 2000 and April 2006. BCON patients received 55 Gy in 20 fractions in four weeks or 64 Gy in 32 fractions in 6.5 weeks daily, five times per week. Carbogen was given five minutes before and during radiotherapy. Nicotinamide (40–60 mg/kg) was given 1.5 to 2 hours before each fraction.

A power calculation determined that 150 samples were required to detect difference in hazard ratio with 0.01 significance and 80% power (assuming equal size of treatment arms). Supplementary Fig. S1 shows the Consort diagram for the study. Among the 152 BCON patients, 75 received RT and 77 received RT + CON. Table 1 describes the cohort demographics, which are comparable with the original clinical trial (Supplementary Table S1). One patient in the RT + CON arm was excluded from the signature validation as pathologic review was consistent with prostate rather than bladder cancer. Histopathology, RNA extraction, quality control and exon array hybridization are described in Supplementary Methods. The 151 BCON patients used in this study contained nine T1 tumors and one grade 2 tumor, where T2 or greater tumors and grade 3 tumors are associated with higher risk. The high-risk muscle-invasive nature of the cohort represents the target patient where the predictive signature proposed in this work should be the most relevant in clinical application.

Independent cohorts

One bladder cancer training cohort was curated from the cancer genome atlas project (TCGA; ref. 12). TCGA has the RNA-sequencing and clinical information for 408 fresh frozen muscle-invasive samples. Another six bladder cancer cohorts (GSE5287, GSE13507, GSE31684, GSE32894, GSE19915, and GSE1827) were also collected for independent validation. Procedures for normalization of transcriptomic data are provided in Supplementary Methods.

Endpoints and statistical analysis

In the BCON study, bladder tumor control was based on cyctoscopic examination at 6 months after treatment and at 6-monthly intervals for a total of 5 years. Regional and systemic assessment with CT was carried out as clinically indicated. Local relapse-free survival (LRFS) was taken as time to muscle-invasive tumor recurrence in bladder, locoregional failure, or death. Patients with persistent muscle-invasive disease or with no cystoscopy post treatment had their time set to zero. Overall survival (OS), defined as death from any cause, was the main clinical endpoint for surgical cohorts if available. Otherwise, disease-free survival (DFS), defined as death from cancer, was used as indicated in the original publications. Patients were censored at 5 years. The χ^2 test was used to compare proportions across the levels of categorical factors. The Mann–Whitney *U* test was used to compare median values for continuous variables between two groups. Survival estimates were performed using the Kaplan–Meier method and differences compared using the log-rank test. Hazard ratios (HR) and 95% confidence intervals (CI) were obtained using the Cox proportional hazard model. Hazard ratios from different cohorts were combined to produce a meta-score in a fixed effect model with generic inverse variance method. All *P* values were two sided, and statistical significance was set as 0.05.

Table 1. Clinicopathologic details by randomization arm

Variable	*N*	RT *N* = 75	RT + CON *N* = 76	*P*
Gender				
Male	115	55 (73.3%)	60 (78.9%)	
Female	36	20 (26.7%)	16 (21.1%)	0.54
Age (years)	151	75.5 (51.1–87.0)	75.1 (51.5–89.7)	0.66
T stage				
T1	9	0 (0%)	9 (11.8%)	
T2	108	54 (72.0%)	54 (71.1%)	
T3	30	19 (25.3%)	11 (14.5%)	
T4a	4	2 (2.7%)	2 (2.6%)	0.01
Grade				
2	1	0 (0%)	1 (1.3%)	
3	150	75 (100%)	75 (98.7%)	0.99
TURBT				
Biopsy	33	15 (20.0%)	18 (23.4%)	
Partial	58	24 (32.0%)	24 (31.6%)	
Complete	55	32 (42.7%)	33 (43.4%)	
No data	5	4 (5.3%)	1 (1.3%)	0.55
Hb (g/L)	149	13.7 (9.8–16.9)	13.9 (9.5–17.2)	0.42
No data	2	1 (1.3%)	1 (1.3%)	
Concurrent pTis				
Absent	117	50 (67.7%)	67 (88.2%)	
Present	34	25 (33.3%)	9 (11.8%)	0.003
Necrosis				
Absent	71	39 (52.0%)	32 (42.1%)	
Present	80	36 (48.0%)	44 (57.9%)	0.29

NOTE: Data represent median (range) or *n* (%). Most patients (151; 99%) received ≥90% of the prescribed RT. In the experimental arm, 66 patients (86%) received ≥ 90% of the stipulated carbogen doses and 50 patients (65%) received ≥90% of the stipulated nicotinamide doses. All analyses were conducted on an "intention to treat" basis.

Abbreviations: CON, carbogen and nicotinamide; Hb, hemoglobin; pTis, carcinoma *in situ*; RT, radiotherapy; TURBT, transurethral resection of bladder tumor.

Published OnlineFirst April 11, 2017; DOI: 10.1158/1078-0432.CCR-17-0038

Testing the predictive ability of published gene signatures

Seven hypoxia signatures developed for tumors of different origins with prognostic value being demonstrated in independent clinical cohorts were tested in BCON (4, 7, 9, 13–15). Five bladder cancer signatures (1, 16–19) were also evaluated. For each signature, a Cox model was trained with the TCGA cohort and hypoxia scores derived for BCON patients with frozen gene coefficients. BCON patients were stratified into high-hypoxia and low-hypoxia groups based on median cohort scores.

A novel bladder cancer hypoxia gene signature

To derive a bladder cancer–specific hypoxia signature, a candidate list of 611 generic hypoxia regulated genes was created from a recent literature review (3). Most of the curated genes are upregulated under hypoxia across different tumor sites. Similarly to the multi-seed approach used in our previous studies (7, 9, 20), we hypothesized that a candidate gene is likely to be hypoxia regulated in bladder cancer if coexpressing with multiple candidate genes. Therefore, a coexpression network was constructed with two genes connected if positively correlated (Spearman correlation ≥ 0.5) in TCGA (12). Hypoxia signature genes were selected as being prognostic (Cox $P < 0.05$) and associated with poor prognosis (HR > 1). Hypoxia gene signature score was computed as median of gene expressions for each tumor. Details of the method are available in Supplementary Methods. The derived signature was then frozen and tested in independent validation cohorts and BCON patients. Patients were stratified into high-hypoxia and low-hypoxia groups based on the median cohort hypoxia score. In a multivariate analysis of the BCON cohort, the hypoxic status/CON was adjusted for age of diagnosis, gender, tumor stage, necrosis, and presence of carcinoma in situ (CIS).

Results

Testing literature gene signatures

Among the published signatures tested, only the Lendahl hypoxia signature and Riester bladder cancer classifier had prognostic and/or predictive significance (Table 2, Supplementary Table S3).

Table 2. Prognostic and predictive significance of the literature signatures for LPFS analysis of the BCON cohort

Signature	BCON Prognosis[a]	BCON (high-hypoxia patients) prediction of benefit
Ragnum et al. (28)	0.882	0.389
Buffa et al. (7)	0.907	0.827
Winter et al. (9)	0.438	0.434
Betts et al. (13)	0.569	0.330
Toustrup et al. (4)	0.252	0.856
Toustrup et al.[b] (4)	0.653	0.664
Chi et al. (14)	0.120	0.078[c]
Lendahl et al. (15)	0.093[c]	0.038[d]
Mitra et al. (17)	0.960	0.530
Riester et al. (16)	0.002[e]	0.032[d]
Sanchez-Carbayo et al. (18)	0.260	0.140
Kim et al. (19)	0.478	0.541
Kim et al. (1)	0.855	0.880

[a]Prognosis was evaluated in patients receiving radiotherapy only, whereas prediction of benefit of CON was assessed with both high-hypoxia and low-hypoxia groups.
[b]Classification of samples into more or less hypoxic using the centroid method described in Toustrup et al.
Significance codes: 0.001.
[c]0.1; [d]0.05; [e]0.01.

Deriving a bladder cancer–specific hypoxia gene coexpression network

As described in the Materials and Methods, *in vivo* transcriptomic data were integrated with knowledge of gene functions to derive bladder cancer–specific hypoxia coexpression network and signature. The derived network comprised 168 candidate hypoxia genes with 458 significant interactions (Supplementary Fig. S2). The high number of interactions between the candidate genes indicates a good likelihood of their hypoxia relevance in bladder carcinoma. The final bladder cancer hypoxia signature derived comprised 24 genes, high expression of which was significantly associated with poor prognosis (Supplementary Table S4).

Copy number variation and methylation data were analyzed to investigate the underlying associations with the 24-gene panel. Copy number variation and methylation status was correlated with the expression of the 24 genes in our signature using data for TCGA tumor samples. The average correlation between copy number variation of the 24 genes and their corresponding gene expression across the *de novo* gene panel was 0.16. A null distribution was constructed by calculating the mean correlation values of 10,000 random gene sets of the same size from the whole platform. The correlation for the 24-gene panel was not significantly higher than the random gene sets ($P = 0.99$), suggesting that the expression levels of the signature genes were not driven by copy number alteration. A similar analysis was performed for methylation data, where the gene panel had an average correlation of -0.36 between methylation status and gene expression for the 24 genes, which was significantly lower than that of the random gene sets ($P = 0.0008$). This analysis suggests that there is a significant association between methylation status and the expression of the genes in our bladder signature.

Testing the *de novo* hypoxia signature in independent cohorts

The prognostic value of the frozen signature in chemotherapy or cystectomy-treated patients was validated in six publicly available bladder cancer cohorts totaling 679 patients, which were independent from the discovery cohort. Kaplan–Meier plots are provided in Fig. 1. Patients stratified as high hypoxia by the *de novo* signature were significantly associated with poor prognosis in GSE13507, GSE31684, GSE32894, and GSE1827. Similar trends can be observed in the other two cohorts (GSE5287 and GSE19915) where the signature had borderline significance. Meta-analysis of the six cohorts revealed a HR of 2.32 (95% CI, 1.73–3.12, $P < 0.0001$) for hypoxic tumors (Supplementary Fig. S3).

Testing the *de novo* hypoxia signature in BCON patients

In the BCON cohort, patients stratified as high hypoxia by the signature had higher tumor stage ($P = 0.03$; Supplementary Table S5). High hypoxia also corresponded to lower pretreatment hemoglobin levels ($P = 0.04$, Supplementary Table S5). Protein expression data for three well-known hypoxia biomarkers (CAIX, HIF-1α, and GLUT1) were available for 127, 92, and 95 of the BCON gene expression cohort, respectively (21). For each protein marker, tumors were stratified into two groups based on upper, median, or lower quartile. A *t*-test (two-tailed) was applied to determine if the 24-gene signature score was significantly different in high and low protein expression groups. The 24-gene signature score was significantly higher in tumors with high CAIX protein

Published OnlineFirst April 11, 2017; DOI: 10.1158/1078-0432.CCR-17-0038

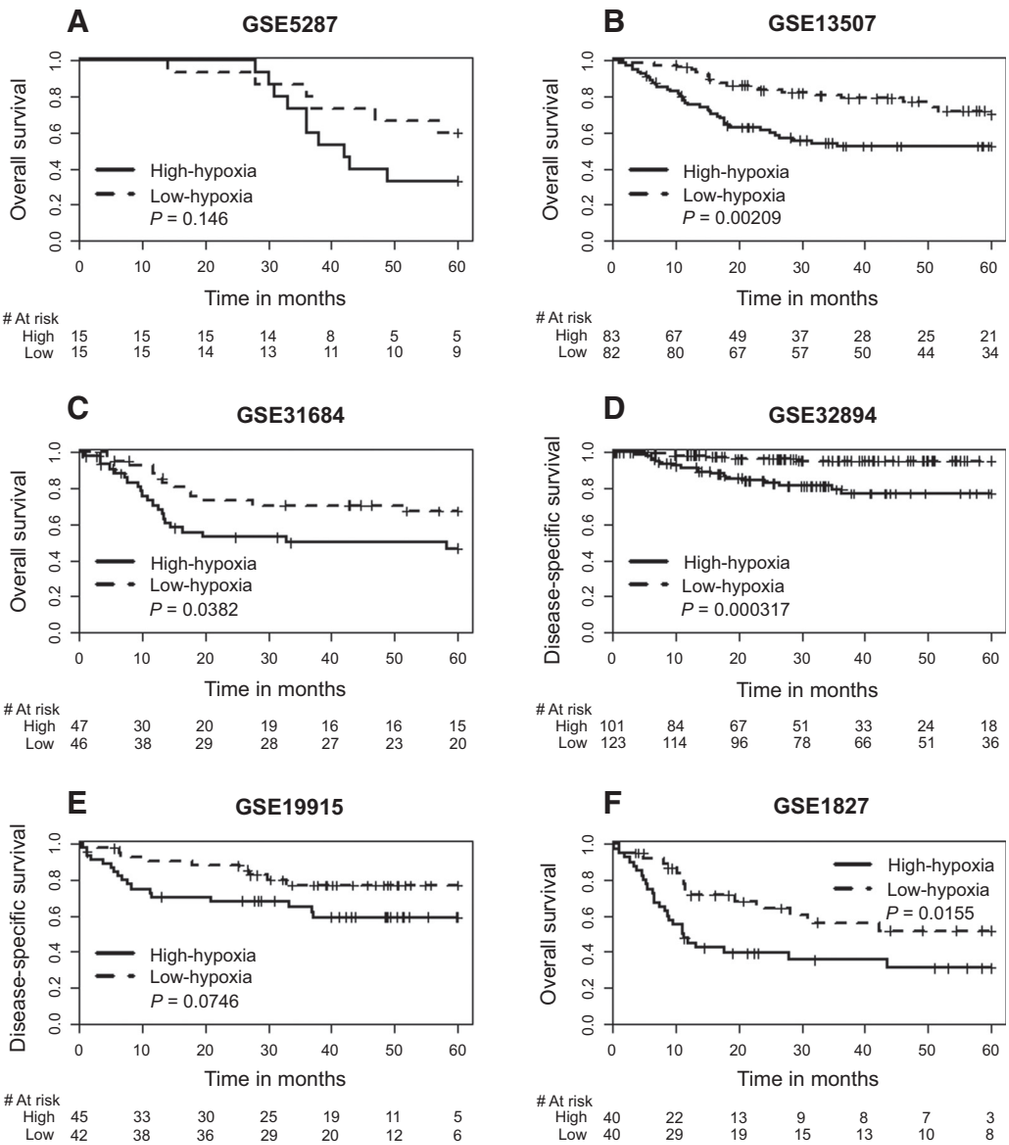

Figure 1.

A–F, Kaplan–Meier plots for independent validations of the 24-gene hypoxia signature in chemotherapy or surgery-treated cohorts.

expression ($P = 0.013$, upper quartile, Supplementary Fig. S4A). Tumors with high HIF-1α protein expression had a trend toward having significantly higher 24-gene signature score ($P = 0.081$, lower quartile, Supplementary Fig. S4B). No significant association was found between the signature and GLUT1 expression.

The prognostic value of the signature was examined in the BCON radiotherapy arm (Fig. 2A). Patients categorized as more hypoxic had a significantly poorer LPFS than those categorized as less hypoxic ($P = 0.0076$, HR 2.37, 95% CI 1.26–4.47). Tumor hypoxia retained prognostic significance in multivariate analysis ($P = 0.017$, HR 2.25; Table 3). The signature had no prognostic significance in patients receiving RT + CON (Fig. 2B).

The 24-gene signature also predicted benefit from adding CON to RT. Patients with high-hypoxia scores receiving RT + CON had an improved LPFS rate than those undergoing RT alone ($P = 0.015$, HR 0.47, 95% CI 0.26–0.86, Fig. 3A). Adding CON into RT was associated with borderline significance in multivariate analysis ($P = 0.058$, HR 0.52, Table 3). Patients with low-hypoxia scores derived no benefit in from CON ($P = 0.21$, Fig. 3B). Hazard ratio, 95 % confidence intervals, and *P* value for prognostic testing of RT-treated patients and patients with high hypoxia signature score were detailed in Table 3. A test for interaction between hypoxia signature and treatment with the entire cohort showed that response to CON was significantly different in more hypoxic tumors than normoxic tumors, for both binary hypoxic status ($P = 0.0094$) and continuous hypoxia score ($P = 0.045$).

The potential benefit of integrating the 24-gene signature with the Riester signature (16) was also investigated. Stratification of BCON patients into four categories based on both 24-gene hypoxia signature and Riester risk signature improved the

Published OnlineFirst April 11, 2017; DOI: 10.1158/1078-0432.CCR-17-0038

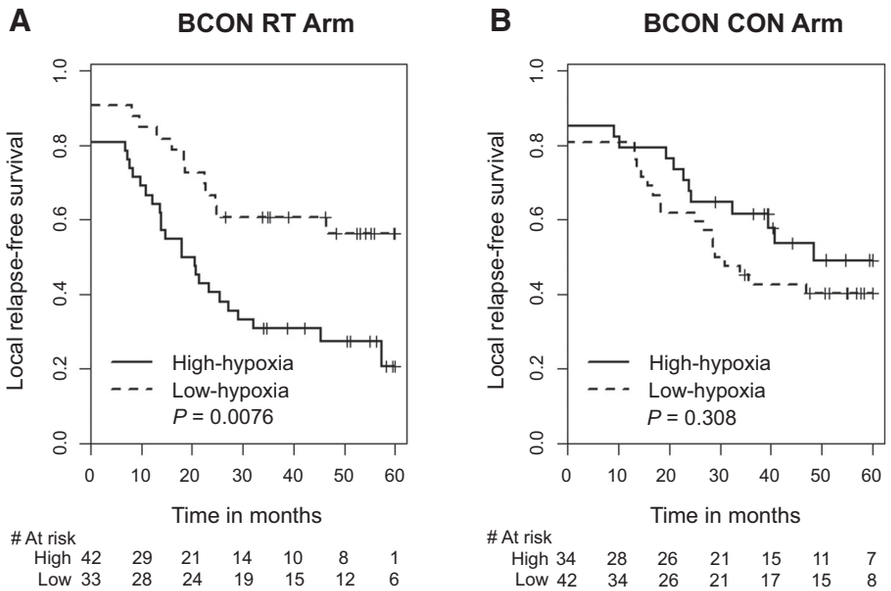

Figure 2.
Kaplan–Meier plots for BCON patients receiving (**A**) RT alone (**B**) RT plus CON. Patients were stratified into high-hypoxia and low-hypoxia by the 24-gene signature. Patients with persistent muscle-invasive disease or with no cystoscopy after treatment had their time set to zero.

prognostic value on patients receiving RT only (Supplementary Fig. S5). Adding the Riester signature into the *de novo* hypoxia signature also led to increased predictive significance in patients with both high hypoxia and high Riester signature scores ($n = 40$, $P = 0.0082$, HR 0.32, 95% CI 0.14–0.75, Supplementary Fig. S6). Adding the Lendahl signature (15) into the 24-gene signature did not improve its prognostic significance (Supplementary Fig. S7) but resulted in higher predictive power ($n = 43$, $P = 0.014$, HR 0.32, 95% CI 0.13–0.79; Supplementary Fig. S8).

The prognostic and predictive performance of CAIX, HIF-1α, GLUT1, and necrosis was assessed in the BCON gene expression cohort used in this study. For each protein marker, tumors were stratified into two groups based on upper, median, or lower quartile. Tumors with high CAIX expression (upper quartile split) had poorer LPFS when treated with RT alone ($n = 64$, $P = 0.022$, HR 2.21, 95% CI 1.12–4.37) and derived benefit from CON ($n = 32$, $P = 0.017$, HR 0.32, 95% CI 0.13–0.82). A significant interaction between CAIX status and intervention was detected ($P = 0.019$). High GLUT1 expression (median split) had no prognostic value in RT-treated patients ($P = 0.20$), while there was a trend for significance in prediction of benefit of CON ($n = 48$, $P = 0.077$, HR 0.51, 95% CI 0.24–1.08). A test of interaction also showed borderline significance ($P = 0.096$). No prognostic or predictive significance was found for HIF-1α expression in this cohort. Necrosis was associated with a poor prognosis ($n = 75$, $P = 0.029$, HR 1.97, 95% CI 1.08–3.60), predictive value ($n = 80$, $P = 0.0051$, HR 0.43, 95% CI 0.24–0.78), and a significant interaction ($P = 0.002$). The potential benefit of combining the 24-gene signature with necrosis was then investigated. CON was associated with lower HR in tumors having both high 24-gene signature score and necrosis ($n = 45$, $P = 0.015$, HR 0.37) than with either high 24-gene signature score ($n = 76$, HR 0.47) or necrosis ($n = 80$, HR 0.43), indicating that combining the two markers identifies the patients that benefit the most from CON. In tumors with either high signature score or necrosis, CON was associated with comparable benefit ($n = 111$, HR 0.49) than in those having either high signature score or necrosis, suggesting that combining the two markers may also identify a wider range of patients that can benefit from hypoxia-targeting therapy.

Discussion

This study showed that the Lendahl hypoxia signature (15) was predictive of benefit from hypoxic modification but not

Table 3. Multivariate analysis of hypoxia signature and pathologic variables for the BCON study

		Univariate		Multivariate	
	Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
RT arm	Hypoxia	2.37 (1.26–4.47)	0.0076[a]	2.25 (1.16–4.39)	0.017[b]
	Stage 3 or 4a	0.59 (0.28–1.23)	0.18	0.44 (0.21–0.95)	0.036[b]
	Male	0.86 (0.45–1.64)	0.65	0.82 (0.42–1.63)	0.58
	Age	1.02 (0.98–1.06)	0.34	1.02 (0.98–1.06)	0.40
	CIS	2.06 (1.13–3.74)	0.018[b]	2.23 (1.18–4.22)	0.013[b]
	Necrosis	2.10 (1.16–3.80)	0.015[b]	2.21 (1.18–4.16)	0.013[b]
High-hypoxia	CON	0.47 (0.26–0.86)	0.015[b]	0.52 (0.26–1.02)	0.058[c]
	Stage 3 or 4a	0.58 (0.29–1.17)	0.13	0.49 (0.24–1.01)	0.052[c]
	Male	0.90 (0.49–1.67)	0.75	0.93 (0.49–1.74)	0.81
	Age	1.03 (0.99–1.08)	0.094[c]	1.03 (0.99–1.08)	0.14
	CIS	2.93 (1.59–5.39)	0.0006[d]	2.63 (1.30–5.32)	0.0069[a]
	Necrosis	1.17 (0.66–2.1)	0.59	1.47 (0.78- 2.77)	0.24

Significance codes: 0.0001.
[a]0.01; [b]0.05; [c]0.1; [d]0.001.

Published OnlineFirst April 11, 2017; DOI: 10.1158/1078-0432.CCR-17-0038

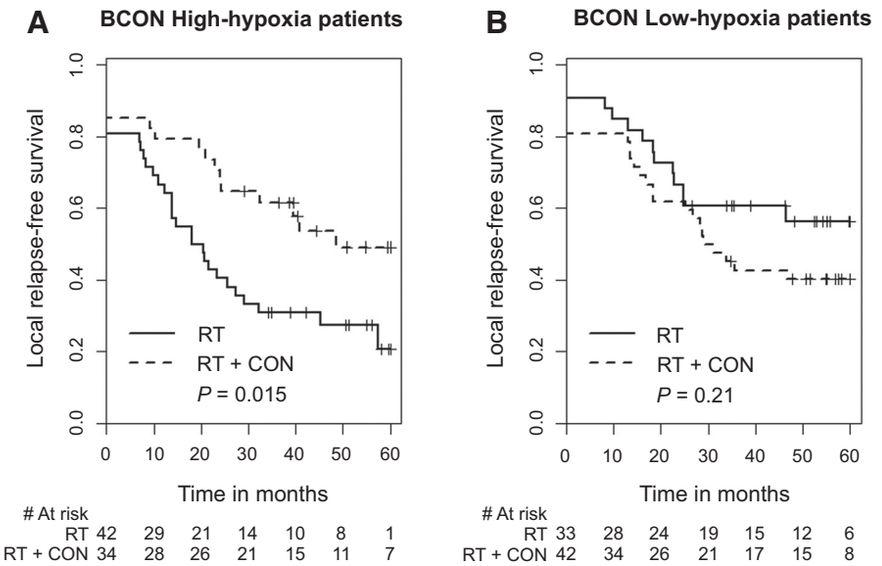

Figure 3.
Kaplan–Meier plots for BCON patients stratified as either (**A**) high-hypoxia or (**B**) low-hypoxia by the 24-gene signature. In each group, patients received either RT or RT plus CON. Patients with persistent muscle-invasive disease or with no cystoscopy after treatment had their time set to zero.

prognostic in muscle-invasive bladder carcinomas. None of the other hypoxia signatures were prognostic or predictive. A *de novo* 24-gene signature was independently validated in multiple publically available cohorts and the new BCON cohort and shown to be prognostic and predictive of benefit from the addition of CON to radiotherapy.

Comparison of the *de novo* 24-gene signature with other published hypoxia signatures showed little overlap. This lack of overlap between genes in hypoxia signatures is well documented and not surprising given the large proportion of the genome that is transcriptionally responsive to changes in oxygenation (3). Four genes (*CAV1*, *P4HA2*, *DPYSL2*, and *SLC2A3*) from the 24-gene signature also appeared in a hypoxia signature published from another group (14). Similarly, two genes (*SLC16A1* and *LDLR*) were common between the 24-gene signature and a head and neck signature prognostic in multiple cancer types (9). There is one overlap (*SLC16A1*) between the 24-gene signature and a 26-gene signature derived in HNSCC by our group (13). This 26-gene signature predicted benefit from CON in patients with laryngeal tumors (ARCON phase III trial) but not bladder tumors (BCON; ref. 6). The heterogeneity between signatures could reflect the importance of different biological pathways involved in the tumor hypoxia response in different cancer types. There is likely to be tissue-dependent patterns of transcription modifiers interacting with HIF, as shown recently for the effects of the BET inhibitor JQ1, which modifies a subset of HIF target genes (22).

The limited overlap could also result from differences in the methods for signature derivation (e.g., different starting seeds), and the use of cell lines versus clinical samples from different tumor types. On the other hand, any commonality observed is likely due to the conserved portion of the transcriptional response to hypoxia which is independent of hypoxia output (7, 14, 15, 23). Harris and colleagues (3) performed a systematic review and identified 32 published hypoxia gene signatures. No gene was found in all signatures, but 20 were identified as being the most prevalent. One of these 20 genes, *P4HA2*, was in the 24-gene signature.

Hypoxia can promote the stabilization of genes that promote somatic copy number gains in tumors. It has been suggested that generation of transient copy number gains could be an adaptive cellular response of cells to stresses such as hypoxia and therefore providing a mechanism for the generation of tumor heterogeneity (24). Our work showed no association between the expression of the 24 genes in our signature with copy number variation in the 24 genes showing no direct association, i.e., the genes do not appear to be modified at the DNA level. In contrast, there appeared to be direct associations with methylation status for the 24 genes with higher methylation associated with lower expression of the individual genes, consistent with a gene silencing effect. Hypoxia leads to epigenetic alterations and some genes are transcriptionally repressed under hypoxia due to histone modification. Hypoxia, however, has also been linked to a global reduction in methylation (25). Further research would be needed to assess the effects of hypoxia in bladder cancer on global copy number alteration and methylation status.

The 24-gene signature was validated in independent publically available cohorts of 679 patients with fresh frozen tissues. The 24-gene signature has translational relevance as it was validated in FFPE samples, a routine technique for preserving tissues in hospitals. It is widely agreed that degradation and chemical modification of nucleic acids in FFPE samples reduce RNA quality (26), which further supports the robustness of the signature in clinical application. The results of the current study clearly indicate that the derived gene signature is worthy of further testing using widely abundant FFPE samples. It is noted that a head and neck hypoxia signature developed by Toustrup and colleagues (4) was validated in FFPE tumor biopsies, and a signature derived from fresh frozen soft tissue sarcoma samples was also prognostic on FFPE samples (27). Second, our group has already validated a hypoxia signature derived for head and neck cancer using a Taqman Low Density Array approach and shown low intra-assay, interassay and intratumor variability (13). Third, although the *de novo* signature was applicable to any hypoxia-targeting therapy, an intervention is available (CON) for use in patients. As our head and neck signature is undergoing prospective qualification in a randomized trial (10), the signature derived here can progress in a similar fashion. The BCON cohort is the first time in the literature that whole tumor transcriptome data have been generated for patients

recruited into a trial randomizing patients to radiotherapy alone or with hypoxia modification. This enables proper evaluation and comparison of the predictive performance of the *de novo* signature together with competing protein markers and clinicopathologic factors. A question of interest in terms of the next steps to take is whether to use the gene signature alone or in combination with another marker. Our analyses showed that a combination of simple histological assessment of necrosis with the 24-gene signature was superior to using either alone. The next step, therefore, would be to carry out a biomarker driven trial using the combination.

The use of median score as cutoff was prespecified in a power calculation to determine the number of BCON samples for gene expression profiling. In validation of the *de novo* signature, median score was therefore chosen as threshold for patient stratification to avoid bias and to provide balanced groups. The *de novo* 24-gene signature has reached the statistical significance in terms of predicting the benefit of CON in patients with high signature scores (Table 3, HR 0.47, 95 % CI 0.26–0.86, $P = 0.015$). A test for interaction between the 24-gene signature and intervention showed significance for the resulting binary hypoxic status (median cutoff, $P = 0.0094$), confirming that response to intervention is indeed different in patients in high and low hypoxia groups. Therefore, the 24-gene signature with the predefined median score cutoff confirms the hypothesis of the study and median score is a clinically relevant cutoff. Other thresholds have been used for hypoxic classification (4, 14, 23). However, there is no consensus on the ideal method to define tumors as hypoxic and no methodological study to date assessing and comparing the performance of the different methods exists. In a prospective clinical trial, the first ~50 patient samples could be used to generate a median threshold in newer FFPE blocks for classification. A strength of the study is the link with an intervention that could pave the way for a future hypoxia signature driven trial.

In conclusion, a hypoxia gene signature for muscle-invasive bladder cancer patients receiving radical radiotherapy was derived. Used as a potential tool for personalized medicine, the signature identifies more hypoxic tumors that have poorer outcome. The signature also predicts benefit from adding concurrent hypoxic modification to radiotherapy. The signature warrants final qualification in a prospective setting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P.J. Hoskin, A.L. Harris, A. Choudhury, C.M.L. West
Development of methodology: L. Yang, H. Denley, A.L. Harris, A. Choudhury, C.M.L. West
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Eustace, P.J. Hoskin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Yang, J. Taylor, J.J. Irlam, J. Alsner, F.M. Buffa, A.L. Harris, A. Choudhury
Writing, review, and/or revision of the manuscript: L. Yang, J. Taylor, A. Eustace, P.J. Hoskin, J. Alsner, F.M. Buffa, A.L. Harris, A. Choudhury, C.M.L. West
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.J. Irlam
Study supervision: P.J. Hoskin, A. Choudhury, C.M.L. West
Other (specialist histological review of patient material): H. Denley

Acknowledgments

The authors thank the Cancer Research UK Manchester Institute Cancer Research Molecular Biology Core Facility and the University of Manchester Clinical Immune and Molecular Monitoring Laboratory for use of Good Clinical Practice facilities. J. Taylor was funded by Cancer Research UK (grant C480/A12328). A. Eustace was funded by the Medical Research Council of the UK (grant G0801525. The work was also supported by Cancer Research UK (grant C1094/A11365), Experimental Cancer Medicine Centre funding. (C1467/A15578), and CRUK Major Centre funding. F.M. Buffa and A.L. Harris were funded by Cancer Research UK, EU Metoxia, and the NIHR Biomedical Research Centre, Oxford. The work was also supported by the NIHR Manchester Biomedical Research Centre.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 8, 2017; revised February 21, 2017; accepted April 5, 2017; published OnlineFirst April 11, 2017.

References

1. Kim W-J, Kim S-K, Jeong P, Yun S-J, Cho I-C, Kim IY, et al. A four-gene signature predicts disease progression in muscle invasive bladder cancer. Mol Med 2011;17:478–85.
2. Choueiri TK, Raghavan D. Chemotherapy for muscle-invasive bladder cancer treated with definitive radiotherapy: persisting uncertainties. Nat Clin Prac Oncol 2008;5:444–54.
3. Harris BHL, Barberis A, West CML, Buffa FM. Gene expression signatures as biomarkers of tumour hypoxia. Clin Oncol 2015;27:547–60.
4. Toustrup K, Sørensen BS, Nordsmark M, Busk M, Wiuf C, Alsner J, et al. Development of a hypoxia gene expression classifier with predictive impact for hypoxic modification of radiotherapy in head and neck cancer. Cancer Res 2011;71:5923–31.
5. Moeller BJ, Richardson RA, Dewhirst MW. Hypoxia and radiotherapy: opportunities for improved outcomes in cancer treatment. Cancer Metast Rev 2007;26:241–8.
6. Eustace A, Mani N, Span PN, Irlam JJ, Taylor J, Betts GNJ, et al. A 26-gene hypoxia signature predicts benefit from hypoxia-modifying therapy in laryngeal cancer but not bladder cancer. Clin Cancer Res 2013;19:4879–88.
7. Buffa FM, Harris AL, West CM, Miller CJ. Large meta-analysis of multiple cancers reveals a common, compact and highly prognostic hypoxia metagene. Br J Cancer 2010;102:428–35.
8. Eustace A, Irlam JJ, Taylor J, Denley H, Agrawal S, Choudhury A, et al. Necrosis predicts benefit from hypoxia-modifying therapy in patients with high risk bladder cancer enrolled in a phase III randomised trial. Radiother Oncol 2013;108:40–7.
9. Winter SC, Buffa FM, Silva P, Miller C, Valentine HR, Turley H, et al. Relation of a hypoxia metagene derived from head and neck cancer to prognosis of multiple cancers. Cancer Res 2007;67:3441–9.
10. Fox NS, Starmans MH, Haider S, Lambin P, Boutros PC. Ensemble analyses improve signatures of tumour hypoxia and reveal inter-platform differences. BMC Bioinformatics 2014;15:1–14.
11. Hoskin PJ, Rojas AM, Bentzen SM, Saunders MI. Radiotherapy With Concurrent Carbogen and Nicotinamide in Bladder Carcinoma. J Clin Oncol 2010;28:4912–8.
12. The Cancer Genome Atlas Research N, Weinstein JN, Collisson EA, Mills GB, Shaw KRM, Ozenberger BA, et al. The cancer genome atlas pan-cancer analysis project. Nat Genet 2013;45:1113–20.
13. Betts GNJ, Eustace A, Patiar S, Valentine HR, Irlam J, Ramachandran A, et al. Prospective technical validation and assessment of intratumour heterogeneity of a low density array hypoxia gene profile in head and neck squamous cell carcinoma. Eur J Cancer 2013;49:156–65.
14. Chi J-T, Wang Z, Nuyten DSA, Rodriguez EH, Schaner ME, Salim A, et al. Gene Expression Programs in Response to Hypoxia: Cell Type Specificity and Prognostic Significance in Human Cancers. PLoS Med 2006;3:e47.

Published OnlineFirst April 11, 2017; DOI: 10.1158/1078-0432.CCR-17-0038

15. Lendahl U, Lee KL, Yang H, Poellinger L. Generating specificity and diversity in the transcriptional response to hypoxia. Nat Rev Genet 2009;10:821–32.
16. Riester M, Taylor JM, Feifer A, Koppie T, Rosenberg JE, Downey RJ, et al. Combination of a Novel Gene Expression Signature with a Clinical Nomogram Improves the Prediction of Survival in High-Risk Bladder Cancer. Clin Cancer Res 2012;18:1323–33.
17. Mitra AP, Lam LL, Ghadessi M, Erho N, Vergara IA, Alshalalfa M, et al. Discovery and validation of novel expression signature for postcystectomy recurrence in high-risk bladder cancer. J Natl Cancer Inst 2014;106:pii: dju290.
18. Sanchez-Carbayo M, Socci ND, Lozano J, Saint F, Cordon-Cardo C. Defining Molecular Profiles of Poor Outcome in Patients With Invasive Bladder Cancer Using Oligonucleotide Microarrays. J Clin Oncol 2006; 24:778–89.
19. Kim W-J, Kim E-J, Kim S-K, Kim Y-J, Ha Y-S, Jeong P, et al. Predictive value of progression-related gene classifier in primary non-muscle invasive bladder cancer. Mol Cancer 2010;9:1–9.
20. Masiero M, Simões Filipa C, Han Hee D, Snell C, Peterkin T, Bridges E, et al. A Core Human Primary Tumor Angiogenesis Signature Identifies the Endothelial Orphan Receptor ELTD1 as a Key Regulator of Angiogenesis. Cancer Cell 2013;24:229–41.
21. Hunter BA, Eustace A, Irlam JJ, Valentine HR, Denley H, Oguejiofor KK, et al. Expression of hypoxia-inducible factor-1[alpha] predicts benefit from hypoxia modification in invasive bladder cancer. Br J Cancer 2014; 111:437–43.
22. da Motta LL, Ledaki I, Purshouse K, Haider S, De Bastiani MA, Baban D, et al. The BET inhibitor JQ1 selectively impairs tumour response to hypoxia and downregulates CA9 and angiogenesis in triple negative breast cancer. Oncogene 2017;6:122–32.
23. Fardin P, Barla A, Mosci S, Rosasco L, Verri A, Versteeg R, et al. A biology-driven approach identifies the hypoxia gene signature as a predictor of the outcome of neuroblastoma patients. Mol Cancer 2010;9:1–15.
24. Mishra S, Whetstine JR. Different Facets of Copy Number Changes: Permanent, Transient, and Adaptive. Mol Cell Biol 2016;36: 1050–63.
25. Ramachandran S, Ient J, Göttgens E-L, Krieg A, Hammond E. Epigenetic therapy for solid tumors: highlighting the impact of tumor hypoxia. Genes 2015;6:935.
26. Hall JS, Taylor J, Valentine HR, Irlam JJ, Eustace A, Hoskin PJ, et al. Enhanced stability of microRNA expression facilitates classification of FFPE tumour samples exhibiting near total mRNA degradation. Br J Cancer 2012;107:684–94.
27. Lesluyes T, Pérot G, Largeau MR, Brulard C, Lagarde P, Dapremont V, et al. RNA sequencing validation of the Complexity INdex in SARComas prognostic signature. Eur J Cancer 2016;57:104–11.
28. Ragnum HB, Vlatkovic L, Lie AK, Axcrona K, Julin CH, Frikstad KM, et al. The tumour hypoxia marker pimonidazole reflects a transcriptional programme associated with aggressive prostate cancer. Br J Cancer 2015; 112:382–90.

Published OnlineFirst April 11, 2017; DOI: 10.1158/1078-0432.CCR-17-0038

AACR American Association for Cancer Research

Clinical Cancer Research

A Gene Signature for Selecting Benefit from Hypoxia Modification of Radiotherapy for High-Risk Bladder Cancer Patients

Lingjian Yang, Janet Taylor, Amanda Eustace, et al.

Clin Cancer Res 2017;23:4761-4768. Published OnlineFirst April 11, 2017.