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Research Paper

Development and Validation of a 28-gene Hypoxia-related Prognostic Signature for Localized Prostate Cancer

Lingjian Yang [a], Darren Roberts [a], Mandeep Takhar [b], Nicholas Erho [b], Becky A.S. Bibby [a], Niluja Thiruthaneeswaran [a,p], Vinayak Bhandari [c,q], Wei-Chen Cheng [d], Syed Haider [d,e], Amy M.B. McCorry [f], Darragh McArt [f], Suneil Jain [f], Mohammed Alshalalfa [b], Ashley Ross [g], Edward Schaffer [h], Robert B. Den [i], R. Jeffrey Karnes [j], Eric Klein [k], Peter J. Hoskin [l], Stephen J. Freedland [m], Alastair D. Lamb [n,o], David E. Neal [o], Francesca M. Buffa [d,e], Robert G. Bristow [p,r], Paul C. Boutros [c,r], Elai Davicioni [b], Ananya Choudhury [a], Catharine M.L. West [a,s,*]

[a] Translational Radiobiology Group, Division of Cancer Sciences, University of Manchester, Manchester Academic Health Science Centre, Christie Hospital, Manchester M20 4BX, UK
[b] GenomeDx Biosciences, Vancouver, BC, Canada
[c] Informatics & Biocomputing Program, Ontario Institute for Cancer Research, Toronto, Canada
[d] Department of Oncology, University of Oxford, Oxford OX3 7DQ, UK
[e] Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DS, UK
[f] Centre for Cancer Research and Cell Biology, Queen's University Belfast, Belfast BT9 7BL, Northern Ireland, UK
[g] James Buchanan Brady Urological Institute, Johns Hopkins Medical Institutions, Baltimore, MD, USA
[h] Northwestern Feinberg School of Medicine, Chicago, IL, USA
[i] Department of Radiation Oncology, Sidney Kimmel Medical College at Thomas Jefferson University, Philadelphia, PA, United States
[j] Department of Urology, Mayo Clinic Rochester, MN, USA
[k] Glickman Urological Institute, Cleveland Clinic, Cleveland, OH, USA
[l] Mount Vernon Cancer Centre, Rickmansworth Road, Northwood, Middlesex HA6 2RN, UK
[m] Department of Surgery, Division of Urology, Center for Integrated Research on Cancer and Lifestyle, Samuel Oschin Comprehensive Cancer Center, Cedars-Sinai Medical Center, Los Angeles, CA, USA
[n] Cancer Research UK Cambridge Institute, University of Cambridge, Cambridge CB2 0RE, UK
[o] Nuffield Department of Surgical Sciences, University of Oxford, Old Road Campus Research Building, Headington, Oxford OX3 7DQ, UK
[p] Princess Margaret Cancer Centre, University Health Network, Toronto, Canada
[q] Sydney Medical School, University of Sydney, Sydney, Australia
[r] Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada
[s] NIHR Manchester Biomedical Research Centre, Central Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre, UK

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ABSTRACT

Background: Hypoxia is associated with a poor prognosis in prostate cancer. This work aimed to derive and validate a hypoxia-related mRNA signature for localized prostate cancer.
Method: Hypoxia genes were identified *in vitro* via RNA-sequencing and combined with *in vivo* gene co-expression analysis to generate a signature. The signature was independently validated in eleven prostate cancer cohorts and a bladder cancer phase III randomized trial of radiotherapy alone or with carbogen and nicotinamide (CON).
Results: A 28-gene signature was derived. Patients with high signature scores had poorer biochemical recurrence free survivals in six of eight independent cohorts of prostatectomy-treated patients (Log rank test $P < .05$), with borderline significances achieved in the other two ($P < .1$). The signature also predicted biochemical recurrence in patients receiving post-prostatectomy radiotherapy ($n = 130$, $P = .007$) or definitive radiotherapy alone ($n = 248$, $P = .035$). Lastly, the signature predicted metastasis events in a pooled cohort ($n = 631$, $P = .002$). Prognostic significance remained after adjusting for clinic-pathological factors and commercially available prognostic signatures. The signature predicted benefit from hypoxia-modifying therapy in bladder cancer patients (intervention-by-signature interaction test $P = .0026$), where carbogen and nicotinamide was associated with improved survival only in hypoxic tumours.

* Corresponding author at: Translational Radiobiology Group, University of Manchester, Manchester Academic Health Centre, Christie Hospital, Wilmslow Road, Manchester M20 4BX, UK.
E-mail address: catharine.west@manchester.ac.uk (C.M.L. West).

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Conclusion: A 28-gene hypoxia signature has strong and independent prognostic value for prostate cancer patients.

1. Introduction

Ninety percent of prostate cancer (PCa) patients are diagnosed with localized carcinoma, which have a highly variable course of disease progression. Hypoxia is a common micro-environmental feature in most solid tumours, which leads to changes in transcriptomic profiles, a higher potential to metastasise and resistance to radiotherapy [1]. Localized prostate cancer has marked and heterogeneous hypoxia [2], and hypoxia is an adverse prognostic feature [3–5]. Combining hypoxia-targeting treatment with radiotherapy was shown to improve local control of tumours and survival of patients in head and neck and bladder cancers [6–9]. There is good evidence from the literature that the most hypoxic tumours benefit the most from hypoxia-modifying therapy. However, there is no clinically validated method of selecting prostate cancer patients who would benefit from hypoxia modifying treatment.

Hypoxia gene signatures were successfully derived for multiple tumour sites including head and neck, bladder, soft tissue sarcoma and cervical cancers, which were not only independently prognostic but also predictive of benefit from hypoxia-modifying therapy in head and neck and bladder cancers [10–18]. Work in prostate cancer showed some prognostic significance for signatures derived in other tumour types [3] or associated with the hypoxia marker pimonidazole [15].

We previously showed that hypoxia gene signatures are better if tumour site specific [19]. The aim of this study was to generate and validate a prostate cancer-specific transcriptomic signature. *In vitro* analysis of genes regulated by hypoxia was combined with *in vivo* analysis of a gene co-expression network and patient survival using data from a retrospective cohort. Following signature derivation, validation was performed in multiple independent cohorts.

2. Methods

2.1. Patient Cohorts

This study included patients with prostate carcinoma from twelve cohorts, including the cancer genome atlas project (TCGA) [20], GSE54460 [21], GSE21032 [22], CPC-GENE [23], Cambridge [24,25], six retrieved from the Decipher GRID™ prostate cancer database (NCT02609269) [26–31], and one from Belfast [32]. A summary of the cohorts and procedures for pre-processing of transcriptomic data are provided in Supplementary Table 1 and Supplementary Methods. Patient cohort characteristics are summarised in Supplementary Table 2. The TCGA cohort was used as the training cohort. In addition, bladder cancer patients enrolled in a randomized trial of hypoxia-modifying therapy were available in the BCON cohort [6,18]. Informed consent protocols were approved by local Institutional Review Boards.

2.2. Endpoints and Statistical Analysis

Biochemical recurrence (BCR) free survival was the clinical endpoint of this study in all but one cohort. Distant metastasis (DMET) free survival was the clinical endpoint of the other cohort. Patient follow up data were censored at 5-year. The signature derived from this work assigned one hypoxia signature score for each individual tumour and in each cohort patients were stratified into high vs. low hypoxia based on the median cohort signature score. Survival estimates were performed using the Kaplan-Meier method. The Log-rank test was used to test the null hypothesis of equality of survival distributions. Hazard ratios (HR) and 95% confidence intervals (CI) were obtained using the Cox proportional hazard model. In case-cohort studies (originally designed to sample the adverse pathology population to estimate risk of metastasis after radical prostatectomy), randomly sampled sub-cohorts were used in order to reduce over-estimation of events in evaluation of the BCR endpoint. Odds ratios (OR) were obtained using logistic regression models. Association between hypoxia signature score and Gleason group, tumour stage was estimated using simple linear regression. All *P*-values were two sided and statistical significance was set as 0.05.

2.3. Prostate Cancer Hypoxia Signature Development

A network-based methodology was applied for generation of a prostate cancer-specific hypoxia signature, hypothesising that *in vitro* hypoxia regulated genes co-expressing with each other *in vivo* collectively indicate tumour hypoxia. Briefly, genes up- and down-regulated by hypoxia (1% O_2, 24 h) were identified with RNA sequencing in four PCa cell lines (PNT2-C2, LNCaP, DU-145, and PC-3). The four cell lines were chosen as they were derived from human tissues and are widely used as *in vitro* PCa models [33]. A gene co-expression network was constructed in a TCGA training cohort and a putative gene module enriched with in vitro hypoxia genes was identified. Genes within the putative module were ranked by their connectivity and added iteratively into the signature. The final signature was selected based on prognostic significance. The signature scores are a continuous variable, which were then binarised into high and low categories using the median value for each cohort. The hypoxia signature derived here was then independently validated in the other eleven PCa cohorts. In multivariable analysis, the hypoxia gene signature was adjusted for standard clinic-pathological factors and a genomic classifier Decipher [27]. More details were given in Supplementary Methods.

2.4. Comparison with Literature Transcriptomic and Genomic Signatures

Seven hypoxia [10,11,14–17] and eleven prostate cancer transcriptomic signatures [34–44]were curated from the literature and their prognostic significances were evaluated for comparison (Supplementary methods). Furthermore, the potential benefit of combining the de novo hypoxia signature with a prognostic 31-loci DNA classifier [45] was also investigated by entering both into a Cox model.

3. Results

3.1. In Vitro Hypoxia Responsive Genes

Genes up or down regulated under hypoxia in four PCa cell lines were identified. 84, 306 and 848 genes were differentially expressed in greater than four, three and two cell lines, respectively. A seed gene database was constructed consisting of the 848 genes differentially expressed in two or more cell lines (Supplementary Table 3). Eight cell cycle and metabolism pathways were enriched in the hypoxia seed gene dataset (Supplementary Table 4). For many of the above identified genes, their regulation under hypoxia was well documented [46,47].

3.2. In Vivo Prostate Cancer-specific Hypoxia Gene Module

A PCa gene co-expression network was built from pre-treatment tumour biopsies in the TCGA cohort. The network, containing 1856 genes (including 113 seed genes) of good variability was partitioned into 34

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