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Comparison of multiple gene expression platforms for measuring a bladder cancer hypoxia signature

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Abstract. Tumour hypoxia status provides prognostic information and predicts response to hypoxia-modifying treatments. A previous study by our group derived a 24-gene signature to assess hypoxia in bladder cancer. The objectives of the present study were to compare platforms for generating signature scores, identify cut-off values for prospective studies, assess intra-tumour heterogeneity and confirm hypoxia relevance. Briefly, RNA was extracted from prospectively collected diagnostic biopsies of muscle invasive bladder cancer (51 patients), and gene expression was measured using customised Taqman Low Density Array (TLDA) cards, NanoString and Clariom S arrays. Cross-platform transferability of the gene signature was assessed using regression and concordance analysis. The cut-off values were the cohort median expression values. Intra- and inter-tumour variability were determined in a retrospective patient cohort (n=51) with multiple blocks (2-18) from the same tumour. To demonstrate relevance, bladder cancer cell lines were exposed to hypoxia (0.1% oxygen, 24 h), and extracted RNA was run on custom TLDA cards. Hypoxia scores (HS) values showed good agreement between platforms: Clariom S vs. TLDA (r=0.72, P<0.0001; concordance 73%); Clariom S vs. NanoString (r=0.84, P<0.0001; 78%); TLDA vs. NanoString (r=0.80, P<0.0001; 78%). Cut-off values were 0.047 (TLDA), 7.328 (NanoString) and 6.667 (Clariom S). Intra-tumour heterogeneity in gene expression and HS (coefficient of variation 3.9%) was less than inter-tumour (7.9%) variability. HS values were higher in bladder cancer cells exposed to hypoxia compared with normoxia (P<0.02). In conclusion, the present study revealed that application of the 24-gene bladder cancer hypoxia signature was platform agnostic, cut-off values determined prospectively can be used in a clinical trial, intra-tumour heterogeneity was low and the signature was sensitive to changes in oxygen levels *in vitro*.

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Introduction

Patients with muscle-invasive bladder cancer (MIBC) are treated with either bladder-preserving radiotherapy or cystectomy (1,2). Whilst cystectomy patients have about a 50% probability of surviving for 5 years, radiotherapy can achieve complete response in up to 70% of patients (3). However 50% of patients will develop metastases and their 5-year survival rate is between 20 and 30%. Survival is improved by adding chemotherapy (1,2).

High levels of hypoxia in solid tumours are associated with an adverse prognosis, which led to interest in developing approaches for its measurement and targeting (4). The UK BCON (bladder carbogen nicotinamide) trial showing that giving hypoxia-modifying carbogen and nicotinamide (CON) with radiotherapy improved the 3-year overall rate by 13% for patients with bladder cancer (5,6). As there are standard-of-care options for MIBC patients, our group developed a signature to assess tumour hypoxia and explored its potential for identifying patients likely to benefit from having radiotherapy plus CON.

The derivation of the signature and validation in multiple bladder cancer patient cohorts is described in an earlier publication (7). A gene expression network was built in a discovery cohort using a list of 611 seed genes shown to be upregulated in hypoxia across multiple tumour types (7). A seed gene was considered most likely to be relevant for bladder cancer if co-expressed with other seed genes. A final list of 24 genes was selected from the network as those that were prognostic and associated with a poor prognosis in the discovery cohort. The signature was then validated for prognostic significance in multiple independent cohorts.

Hypoxia scores were calculated as the median expression value of the 24 signature genes in each sample. Individual cohort median hypoxia scores were used as a cut-off as it was pre-specified in a power calculation (7). Using the pre-defined median cut-off value the signature was shown to be prognostic in a meta-analysis of six cohorts (n=679). We then showed the signature predicted benefit from having CON with radiotherapy in patients recruited into the BCON trial (7). Our published signature validation work used gene expression data generated from fresh frozen or archival formalin fixed paraffin embedded (FFPE) tissue. As RNA levels in FFPE samples decline over time (8), implementing a hypoxia signature clinically requires deriving a cut-off using recently obtained FFPE material. We also needed to identify a platform for generating hypoxia scores prospectively in a future biomarker-driven clinical trial, identify whether intra-tumour heterogeneity might be a barrier to implementation and confirm that hypoxia scores increased in bladder cancer cell lines in response to hypoxia. The objectives of the work reported here, therefore, were to: i) derive cut-off values using RNA extracted from recently diagnosed MIBC patients that could be used in a clinical trial; ii) demonstrate that the signature can be implemented using several platforms; iii) determine the reliability of signature scores generated from multiple samples from the same patient; and iv) investigate whether hypoxia scores increased *in vitro* in cell lines exposed to low oxygen. The three platforms we selected to compare for our 24-gene signature were qPCR (TLDA), NanoString (nCounter) and microarray (Affymetrix Clariom S).

Materials and methods

Patient samples. Samples were obtained for two cohorts of MIBC patients. Ethics approval was obtained from the institute where the experiments were performed (Manchester Cancer Research Centre Biobank; research tissue bank ethics reference: 18/NW/0092). All patients provided written consent for use of their pre-treatment FFPE MIBC tissue in research. The ethics application was fully reviewed permitting publication of the research findings. The mean (range) sample age of a prospectively collected cohort (n=51) at the time of processing was 6 (3-8) months. The second, retrospective, cohort (n=51) provided multiple pre-treatment FFPE MIBC samples (2-18 per patient) from 10 patients. The mean (range) sample age was 7 (5.7-9.3) years.

Two 10 μm sections were taken per sample for RNA extraction. An adjacent 4 μm section underwent histological review by an experienced specialist uropathologist (HD) and used to ensure tumour cellularity was >30%. RNA was extracted from the two 10 μm sections using the Roche High Pure FFPET RNA isolation kit. RNA quantification and purity were determined on a NanoDrop UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc.) and a Qubit fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.). RNA extracted from the recent samples was run on TLDA cards, NanoString nSolver and Clariom S array. The older samples were assayed using Clariom S arrays.

Reverse transcription and pre-amplification. Complementary DNA (cDNA) was generated using a high-capacity RNA-to-cDNA kit (Applied Biosciences; Themo Fisher Scientific, Inc.). One sample of cDNA was subject to pre-amplification using a custom preamp pool mix. This consisted of primers to genes present on the TaqMan human EC card array (Applied Biosystems) and a preamp TaqMan Fast Advanced Master Mix (2X; Thermo Fisher Scientific, Inc.). The pre-amplification step involved 14 cycles on a 2720 thermal cycler.

Applied Biosystems TLDA cards. Custom TLDA cards were generated using a gene selector tool (Thermo Fisher Scientific, Inc.) that identified probes for the signature genes (Table I), ECs (9) and the hypoxia-sensitive marker, carbonic anhydrase 9 (CA9). Following reverse transcription and pre-amplification, cDNA was loaded onto the TLDA cards and subject to qPCR on a Quantstudio12 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each HS was generated using the following formula: $2^{-(\text{median of signature genes-geomean of the endogenous controls})}$.

NanoString nSolver customised panel. A customised codeset was designed by NanoString technologies (NanoString Technologies) for a gene panel comprising the 24 hypoxia signature genes, eight ECs selected as previously described (9) and CA9. Samples were prepared at a concentration of 20 ng/μl from stock RNA. The samples (5 μl) were loaded into individual wells in 12 tube PCR hydridisation strips containing 3 μl reporter codeset, 5 μl hybridisation buffer and 2 μl capture probeset. The strips were then frozen and submitted to the University of Manchester Genomics Core Facility Hub for processing. Sixty ROC and RLF files were uploaded into nSolver 4.0, downloaded from NanoString Technologies website (NanoString.com). Positive control and codeset normalisations were carried out by calculating a scaling factor based on the geomean of the positive controls (A-E) and the ECs respectively.

Affymetrix Clariom S full transcriptome arrays. RNA extracted from both the recent and older sample cohorts were subject to Clariom S analysis. RNA (8 ng/μl in a 9 μl volume) was prepared for gene expression arrays with the Clariom S pico HT human assay (Thermo Fisher Scientific, Inc.). Sample hybridisation on Clariom S arrays was carried out by Yourgene Health (Manchester, UK). Batches of CEL files were GC SST (Signal Space Transformation with probe Guanine Cytosine Count Correction) RNA normalised using Affymetrix Array Power Tools (https://www.thermofisher.com/uk/en/home/life-science/microarray-analysis/microarray-analysis-partners-programs/affymetrix-developers-network.html). The $\log_2$ summarised gene level expression values generated were batch corrected using the ComBat function from the Bioconductor package sva. Hypoxia scores were calculated as the median expression of the 24 signature genes.

Cell lines and hypoxia experiments. Authenticated bladder cancer cell lines J82 and RT112 (American Type Culture Collection) were screened routinely for mycoplasma. Cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum (FBS) (Gibco) and 2 mM L-glutamine in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37˚C. Although ATCC recommends RPMI-1640 (9 mM glucose) for RT112, EMEM was used for both cell lines because: i) it has a glucose content of 4.5 mM, which is similar

Table I. Signature genes with the Thermo Fisher gene probe.

Signature gene	Gene probe
CAV1	Hs00971716_m1
COL5A1	Hs00609133_m1
ITGA5	Hs01547673_m1
P4HA2	Hs00990001_m1
SLC16A1	Hs01560299_m1
TGFB1	Hs00998133_m1
DPYSL2	Hs00265851_m1
SRPX	Hs00959148_m1
TRAM2	Hs00950945_m1
SYDE1	Hs00973080_m1
LRP1	Hs00233856_m1
PDLIM2	Hs00917389_m1
SAV1	Hs00560416_m1
AHNAK2	Hs00292832_m1
CAD	Hs00983188_m1
CYP1B1	Hs00164383_m1
DAAM1	Hs00982998_m1
DSC2	Hs00951428_m1
SLC2A3	Hs00359840_m1
FUT11	Hs00543033_m1
GLG1	Hs00939452_m1
GULP1	Hs01061497_m1
LDLR	Hs01092524_m1
THBS4	Hs00170261_m1

to the concentration found in human plasma; and ii) excess glucose affects the expression of glycolytic enzymes and other hypoxia-response genes (10,11). For each cell line, 10 cm diameter petri-dishes containing 10 ml of medium were seeded using $0.5x10^6$ cells and incubated at 37˚C in a humidified 5% CO_2:95% air atmosphere (normoxia). After 24 h, medium was replaced in one dish of each cell line under normoxic conditions. Media in the other dishes were replaced with pre-equilibrated hypoxic medium in a Whitley 35 Hypoxystation (Don Whitley Scientific Limited) at 37˚C at 0.1% O_2:5% CO_2. After 24 h of incubation under normoxic and hypoxic conditions, the cells were washed twice with PBS (PBS pre-equilibrated in hypoxia was used for cells maintained under hypoxic conditions) then harvested by rotating and scraping in PBS. The detached cells were transferred to 1 ml RNAse free microfuge tubes and the tubes centrifuged at 4˚C for 10 min at 10,000 rpm. The supernatant was removed and the cell pellets frozen at -80˚C for RNA extraction. RNA was extracted from the pelleted cell lines using RNeasy Plus Mini Kit (Qiagen) and Qiagen QIAshredder Kit following the manufacturer's instructions. Reverse transcription was performed as for the clinical samples. Pre-amplification was carried out using a custom preamp pool mix. This consisting of primers to genes present on the TaqMan human EC card array (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a preamp TaqMan Fast Advanced Master Mix (2X; Thermo Fisher Scientific, Inc.). The pre-amplification step involved 14 cycles on a 2720 thermal cycler.

Statistical analyses. For each sample, the HS was considered high if greater or equal to the median HS for the whole cohort. A pre-study power calculation determined that a minimum of 34 samples should be analysed to generate an absolute value for a cut-off for clinical application with a 95% confidence interval and accounting for 10% error. The study was not powered or set up to study associations with prognosis. Cross platform correspondence was determined by Spearman regression (correlation) analysis and concordance. Concordance was defined as the proportion of cases where hypoxia status concurred between each platform. Inter- and intra-tumour heterogeneity was assessed as coefficient of variation (CoV). Significant differences between mean values of HS generated in the cell data were compared using the Student's t-test (unpaired).

Results

Comparing hypoxia scores obtained using different platforms. The mean (range) yields of RNA were 338 (64-956) ng/μl for the 51 recent samples. The mean (range) RNA quality ratios were 1.92 (1.63-2.19) for 260/280 and 1.73 (0.97-2.19) for 260/230. Tumour cellularity was <30% in 4 of the 51 samples from the recent cohort, which were then excluded from further analysis. Table II summarises the results of the cross-platform comparisons in 47 patients. There were strong correlations ($r>0.70$, $P<0.0001$; Fig. 1) between HS values generated using the three platforms. The levels of concordance were also high: 73% for TLDA and Clariom; 78% for Clariom and NanoString; and 78% for TLDA and NanoString. As the most significant correlation and highest concordance were with NanoString, further analyses were carried out using this platform.

Expression levels of signature EC genes determined by NanoString are shown in Fig. 2A. Four of the EC genes had higher expression than the highest expressed signature gene. To ensure that the HS was not biased by including four highly expressed EC genes, normalisation was carried out using four genes expressed at a level similar to the signature genes and also using all eight EC genes. As HS values derived by either normalisation were highly correlated (rho=0.98, $P<0.0001$; Fig. 2B), HS was generated using all 8 EC genes. The CoV for the signature genes ranged from 4.5 to 36.6% (Fig. 3) and, with the exception of *CAD*, were greater than for the EC genes (range 2.1% for *UBC* to 4.68% for *POLR2A*).

The median HS values generated for the 47 samples using Clariom S, TLDA and NanoString were 6.667, 0.047 and 7.328 respectively.

Intra- and inter-tumour variability in hypoxia score. Three blocks from the older patient cohort with tumour cellularity was <30% were excluded from further analysis. Intra- and inter-tumour variation in the expression of each gene in the bladder-cancer hypoxia signature was determined in blocks from 48 patients. Multiple blocks were available for 10 patients. Variability in gene expression and HS was assessed using the Clariom S platform. Inter-tumour variation in HS values was higher (CoV=7.95) than intra-tumour variation (mean CoV=3.93%; Fig. 4A). Hypoxia status was consistent in all blocks for 8 patients, but differed for two patients (Fig. 4B).

Effect of hypoxia on HS values in bladder cancer cell lines. Hypoxia scores generated using TLDA for RT112 and J82

Table II. Platform comparisons in recent samples from 47 patients.

Platforms compared	Correlation coefficient[a] (significance)	Concordance
Clariom S vs. TLDA	0.72 (P<0.0001)	73%
Clariom S vs. NanoString	0.84 (P<0.0001)	78%
TLDA vs. NanoString	0.80 (P<0.0001)	78%

[a]The Spearman's rank correlation coefficients.

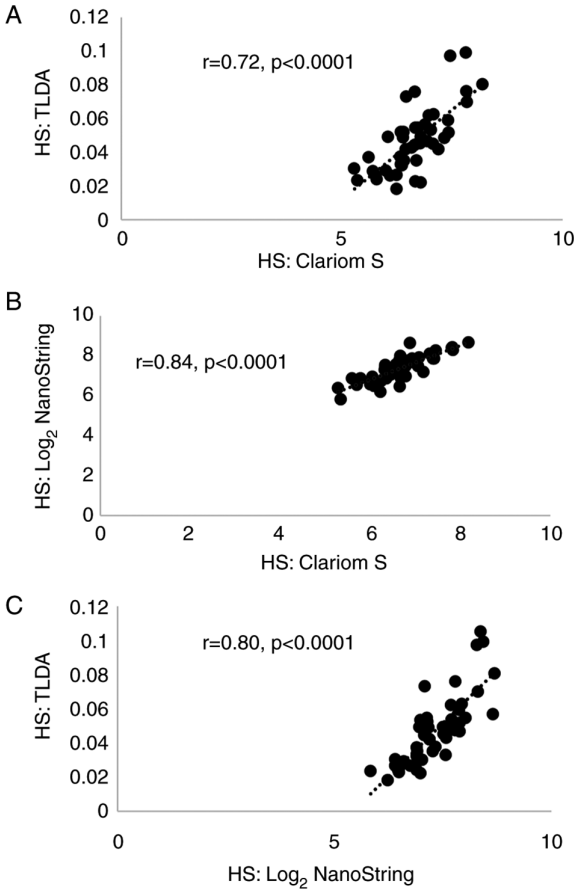

Figure 1. Scatter plots of hypoxia scores for 47 samples from a prospective cohort of 51 patients with MIBC. Scores were derived using Clariom S, TLDA based PCR and NanoString platforms. Correlation coefficients: (A) Clariom S vs. TLDA (r=0.72; P<0.0001); (B) Clariom S vs. NanoString (r=0.84; P<0.0001) and (C) TLDA vs. NanoString (r=0.80; P<0.0001). TLDA, Taqman low density array; MIBC, muscle invasive bladder cancer.

bladder cancer cell lines exposed to hypoxia (0.1% O_2) for 24 h and compared with cells maintained in the presence of 21% O_2. The mean and SD of the HS values in hypoxic and normal O_2 levels are shown in Fig. 5. For both cell lines HS values were higher (P<0.02) in cells exposed to hypoxia demonstrating that the signature responds to low O_2.

Discussion

Our study derived cut-off values for a 24-gene bladder-specific hypoxia signature that could be used in a future biomarker driven clinical trial. We also showed that the signature can be implemented using several platforms and would not be limited by intra-tumour heterogeneity; and then confirmed the hypoxia relevance of our signature in bladder cancer by showing increased hypoxia scores *in vitro* in cell lines exposed to low oxygen.

Molecular testing is a rapidly evolving area, particularly for breast cancer (e.g. Oncotype DX, MammaPrint, Prosigna, Breast Cancer Index), and clinical implementation requires selecting an appropriate platform. Platforms for measuring RNA expression include full transcriptomic arrays/RNAseq and targeted panels. Microarrays/RNAseq provide information on all transcribed genes, which is useful in developing gene signatures but is expensive and requires complex analysis when used for a small number of genes in routine clinical practice. Quantitative polymerase chain reaction (qPCR) measures relative gene expression and is the gold standard method for bench marking gene expression. Applied Biosystems customised TLDA cards can measure gene expression for a small number of genes using qPCR (12,13). The approach uses a detection system based on probes where complementary binding to cDNA results in probe degradation and loss of quenching of a fluorescent beacon. Each reaction well contains only one probe and so gene numbers analysed are in the low twenties. NanoString nCounter technology has the advantage that reverse transcription is not needed and a larger number of genes (up to 800) can be analysed. QuantiGene assays and targeted next generation sequencing are also used to measure RNA expression.

Cross platform comparisons are required to demonstrate transferability of signatures developed using full transcriptomics onto targeted panels. For example, we previously derived a 26-gene signature for measuring hypoxia in head and neck cancer and showed a good correlation between microarray and TLDA derived hypoxia scores (14). We then used TLDA cards in a prospective clinical trial (15). A recent study (16) also demonstrated transferability between array, nCounter and PCR-based platforms for a 38-gene signature stratifying head and neck cancer response to primary or adjuvant radiochemotherapy. In agreement with previous studies (16,17), the bladder cancer hypoxia signature expressed on the Clariom S microarray correlated closely and showed greater concurrence with NanoString compared with PCR-based techniques. It has also been shown that NanoString demonstrates better correspondence between RNA from FFPE and fresh-frozen tissue than does PCR (18). Reis *et al* (18) demonstrated that gene expression data by NanoString showed a higher mean correlation (r=0.94) between individual fresh-frozen and FFPE sample pairs compared to real-time quantitative PCR (r=0.53)

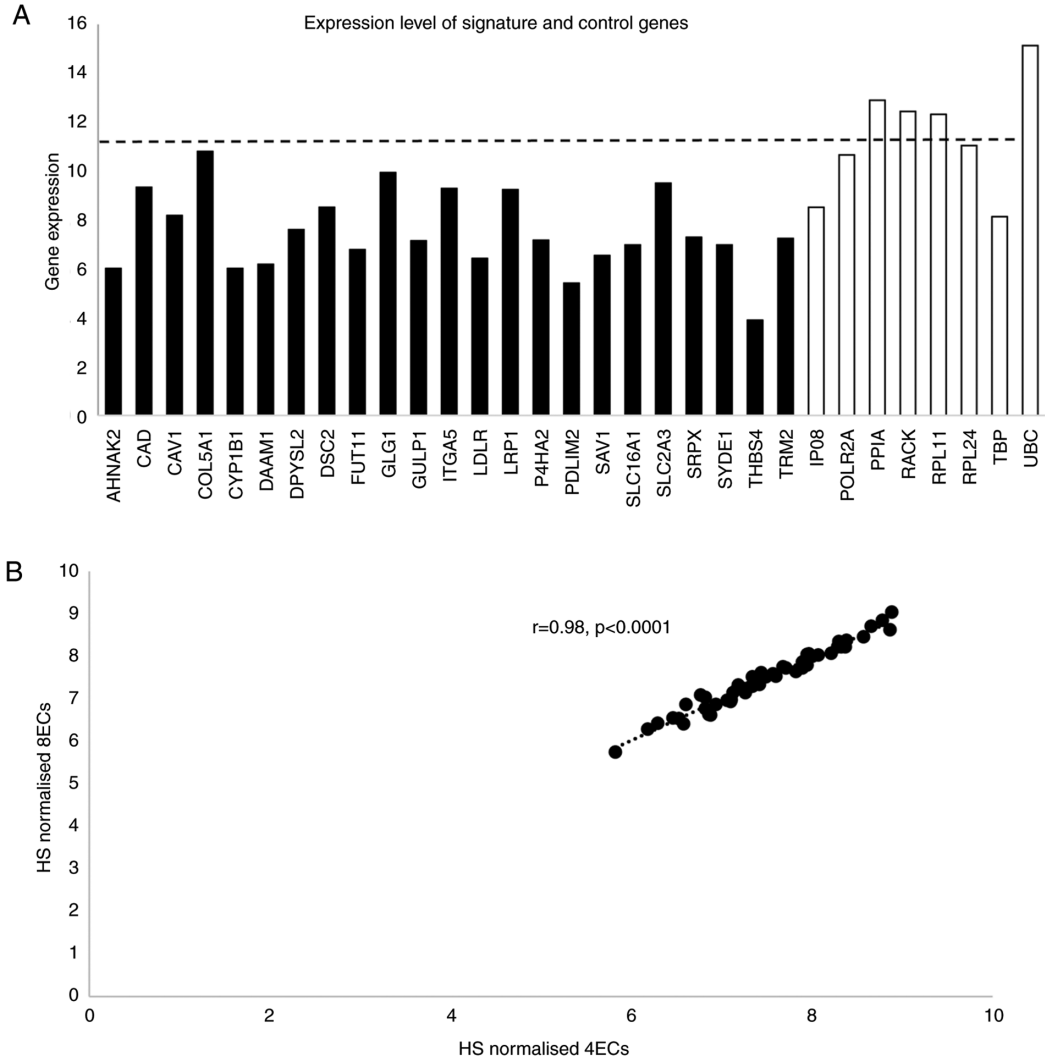

Figure 2. (A) Expression level of signature and endogenous control genes derived on NanoString. (B) Scatter plot of HS determined on NanoString normalised by all 8 or the 4 lowest expressed endogenous control genes (r=0.98; P<0.0001). HS, hypoxia score.

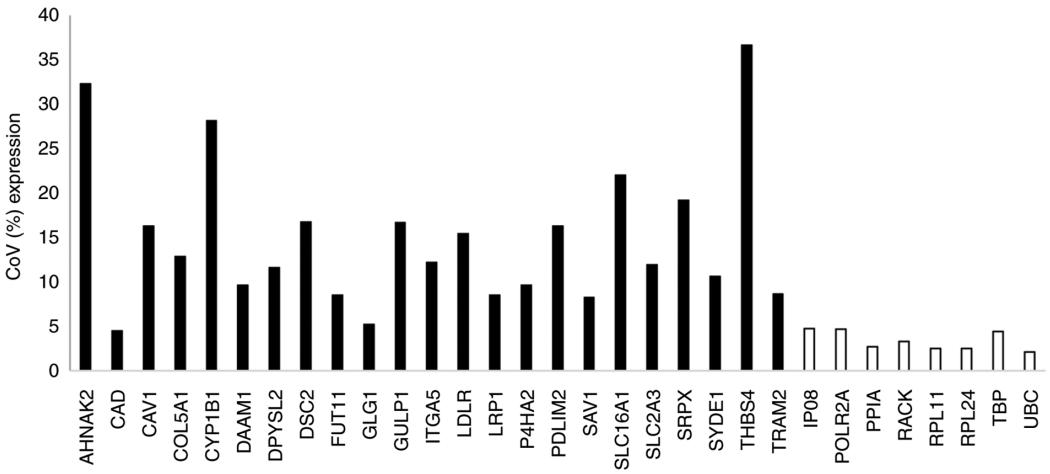

Figure 3. Inter-tumour variability (CoV) of signature (solid) and endogenous control (empty) gene expression derived using NanoString using tumour tissue from a prospective cohort of 47 patients with MIBC. CoV, coefficient of variation; MIBC, muscle invasive bladder cancer.

in a cohort of 19 patients with oral carcinoma. Together these finding illustrate good transferability of gene expression signatures across platforms.

We also examined the level of intra-tumour variability in the bladder cancer hypoxia signature. Tumours commonly exhibit histological and intratumour diversity that can impact

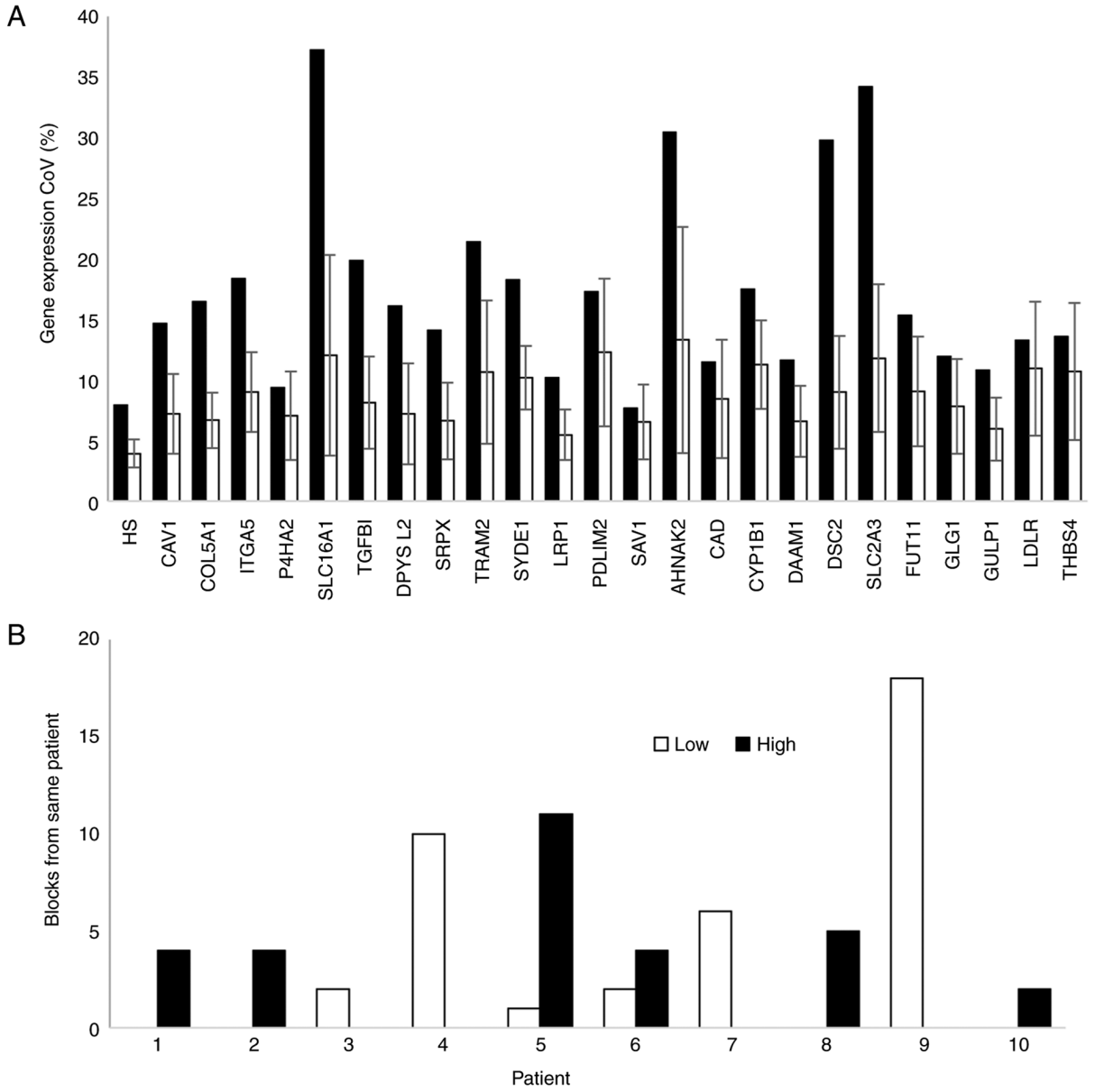

Figure 4. Intra- and inter-tumour variability in hypoxia score and bladder cancer hypoxia signature gene expression obtained in a retrospective cohort of samples from patients with MIBC (n=48). (A) Inter-tumour CoV (solid bars, n=48) and intra-tumour CoV for multiple samples from 10 patients (error bars: SD). (B) Variation in hypoxia status in the 10 patients with multiple blocks. MIBC, muscle invasive bladder cancer; CoV, coefficient of variation; SD, standard deviation.

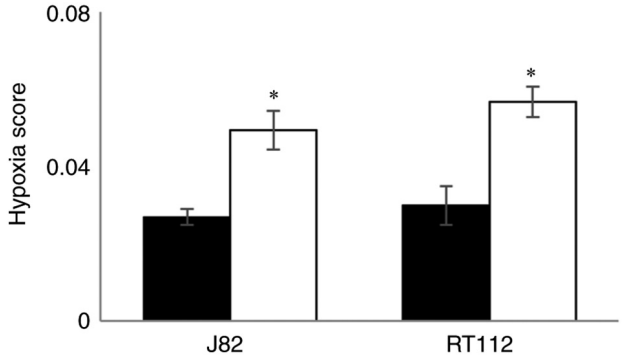

Figure 5. Mean (SD) of HS values in RT112 and J82 cells maintained in 21% O_2 (black bars) or exposed to 0.1% O_2 for 24 h (white bars). In both cell lines HS were higher in cells subject to hypoxia for 24 h (*P<0.02). Results are mean (±SD) of three independent cell experiments, each analysed by qPCR with three technical replicates. HS, hypoxia score.

the powering of clinical trials (19). When a biomarker is heterogeneously distributed within tissue, the ability to detect a specific effect is diminished (20) requiring larger cohort sizes in clinical trials. As expected, we found less variation in the expression of our 24 signature genes and hypoxia scores within than between tumours. Event number requirements are related to gene signature length (21) probably due to the increased susceptibility of shorter signatures to tumour heterogeneity. Dibben *et al* (21) demonstrated, that less events were required to achieve a power of 80% as the number of signature genes increased. The effect was particularly apparent between 4 and 10 genes but reduced when signature size increased to between 10 and 50 genes (21). Therefore, the 24 genes in our signatures will limit heterogeneity.

Our group and collaborators develop hypoxia gene signatures from seed genes taken from the literature or generated

by us in tumour-specific cell lines (22-24). The bladder gene signature was developed using an *a-priori* approach based on established hypoxia-sensitive genes and evaluated by demonstrating prognostic capability. The resulting 24 gene signature was then validated using gene expression data and outcome data from a further seven patient cohorts. Here we confirm that hypoxia scores were higher in bladder cancer cell lines exposed to hypoxia.

A potential limitation of our study is that we did not examine relationships between hypoxia scores with clinicopathological variables and patient outcomes. However, these relationships have been reported previously. In a microarrayed sub-group of the BCON cohort (n=151), patients stratified as high hypoxia by the signature (i.e. those with hypoxia scores greater than the cohort median) had higher tumour stages (P=0.03) and lower pre-treatment haemoglobin levels (P=0.04). There were no associations with sex, age, growth pattern and presence of carcinoma in situ. Also, the prognostic and predictive value of the 24-gene signature was independent of clinicopathologic variables and retained significance in multivariable analysis (7). We did not power the study reported here for testing for prognostic significance or relationships with clinical variables. However, the work generated here enables us to test the signature prospectively using one of the platforms tested.

In conclusion, our 24-gene bladder cancer hypoxia signature is platform agnostic, i.e., hypoxia scores can be generated using several gene expression approaches. There was a better correlation between hypoxia scores generated using NanoString and Clariom S gene arrays than that obtained using TLDA cards. As NanoString can also accommodate probes for multiple clinically relevant signatures (e.g. bladder cancer subtype signatures), the platform could be used in a clinical trial. The cut-off value determined prospectively in recent FFPE samples could be used to identify patients with high levels of tumour hypoxia who are most likely to benefit from having radiotherapy plus CON.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The transcriptomic datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus repository, under accession number GSE203149 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE203149; publicly accessible, December 31, 2022).

Authors' contributions

CMLW, AC, PJH and TADS designed the study. RS and SV performed cell studies (each can verify the data). Clariom S data were normalised by BL and analysed by TADS, BL and TS confirm the authenticity of all the raw data. KJR acquired clinical material and analysed data. HD (pathologist) reviewed the histology for tumour cellularity. SL, EM, JIJ, OAA, HV and TADS prepared samples, extracted RNA and performed PCR. TADS, AC and CMLW wrote the paper. All authors read, edited and approved the final version of the manuscript.

Ethics approval and consent to participate

Samples were obtained via the Manchester Cancer Research Centre Biobank under research tissue bank ethics (ref. 18/NW/0092). Pre-treatment FFPE MIBC samples were collected from recently treated patients who consented for use of their tissue in research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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