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Expression of hypoxia-inducible factor-1α predicts benefit from hypoxia modification in invasive bladder cancer

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Background: The addition of carbogen and nicotinamide (CON) to radiotherapy (RT) improves overall survival in invasive bladder cancer. We explored whether expression of the hypoxia marker hypoxia-inducible factor-1α (HIF-1α) alone or in combination with other markers predicted benefit from CON.

Methods: A retrospective study was carried out using material from patients with high-grade invasive bladder carcinoma enrolled in the BCON phase III trial of RT alone or with CON (RT + CON). HIF-1α expression was studied in 137 tumours using tissue microarrays and immunohistochemistry. Data were available from other studies for carbonic anhydrase IX and glucose transporter 1 protein and gene expression and tumour necrosis.

Results: Patients with high HIF-1α expression had improved 5-year local relapse-free survival with RT + CON (47%) compared with RT alone (21%; hazard ratio (HR) 0.48, 95% CI 0.26–0.8, $P = 0.02$), no benefit was seen with low HIF-1α expression (HR 0.81, 95% CI 0.43–1.50, $P = 0.5$). Combinations of markers including necrosis also predicted benefit but did not improve on prediction using necrosis alone.

Conclusions: HIF-1α may be used to predict benefit from CON in patients with bladder cancer but does not improve on use of necrosis.

Bladder cancer is common, with over 10 000 new diagnoses in the United Kingdom in 2010 (Kreimer *et al*, 2005; CRUK, 2013). Conventional treatment for muscle-invasive disease involves radiotherapy (RT) or radical cystectomy, which have similar survival rates (Dunst *et al*, 2001). Five-year overall survival (OS) is only around 58% for men and 50% for women (Klussmann *et al*, 2003). There is therefore a need for improved treatment strategies. The BCON (bladder carbogen and nicotinamide) phase III clinical trial showed that addition of carbogen and nicotinamide (CON) to RT improved OS (Hoskin *et al*, 2010). Adding gemcitabine (Choudhury *et al*, 2011) or fluorouracil and mitomycin C (James *et al*, 2012) to RT also improves outcomes. Biomarkers are required to predict benefit from the different approaches to individualise treatment.

Hypoxic tumours benefit most from hypoxia modification (Kaanders *et al*, 2002; Rischin *et al*, 2006; Janssens *et al*, 2012; Toustrup *et al*, 2012). As direct measurement of hypoxia is invasive and impractical, analysis of pathological features and surrogate

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hypoxia markers may be preferable. In support, we showed previously that tumour necrosis is an independent prognostic factor in invasive bladder cancer, and both necrosis and expression of the hypoxia-inducible enzyme carbonic anhydrase IX (CAIX) predicted benefit from hypoxia modification (Eustace *et al*, 2013a). In contrast, a 26-gene hypoxia signature did not predict benefit from RT + CON in BCON (Eustace *et al*, 2013b).

In this study, we explored hypoxia-inducible factor (HIF). HIF-1 is a transcription factor that transactivates genes implicated in cancer development, including contributors to angiogenesis and anaerobic metabolism (Shweiki *et al*, 1992; Elbert *et al*, 1996). The subunit hypoxia-inducible factor-1α (HIF-1α), which accumulates in response to cellular hypoxia, correlates with stage, grade and metastatic potential of bladder cancers (Wang *et al*, 1995; Deniz *et al*, 2010). Moreover, HIF-1α and the downstream proteins CAIX and glucose transporter 1 (GLUT1) are all independent prognostic factors in bladder cancer (Hoskin *et al*, 2003; Palit *et al*, 2005; Theodoropoulos *et al*, 2005; Ord *et al*, 2007; Chai *et al*, 2008; Deniz *et al*, 2010).

As tumour necrosis represents an attractive prognostic and predictive factor that is relatively simple to assess, it remains unclear whether the addition of hypoxia markers will improve our ability to predict benefit from CON. We hypothesised that as HIF-1α has an important role in mediating the cellular response to tumour hypoxia, it may improve patient stratification. Therefore, we performed a retrospective study to explore whether expression of combinations of necrosis, HIF-1α, CAIX and GLUT1 predict benefit from hypoxia modification. We also compared the predictive ability of quantitative CAIX or GLUT1 gene expression with immunohistochemistry. Samples were taken from patients enrolled in the BCON trial. REMARK guidelines for prognostic tumour marker studies were followed (McShane *et al*, 2005).

MATERIALS AND METHODS

Patients and tissue samples. A retrospective cohort study was carried out in 137 patients with high-grade, non-metastatic transitional cell carcinoma of the bladder. Patients participated in the BCON phase III trial and were randomised between November 2000 and April 2006. The study was approved by the local research ethics committee (LREC 09/H1013/24) and informed consent for sample collection and analysis was obtained.

Trial protocol and sample acquisition were described previously (Hoskin *et al*, 2010; Eustace *et al*, 2013a). In brief, patients were randomised to RT alone or RT plus carbogen (2% CO_2 + 98% O_2) and nicotinamide (40 or 60 mg kg^{-1}). Pre-treatment tissue samples were obtained via transurethral resection of the bladder tumour and formalin-fixed and paraffin-embedded blocks were constructed.

Immunohistochemistry. Methods for tissue microarray construction were previously described (Eustace *et al*, 2013a). Immunohistochemistry was carried out for CAIX and GLUT1 as per a previous protocol (Hoskin *et al*, 2003). HIF-1α staining was carried out using the Bond-Max Automated staining system (Leica Biosystems, Newcastle, UK). Samples were de-waxed and rehydrated, followed by antigen retrieval at pH 9.0 for 40 min at 100 °C. Endogenous peroxidase was blocked using 3% hydrogen peroxide solution. Primary antibody (mouse monoclonal HIF-1α, BD Biosciences 610959, Oxford, UK) was diluted to a 1 : 20 solution with diluent and incubated with samples for 15 min at room temperature. A negative control of IgG1 (Dako X0931, Cambridge, UK) was also used. Post-primary rabbit anti-mouse link reagent was applied (Bond Polymer Refine Detection System,

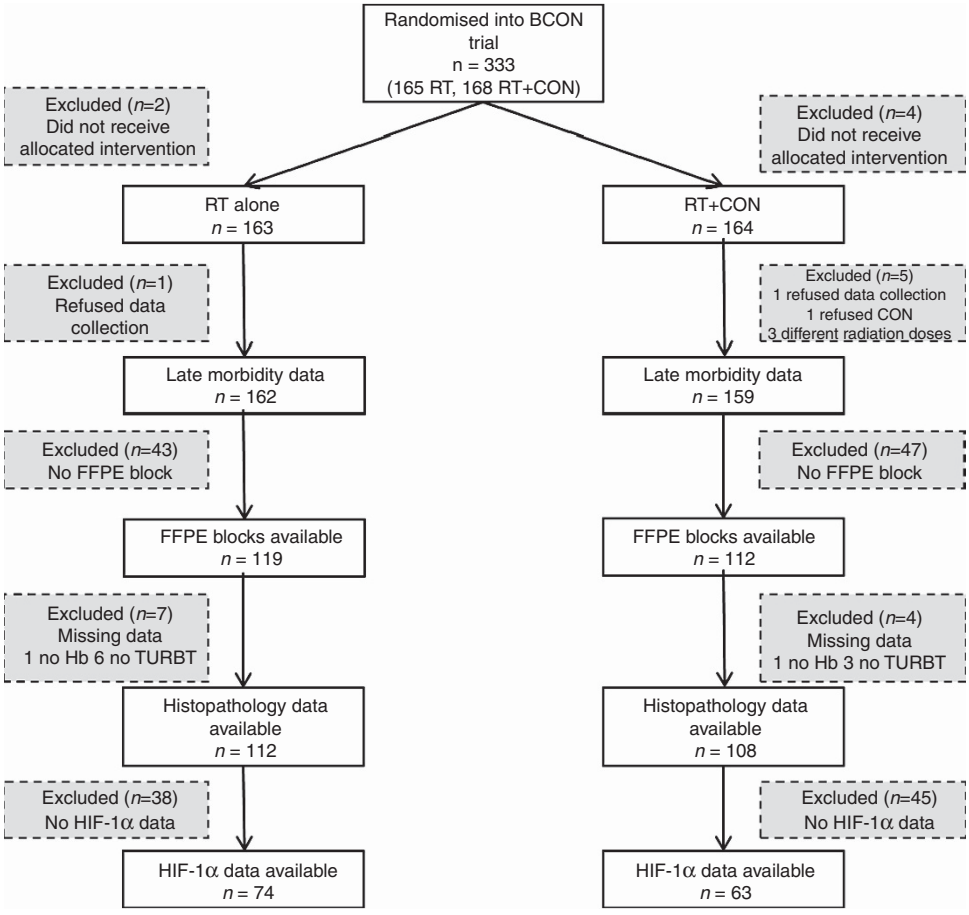

Figure 1. Study CONSORT diagram. Data for HIF-1α expression were available for 137 patients enrolled in the BCON trial.

Leica DS9800, Newcastle, UK), and samples were incubated for 8 min at room temperature. The anti-rabbit polymer-HRP detection reagent (Bond Polymer Refine Detection System, Leica) was then added and samples were incubated at room temperature for a further 8 min. 3,3′-diaminobenzidine tetrahydrochloride was added, and after a further 10 min of incubation samples were counterstained with haematoxylin.

Immunohistochemical analysis. Data for necrosis, CAIX and GLUT1 were available from another study (Eustace *et al*, 2013a). HIF-1α, GLUT1 and CAIX expression were determined using an H-score: a combination of the intensity (0–3) and percentage of cells stained, with a range of 0–300. Only nuclear expression of HIF-1α was scored. For CAIX, scoring included both nuclear and cytoplasmic staining. Supplementary Figure 1 shows examples of staining for HIF-1α, CAIX and GLUT1. For HIF-1α, cores were scored twice by the same scorer (BH) on different days. Cores (10%) were scored independently by a consultant histopathologist (HD). Scorers were blinded to clinical outcome data. Duplicate scores and independent histopathologist scores correlated well (Spearman $\rho > 0.91$). There was no statistically significant intra-observer ($P = 0.47$) or inter-observer ($P = 0.06$) variability in histological scores. Where scores were discordant, the score of the consultant histopathologist was used. Median HIF-1α, CAIX and GLUT1 scores were 19 (range 0–199), 2.0 (range 0–208.4) and 106 (range 0–300), respectively. Median HIF-1α H-scores were used to provide an objective cutoff and facilitate comparison with other studies. Owing to the distribution of scores, cutoff values were 0 for CAIX and 100 for GLUT1. High CAIX (>0) was seen in 79 (58%) patients and high GLUT1 (>100) in 65 (47%) patients.

Qualitative PCR and gene analysis. Data for quantitative gene expression of CAIX ($n = 111$) and SLC2A1 ($n = 147$) were available from a previous study (Eustace *et al*, 2013b). Methods for RNA extraction, cDNA synthesis and gene quantification were previously described (Eustace *et al*, 2013b).

Statistical analysis. Analyses were performed using SPSS (IBM, version 12, Portsmouth, UK) and Prism (Graphpad, version 6, La Jolla, CA, USA). Five-year OS time was taken as time from randomisation to any cause of death; patients still alive were censored to date of the last follow-up or at 5 years, depending on which was earlier. Local relapse-free survival (LRFS) was taken as time to tumour recurrence in bladder, locoregional failure or death from any cause. Those alive and free of local disease were censored at their last follow-up. Patients with persistent muscle-invasive disease or with no cystoscopy post treatment had their time set to zero. Survival estimates were performed using the Kaplan–Meier analysis; data were compared using the Mantel–Cox log-rank test. Hazard ratios (HRs) and 95% confidence intervals for OS and LRFS were obtained using Cox regression analysis. Differences in treatment effect according to HIF-1α expression were addressed using stratum-specific treatment variables. Correlations were assessed using Spearman's correlation, and variability analysed using the Wilcoxon matched pairs test. All *P*-values were two sided and agreed statistical significance was 0.05. No corrections were made for multiple testing and *P*-values should be interpreted accordingly. The χ^2-test with Yates correction was used to compare proportions across the levels of categorical variables.

RESULTS

Figure 1 shows the study CONSORT diagram. Analysis of HIF-1α expression was possible in a subset of 137 patients enrolled in the BCON trial. HIF-1α data were not obtained for some patients because of poor stain uptake or TMA degradation. Data for CAIX and GLUT1 expression were available for 138 and 127 patients, respectively. Table 1 shows the distribution of clinicopathological parameters by HIF-1α expression. Patients with high tumour expression of HIF-1α also had high expression of CAIX ($P = 0.004$) and GLUT1 ($P = 0.006$), and tended to have more necrosis ($P = 0.07$).

Analyses for OS and LRFS were performed, but owing to similarity in results, data for LRFS only are presented. Univariate and multivariate prognostic analyses for patients receiving the standard treatment of RT alone showed that neither HIF-1 α ($n = 74$), CAIX ($n = 73$) nor GLUT1 ($n = 70$) were prognostic in patients receiving RT alone ($P > 0.29$). Age ($P = 0.04$) and necrosis ($P = 0.01$) were the only prognostic factors in the univariate analysis, but neither was significant in multivariate analysis.

Multivariate analyses were performed on a smaller cohort of 133 patients, as those with missing data for any clinicopathological or

Table 1. Distribution of clinicopathological features by HIF-1α expression

Variable	HIF-1α < 19	HIF-1α ⩾ 19	*P*
Gender			
Male	54 (47)	60 (53)	0.18
Female	15 (65)	8 (35)	
Age (years)			
<75	38 (58)	27 (42)	0.1
⩾75	31 (43)	41 (57)	
Stage			
1	2 (40)	3 (60)	0.91
2	51 (50)	50 (50)	
3	14 (54)	12 (46)	
4a	2 (40)	3 (60)	
TURBT			
Complete	24 (46)	28 (54)	0.31
Partial	22 (47)	25 (53)	
Biopsy	21 (62)	13 (38)	
Necrosis			
Absent	38 (59)	26 (41)	0.07
Present	31 (42)	42 (58)	
Growth pattern			
Papillary	7 (50)	7 (50)	0.4
Solid	37 (56)	29 (44)	
Both	25 (44)	32 (56)	
CIS			
Absent	45 (46)	52 (54)	0.21
Present	24 (60)	16 (40)	
Hb (g dl^{-1})			
<14	43 (57)	33 (63)	0.15
⩾14	26 (43)	35 (57)	
CAIX			
0	34 (65)	18 (35)	0.004
>0	30 (38)	49 (62)	
GLUT1			
<100	36 (60)	24 (40)	0.006
⩾100	22 (34)	43 (66)	

Abbreviations: CAIX = carbonic anhydrase IX; CIS = carcinoma *in situ*; Hb = haemoglobin; HIF-1α = hypoxia-inducible factor-1α; TURBT = transurethral resection of bladder tumour.

immunohistochemical variable were excluded. Forward stepwise analysis including both treatment arms stratified by HIF-1α expression showed that age (1.65, 95% CI 1.06–2.57, $P=0.03$) and treatment in the presence of high HIF-1α (HR 0.49, 95% CI 0.27–0.90, $P=0.02$) were statistically significant prognostic factors (Table 2). Treatment was not a significant prognostic factor in those with low HIF-1α expression ($P=0.55$).

Kaplan–Meier survival analyses are presented in Figures 2–4. Hazard ratios for RT + CON compared with RT alone for patients with high or low HIF-1α, CAIX and GLUT1 expression are presented in Table 3. Also shown are HRs for combinations of these markers and combinations with necrosis.

Five-year LRFS was 46% in patients receiving RT + CON and 38% in those receiving RT alone. In patients with high HIF-1α expression, there was a statistically significant improvement in 5-year LRFS in those receiving RT + CON (47%) compared with RT alone (21%) ($P=0.02$). Similarly, in those with high CAIX expression, 5-year LRFS was improved with RT + CON (52%) *vs* RT alone (25%) ($P=0.006$). Patients with combinations of high HIF-1α and CAIX expression or high CAIX and GLUT1 expression had improved 5-year LRFS with RT + CON (61% and 47%, respectively) compared with RT alone (16% and 18%, respectively, $P=0.01$ and 0.03). Patients with necrosis plus high HIF-1α, CAIX or GLUT1 expression all had significant improvements in LRFS with RT + CON (57%, 54% and 66%, respectively) *vs* RT alone (19%, 28% and 25%, respectively, $P=0.01$, 0.02 and 0.01).

There was no improvement in LRFS in patients with high expression of the *CAIX* gene receiving RT + CON compared with RT (HR 0.92, 95% CI 0.49–1.75, $P=0.81$). Similarly, no benefit from CON was seen in those with high *GLUT1* gene expression (HR 0.66, 95% CI 0.38–1.14, $P=0.14$). There was no correlation between *CAIX* gene and protein expression ($r=-0.02$) and only weak correlation between *GLUT1* gene and protein expression ($r=0.39$).

DISCUSSION

Hypoxia is of critical importance in cancer treatment. It contributes to tumour progression, invasiveness, metastasis and resistance to both chemotherapy and RT (Gray *et al*, 1953; Deschner and Gray, 1959; Brown, 2000; Harris, 2002). In bladder cancer, there is evidence that hypoxia modification improves locoregional control after RT (Overgaard and Horsman, 1996) and the BCON trial showed significant improvements in OS, risk of death and local relapse for patients receiving RT + CON (Hoskin *et al*, 2010). Furthermore, significant improvements in 5-year regional control with RT + CON were observed in patients with hypoxic laryngeal cancers; those with well-oxygenated tumours received no benefit (Janssens *et al*, 2012). Our findings support the notion that patients without evidence of tumour hypoxia do not receive benefit from hypoxia modification. For such patients, alternative methods of radiosensitisation such as concurrent gemcitabine (Choudhury *et al*, 2011) or fluorouracil and mitomycin C (James *et al*, 2012) should be considered. There is a clear need to stratify patients according to the presence of tumour hypoxia. Future work is planned to investigate the relationship between necrosis and outcomes in patients enrolled in the BC2001 trial that randomised to RT alone or with 5-fluorouracil and mitomycin. The latter study will investigate whether patients with necrosis in their tumours benefit from the addition of fluorouracil and mitomycin C to RT.

Direct measurement of hypoxia via Eppendorf electrodes is difficult in bladder cancer owing to tumour inaccessibility, and while pimonidazole binding is a widely accepted surrogate it is invasive and impractical. Our previous work showed that tumour necrosis is an attractive and easily assessable factor that may be used to predict benefit from hypoxia modification in bladder cancer (Eustace *et al*, 2013a). Coagulative tumour necrosis, the subtype studied here is thought to be specific to tumour hypoxia. In addition, analysis of hypoxia-associated proteins is attractive because of the simplicity of obtaining paraffin-embedded material. Before HIF-1α, CAIX or GLUT1 can be used clinically they must be validated as intrinsic markers of tumour hypoxia. In support, their expression correlates with pO_2 and co-localises with pimonidazole in cervical cancer (Airley 2001, Haugland 2002, Jankovic 2006 and Dellas 2008). There is also significant co-localisation of CAIX and GLUT with pimonidazole in bladder cancer Hoskin *et al*, 2003. Although there are no current data correlating HIF-1α expression with pimonidazole binding in bladder cancer, our work did suggest that patients with high HIF-1α expression also tended to have higher expression of CAIX and GLUT1.

Analysis of a larger cohort of the BCON patients showed tumour necrosis is a prognostic factor in bladder cancer (Eustace *et al*, 2013a). The loss of significance in multivariate analysis in the current analysis is due to reduced patient numbers owing to adding another variable. Although our results suggest that HIF-1α, GLUT1 and CAIX are not prognostic, this is not consistent with other studies and may be due to differences in analytical techniques, sample heterogeneity, the limitations of TMA analysis or most likely the small number of patients in the RT only arm (Hoskin *et al*, 2003; Theodoropoulos *et al*, 2005).

Tumour necrosis was strongly predictive of benefit from CON and is clinically very appealing owing to its simplicity and wide availability. HIF-1α is a hypoxia-inducible marker, which we show for the first time predicts benefit from CON. Kaplan–Meier analyses showed that CAIX was also predictive in the cohort studied here, as we reported previously with a larger group (Eustace *et al*, 2013a). The findings that combined expression of HIF-1 or GLUT1 with CAIX retained predictive ability supports the notion that tumour hypoxia may be best assessed using multiple markers. Furthermore, analysis of necrosis in combination with HIF-1α, CAIX or GLUT1 may be a viable option, as this improved the predictive value of HIF-1α and CAIX. However, it is important to note that combinations of hypoxia-specific markers alone or with necrosis did not improve upon the prognostic or

Table 2. Multivariate forward stepwise analysis stratified by HIF-1α expression

Step	Variable	*n*	LRFS HR	95% CI	*P*	OS HR	95% CI	*P*
1	**HIF-1α: treatment**							
	RT alone or HIF-1α < 19 + CON	97						
	HIF-1α > 19 + CON	36	0.49	0.27–0.90	0.02	0.49	0.27–0.91	0.02
2	**Age**							
	< 75	62						
	⩾75	71	1.65	1.06–2.57	0.03	1.64	1.06–2.56	0.03

Abbreviations: CI = confidence interval; HIF-1α = hypoxia-inducible factor-1α; HR = hazard ratio; LRFS = local relapse-free survival; OS = overall survival.

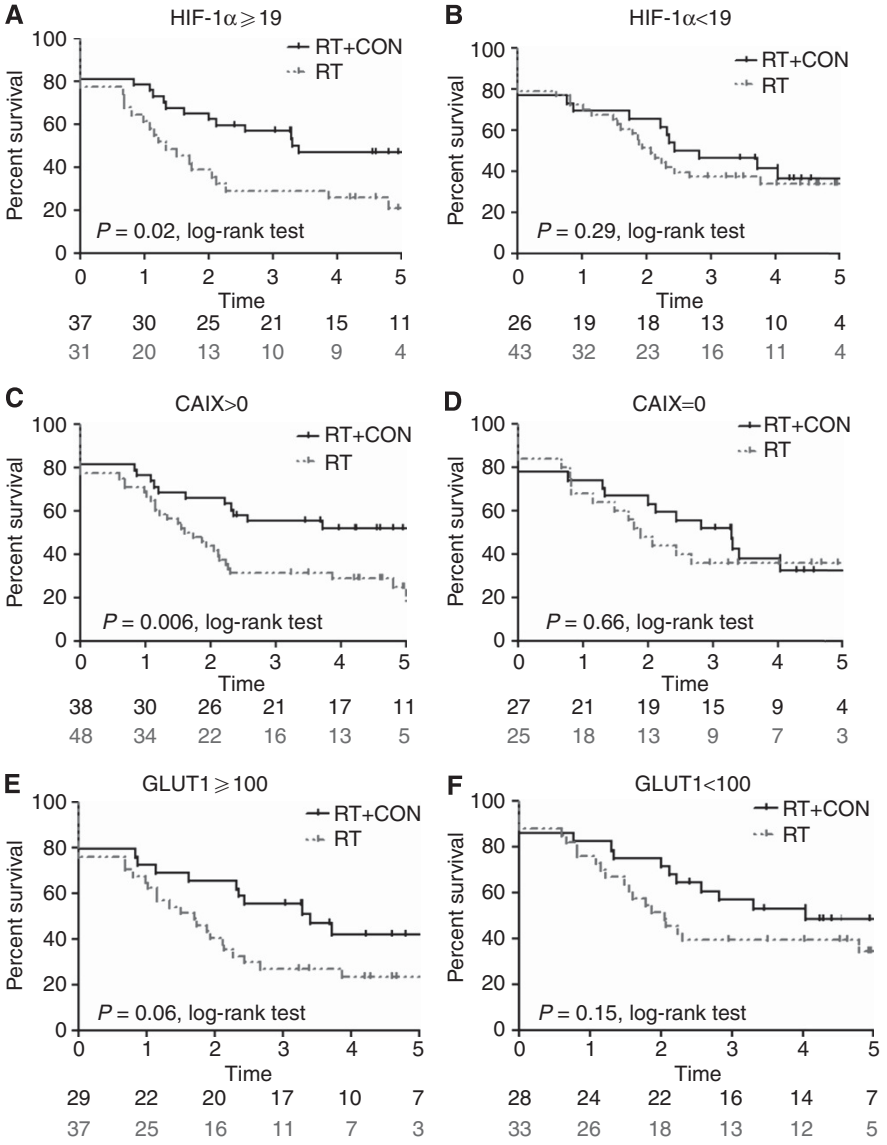

Figure 2. Kaplan–Meier survival plots for local relapse-free survival after radiotherapy alone or with carbogen and nicotinamide and stratified according to (**A**) HIF-1α score ⩾19 or (**B**) <19, (**C**) CAIX score >0 or (**D**) = 0, or (**E**) GLUT1 ⩾100 or (**F**) <100. Log-rank *P*-values and number of patients at risk in each yearly interval are shown.

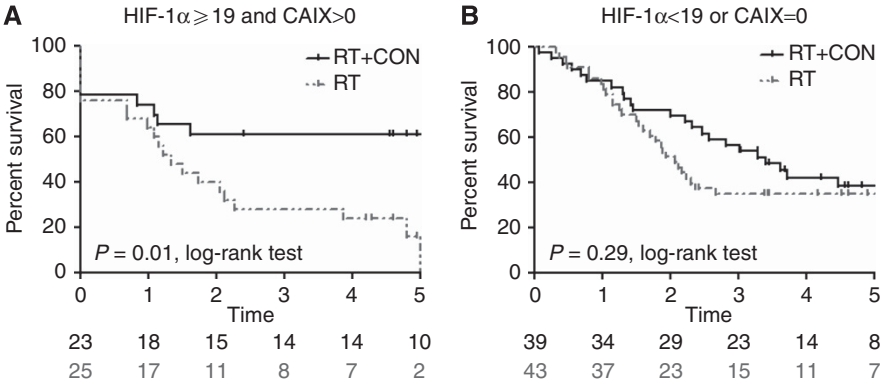

Figure 3. Kaplan–Meier survival plots for local relapse-free survival after radiotherapy alone or with carbogen and nicotinamide and stratified according to (**A**) HIF-1α score ⩾19 and CAIX score >0 or (**B**) <19 or = 0. Log-rank *P*-values and number of patients at risk in each yearly interval are also shown.

predictive ability of necrosis alone. A limitation of our study was the lack of a validation cohort to confirm our findings, but unfortunately there is no other trial that randomised patients to RT alone or with CON.

Previous work established a hypoxic gene signature that may predict response to hypoxia-modifying therapy (Eustace *et al*, 2013b). The signature predicted response to CON in laryngeal cancers but not in bladder tumours, which may reflect highly

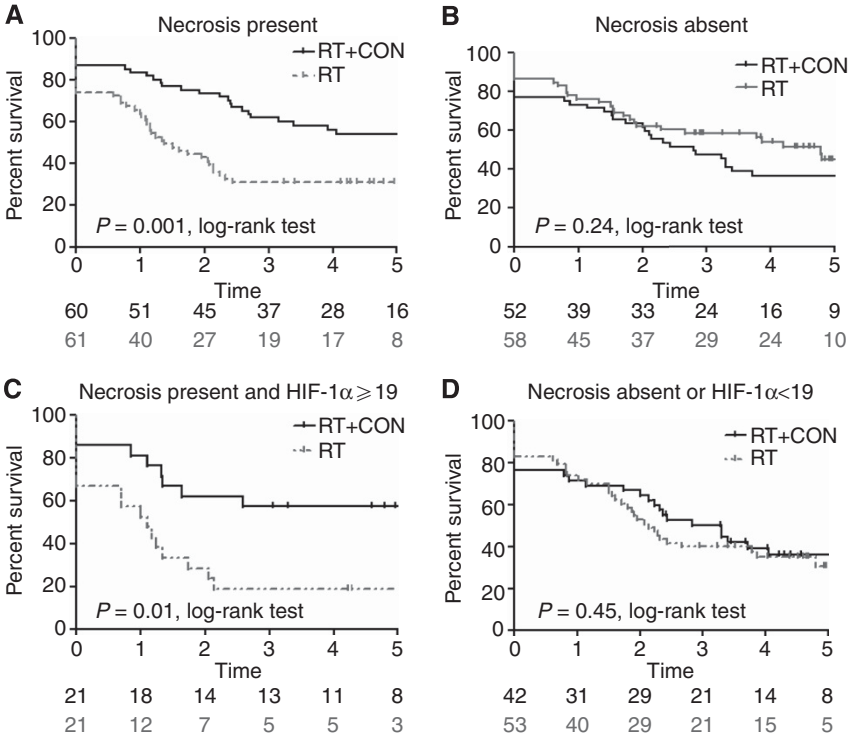

Figure 4. Kaplan–Meier survival plots for local relapse-free survival after radiotherapy alone or with carbogen and nicotinamide and stratified according to (**A**) necrosis present, (**B**) necrosis absent, (**C**) necrosis present and HIF-1α >19 and (**D**) necrosis absent or HIF-1α<19. Log-rank *P*-values and number of patients at risk in each yearly interval are also shown.

Table 3. Hazard ratios for 5-year local recurrence-free survival after radiotherapy plus carbogen and nicotinamide compared with radiotherapy alone

Variable	HR	95% CI	*P*-value
HIF-1α ⩾19	**0.48**	**0.26–0.80**	**0.02**
HIF-1α <19	0.81	0.43 – 1.50	0.5
CAIX >0	**0.47**	**0.28–0.81**	**0.006**
CAIX = 0	0.81	0.43 – 1.50	0.5
GLUT1 ⩾100	0.56	0.31 – 1.03	0.06
GLUT <100	0.62	0.32 – 1.20	0.15
HIF-1α ⩾19 + CAIX >0	**0.38**	**0.18–0.79**	**0.01**
HIF-1α <19 or CAIX = 0	0.74	0.43 – 1.28	0.29
CAIX >0 + GLUT1 ⩾0	**0.47**	**0.23–0.95**	**0.03**
CAIX = 0 or GLUT1 <100	0.64	0.35 – 1.15	0.13
HIF-1α ⩾19 + GLUT1 ⩾100	0.49	0.23 – 1.05	0.06
HIF-1α <19 or CAIX = 0	0.69	0.40 – 1.21	0.2
Necrosis present	**0.46**	**0.29–0.73**	**0.001**
Necrosis absent	1.37	0.81 – 2.30	0.24
Necrosis present + HIF-1α ⩾19	**0.36**	**0.16–0.79**	**0.01**
Necrosis absent or HIF-1α <19	0.82	0.49 – 1.38	0.45
Necrosis present + CAIX >0	**0.47**	**0.25–0.89**	**0.02**
Necrosis absent or CAIX = 0	0.77	0.43 – 1.38	0.38
Necrosis present + GLUT1 ⩾100	**0.37**	**0.17–0.80**	**0.01**
Necrosis absent + GLUT1 <100	0.89	0.50 – 1.58	0.69

Abbreviation: CAIX = carbonic anhydrase IX; CI = confidence interval; GLUT1 = glucose transporter 1; HIF-1α = hypoxia-inducible factor-1α; HR = hazard ratio. Hazard ratios for necrosis were assessed in the 137 patients with available HIF-1α data. Statistically significant differences are highlighted in bold.

conserved genetic alterations specific to bladder cancer (Eustace *et al*, 2013b). Our findings that *CAIX* gene expression did not correlate with protein expression may offer some explanation as to why the signature was not predictive. The finding differs from our observations in head and neck cancers, where CAIX protein and gene expression correlated (Winter *et al*, 2007). At present immunohistochemical analysis of protein expression seems more favourable than gene expression analysis in bladder cancer, but neither of them seem to improve upon the utility, simplicity and availability of tumour necrosis.

BCON is currently a routine treatment for bladder cancer in some UK centres. Our data suggest that BCON is more effective in those with evidence of necrosis or hypoxia marker expression. Although HIF-1α could be used to predict benefit from CON, it does not improve on the simple assessment of necrosis alone.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

BAH carried out the HIF work, the analyses and wrote the paper; AE, AC, HRV, JJL and CMW supervised the project; HD carried out pathology assessments; RS oversaw or carried out statistical analyses; all authors read the paper and provided feedback.

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