Glucose transporter-1 protein (GLUT1) and carbonic anhydrase IX (CAIX) are regulated by hypoxia inducible factor-1 (HIF-1) and have been studied as putative intrinsic cellular markers for hypoxia. This study directly compares CAIX and GLUT1 with pimonidazole binding in a prospective series of bladder cancer patients and also studies the prognostic significance of the markers, in combination with vascularity and proliferation, in a retrospective series of bladder cancer patients treated in a phase II trial of radical radiotherapy with carbogen and nicotinamide (ARCON). A total of 21 patients with a diagnosis of transitional cell carcinoma of the bladder received 0.5 g m$^{-2}$ pimonidazole. Serial tumour sections were stained for pimonidazole, GLUT1 and CAIX and compared. Tissue sections obtained from a series of 64 patients previously treated for invasive bladder cancer using ARCON were stained for GLUT1 and CAIX together with Ki-67 and CD31/34. There was a good geographical colocalisation of both intrinsic markers with pimonidazole. Serial sections of a series of 64 patients previously treated for invasive bladder cancer using ARCON were stained for GLUT1 and CAIX together with Ki-67 and CD31/34. There was a good geographical colocalisation of both intrinsic markers with pimonidazole. There was a good geographical colocalisation of both intrinsic markers with pimonidazole.}

Keywords: hypoxia; bladder cancer; pimonidazole; carbonic anhydrase IX; glucose transporter protein 1 (GLUT1)

MATERIALS AND METHODS

Patients and treatments

The prospective cohort comprised 21 patients with a diagnosis of transitional cell carcinoma of the bladder who were to undergo radiotherapy. The significance of hypoxia in bladder cancer has been reviewed and stained with GLUT1, CAIX, Ki-67 and the combined vascular markers CD31 and CD34. The results of this have been explored in relation to treatment outcome.
with appropriate approval from the Local Ethics Committee for Mount Vernon Cancer Centre, patients received 0.5 g m\(^{-2}\) pimonidazole (Hydroxyprobe-1\(^{TM}\), Natural Pharmacica Inc., Belmont, MA, USA) administered in 100 ml of normal saline by intravenous infusion over 5 min, 8–18 h prior to biopsy. Tumour samples taken at TURBT were fixed in formalin, protected from light and stored at 4°C.

The retrospective series of patients had been treated at Mount Vernon Hospital in a phase II trial of radical radiotherapy with carbogen and nicotinamide. The patients received 50–55 Gy in 20 daily fractions over 4 weeks with carbogen breathing delivered at a rate of 151 min\(^{-1}\) through a sealed face mask and a closed breathing system with a one-way valve, started 5 min prior to radiotherapy and maintained throughout treatment; only four patients failed to tolerate the whole course of carbogen breathing. A total of 33 patients also received nicotinamide (80 mg kg\(^{-1}\)) given orally at least 1 h after eating and 1.5 h prior to radiotherapy; 31 patients did not receive nicotinamide either due to coexisting vascular disease or patient refusal. Approval from the Local Ethics Committee for Mount Vernon Cancer Centre was granted to approach these patients for permission to use samples of their stored original biopsy material for further analysis. A total of 64 samples was finally obtained and analysed. The characteristics of these patients are shown in Table 1.

### Immunohistochemistry

In the prospective study, pimonidazole, CAIX and GLUT1 were stained on serial sections using single-staining procedures. Staining for pimonidazole both alone and in combination with proliferation and vascular markers have been reported separately (Hoskin et al., 2003 (submitted)). Briefly, after dewaxing and rehydration, the sections were pretreated with 0.03% H\(_2\)O\(_2\) for 5 min at room temperature, this was followed, after washing, by Envision HRP and DAB combination followed by CD31/34 using the Envision AP and Vector red combination as described above. Either rabbit anti-human Ki-67 antibody (Dako) diluted 1/200 or mouse anti-CD31/CD34 antibody cocktail (Dako M0823/M7080) diluted 1/50 and 1/100 was incubated in TBS for 1 h at room temperature. Then, the appropriate Envision polymer and substrate was added as described above to visualise the second antigen. The sections were rinsed in TBS and washed well in running tap water. The sections were counterstained lightly in Gills haematoxylin between 10 and 60 s and then washed well in tap water. Finally, the sections were dehydrated through graded alcohols, cleared by xylene and sections mounted in DPX.

### Immunohistochemical analysis

As hypoxia-related markers tend to be expressed in contiguous cells, the area of pimonidazole, CAIX and GLUT1 staining was assessed at \(\times 100\) (\(\times 10\) eyepiece and objective) using a tool that enabled manual drawing of regions around the stained cells, the overall tumour area and necrosis; this made possible the exclusion of stroma. Two sections from each tumour were systematically assessed and between 3 and 45 fields were viewed for each specimen. The fraction of cells stained for hypoxia was expressed as a percentage of the total tumour area.

Geographic colocalisation was assessed semiquantitatively by visual inspection of stained areas in serial sections.

The proliferative index was assessed from Ki-67 staining in the retrospective series by computer-aided manual counting of multiple fields (\(\times 400\)) captured from two sections of each tumour.

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**Table 1** Patient characteristics

| Stage | Prospective pimonidazole study | Retrospective carbogen patients |
|-------|-------------------------------|-------------------------------|
| Age (years) | 70 (49–87) | 73 (38–88) |
| Median(range) | | |
| Grade | | |
| G1 | 1 | 2 |
| G2 | 10 | 17 |
| G3 | 55 | |
| Total | 21 | 64 |

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Vascularity was assessed by 'hot spot' counting and overall vascular density. The sections were systematically surveyed for a 'hot spot' of vessels using a × 5 objective. The 'hot spot' was then centralised in the microscope field, viewed under a final magnification of × 200 and individual vessels were counted. Between three and 10 hot spots were counted for each tumour, depending on the size of the section. The highest hot spot count was assigned to the tumour. Vascular density involved simply counting all vessels at × 100 magnification in multiple fields from two sections of each tumour. This represented a field size of 0.710 mm². The vascularity was expressed as the number of vessels per mm² of tissue section.

**Statistical analysis**

The relationships between biological parameters (as continuous variables) and T stage and grade were tested using one-way analysis of variance and the χ² test. The biological parameters were compared by either the Spearman rank correlation coefficient for continuous variables or by χ² statistic. Survival intervals were calculated using the Kaplan–Meier product limit method. In the calculation of local recurrence-free survival and metastasis-free survival, nonfailures were censored at last follow-up or at death. For cause-specific survival, patients who died as a result of bladder cancer were classed as failures and nonfailures and were censored at death from other causes or at the last follow-up. For overall survival, patients who died of any cause were classed as failures and non-failures and were censored at the last follow-up. All time intervals were calculated from the date of first radiotherapy treatment. Individual factors were tested using the log rank test, where P<0.05 was considered significant. The Cox proportional hazard model was used for multiple regression analysis.

**RESULTS**

**Staining patterns for pimonidazole, CAIX and GLUT1 in the prospective study**

There was considerable intra- and intertumour variation in both the amount and intensity of staining for each of the three hypoxia-associated markers. Staining with pimonidazole was both cytoplasmic and nuclear and tended to be contiguous, but some focal hypoxia was noted, and even single-cell staining was seen. The darkest staining intensity was consistently seen adjacent to necrotic regions, but there were also other regions of dark staining unassociated with necrosis within tumour sections. CAIX and GLUT1 staining was predominantly membranous and was strongly associated with areas adjacent to necrosis and there was a paucity of staining near blood vessels. Geographical colocalisation of pimonidazole, CAIX and GLUT1 was assessed by visual inspection. The majority of tumours showed similar staining patterns for the three markers with the exception of one tumour that stained extensively for pimonidazole but minimally for both GLUT1 and CAIX, while another showed no pimonidazole binding but substantial GLUT1 and CAIX staining. Concordant localisation is exemplified in Figure 1, which demonstrates closely matched areas where CAIX and GLUT1 showed identical expression within a region that bound pimonidazole. In this case, the binding of pimonidazole was more extensive than the intrinsic markers, but this was variable between tumours. Colocalisation was prevalent but there were also regions of mismatch, more often where pimonidazole and GLUT1 were both positive and CAIX was negative.

In the prospective series of patients, the median stained fraction and range of values for pimonidazole, GLUT1 and CAIX was 9% (0–37%), 15% (0–45%) and 12% (0–35%), respectively. Three tumours failed to demonstrate any pimonidazole binding, while only one was negative for GLUT1 and two did not express CAIX. The markers were all highly correlated with each other (Table 2, Figure 2), but Figure 2 highlights that in box 2 there were tumours that showed expression of the extrinsic marker in the absence of the intrinsic markers and vice versa in box 3.

**Biological parameters in the retrospective study**

In the retrospective group of patients the median hypoxic fraction as defined by GLUT1 was 6.5% (range 0–62%) compared to 3.5% (range 0–67%) with CAIX (Table 3). Again, there was a strong correlation between CAIX and GLUT1 (Table 2, Figure 3). However, there were differences between this series and the prospective patients notably in the proportion of tumours that
showed no evidence of expression of the intrinsic markers. In this series, there were 22 (34%) tumours that were negative for both CAIX and GLUT1 and a further five were without CAIX staining, but had low levels of GLUT1 expression. Interestingly, the absolute levels of both CAIX and GLUT1 positivity was similar in both the prospective and retrospective series in those tumours which expressed the proteins (Figure 3).

Figure 4 shows examples of dual staining from which the data in Table 3 were obtained. In general, there was close geographical concordance of GLUT1 and CAIX as was noted in the prospective study. Although not a formal part of this study, it was noted that both CAIX and GLUT1 tended to stain closer to blood vessels than pimonidazole and that there was an overlap between these markers and Ki-67. The median positivity of 35.7% for Ki-67 represents a value that is indicative of rapid proliferation characteristics. Interestingly, although there was considerable variation in GLUT1 and CAIX both within and between individual tumours, proliferation and vascular parameters showed less variation. Correlation analysis had already revealed a highly significant association between CAIX and GLUT1 (Table 2), but there was no correlation between these markers and vascularity assessed by either vascular

![Figure 2](image-url) Comparison between intrinsic and extrinsic markers of hypoxia. The data are presented as the intrinsic markers subtracted from the pimonidazole score for each tumour in the prospective study. Box 1 indicates good absolute agreement, while boxes 2 and 3 represent disparity between the markers.

![Figure 3](image-url) Correlation between GLUT1 and CAIX in the prospective (○) and retrospective (△) studies.

![Figure 4](image-url) Examples of double staining. Serial sections showing similar areas stained for CAIX (brown) vs CD31/34 (red) (upper), CAIX (red) vs Ki-67 (brown) (middle), and GLUT1 (brown) vs CD31/34 (red) (lower).

Table 2 Linear regression and correlation analysis of the intrinsic and extrinsic hypoxia markers in the prospective and retrospective studies

| Parameters       | Slope | Intercept | $r^2$ | P-value |
|------------------|-------|-----------|-------|---------|
| Pimo vs CAIX     | 0.69  | +4.8      | 0.74  | <0.0001 |
| Pimo vs GLUT1    | 0.76  | +3.7      | 0.82  | <0.0001 |
| CAIX vs GLUT1 prospective | 0.91 | +1.2      | 0.91  | <0.0001 |
| CAIX vs GLUT1 retrospective | 0.90 | −0.8      | 0.96  | <0.0001 |

Pimo = pimonidazole; CAIX = carbonic anhydrase IX; GLUT1 = glucose transporter-1 protein.
density or ‘hot spot’ analysis. However, there was a positive correlation between GLUT1 and Ki-67 (Spearman’s correlation $= 0.294$, $P = 0.018$) and a near-significant correlation between CAIX and Ki-67 (Spearman’s correlation $= 0.24$, $P = 0.056$). Figure 5 shows that this was not a strong relationship, but proliferation was significantly higher in those tumours with the highest levels ($>10\%$) of both GLUT1 ($P = 0.025$) and CAIX ($P = 0.029$) compared to those tumours that were either negative or had low levels of expression. Vascularity showed no association with proliferation, but ‘hot spot’ count did correlate with overall vascular density (Spearman’s correlation $= 0.65$, $P = 0.0001$).

Correlation with clinicopathological features and outcome

None of the biological parameters, in either study, were related to disease stage, grade or patient age.

Table 4 shows the data obtained using the median value of each parameter as a cutoff for univariate analysis for a series of clinical outcome end points. None of the parameters had a significant influence on local control or time to metastasis; the only trend was that rapid proliferation was associated with worse local control. In contrast, there was a highly significant difference in the overall and cause-specific survival when patients were stratified according to the intrinsic markers of hypoxia. The 5-year overall survival rate in tumours expressing higher than median values of GLUT1 was 32% compared to 72% with low levels; the figures were 35% and 71% for CAIX. This analysis was taken a step further and the patients were delineated into three groups, those that were negative for either GLUT1 or CAIX and the positive tumours were stratified above or below a value of 10%. The data are presented in Figure 6. These curves show that tumours without evidence of hypoxia-associated marker expression have the best survival prospects, suggesting that these are genuinely negative tumours and not attributable to sampling error. Low GLUT1 or CAIX is associated with intermediate survival, while 5-year survival rates of 25% were estimated for those patients in which either hypoxia surrogate marker exceeded 10%; this represented 29 (39%) and 22 (34%) patients for GLUT1 and CAIX, respectively.

Multivariate analysis was undertaken using the biological parameters as continuous variables in combination with age, T stage and grade; CAIX and GLUT1 were entered both individually and together. None of the parameters showed independent significance for local control. For both cause-specific and overall survivals, CAIX and GLUT1 showed high independent significance when entered into the analysis individually. The risk ratios (RR) for the overall survival were 3.21 (95% CI 1.16–10.22; $P = 0.02$) for CAIX and 3.14 (95% CI 1.23–10.09; $P = 0.03$) for GLUT1. Similar results were obtained for cause-specific survival. When both

| Parameter | Local control $\chi^2$ | $P$-value | Time to distant metastasis $\chi^2$ | $P$-value | Cause-specific survival $\chi^2$ | $P$-value | Overall survival $\chi^2$ | $P$-value |
|-----------|------------------------|-----------|----------------------------------|-----------|----------------------------------|-----------|--------------------------|-----------|
| GLUT1     | 1.56                   | 0.21      | 0.22                             | 0.64      | 5.84                             | 0.016     | 8.06                     | 0.0045    |
| CAIX      | 0.82                   | 0.36      | 0.29                             | 0.59      | 4.16                             | 0.041     | 8.57                     | 0.0034    |
| Ki-67     | 2.23                   | 0.14      | 1.39                             | 0.24      | 0.63                             | 0.43      | 0.006                    | 0.94      |
| MVD       | 0.068                  | 0.79      | 0.011                            | 0.92      | 0.002                            | 0.96      | 0.07                     | 0.79      |
| Hot spot  | 1.40                   | 0.23      | 0.013                            | 0.91      | 0.57                             | 0.45      | 0.001                    | 0.98      |

GLUT1 = glucose transporter-1 protein; CAIX = carbonic anhydrase IX. Each parameter was categorised according to the median value; MVD=mean vascular density.
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Influence of GLUT1 (upper panel) and CAIX (lower panel) on the overall survival of patients treated with radical radiotherapy, carbogen and nitricamide. The data were classified into tumours with no expression of the proteins and using a cutoff of 10% for those that were positive.

variables were entered into the analysis the significance was lost, CAIX 2.12 (95% CI 0.39 – 10.94); GLUT1 2.12 (95% CI 0.36 – 10.33), suggesting that they are interchangeable as prognostic markers and that either CAIX or GLUT1 is predictive, but this is not refined by the addition of the second marker.

DISCUSSION

Hypoxia in tumours is primarily a pathophysiological consequence of a structurally and functionally compromised microcirculation and the deterioration of diffusion conditions. Hypoxia appears to be strongly associated with tumour propagation, malignant progression and resistance to therapy, and it has thus become a central issue in tumour physiology and cancer treatment (Brown, 2000; Hockel and Vaupel, 2001; Harris, 2002). An overview analysis showed that modification of tumour hypoxia significantly improved the locoregional tumor control after radiotherapy in head and neck (H&N) cancer and to a lesser extent in bladder tumours (Overgaard and Horsman, 1996). However, unlike H&N cancer (Nordsmark et al, 1996; Brizel et al, 1997), it was not possible to confirm the presence and clinical significance of hypoxia in bladder cancer due to the inaccessibility of the tumour site for oxygen electrode-based measurements. The availability of histological markers of hypoxia has widened the opportunity to study tumours in more remote sites. The binding of pimonidazole adducts is perhaps the most robust and widely accepted marker of cellular hypoxia (Varia et al, 1998; Nordsmark et al, 2001; Raleigh et al, 2001; Kaanders et al, 2002). However, the technique is invasive as it requires an injection of pimonidazole to be scheduled prior to surgical procedures. To circumvent this, there has been a growing interest in studying hypoxia-regulated proteins as potential surrogates for the more invasive techniques. These have included HIF-1α and HIF-2α (Beasley et al, 2002), VEGF (Salven et al, 1997), GLUT1 and GLUT3 (Younes et al, 1997; Airley et al, 2001; Baer et al, 2002) and CAIX (Loncaster et al, 2001; Swinson et al, 2003). In the prospective arm of this study, we have demonstrated that pimonidazole, GLUT1 and CAIX are high degree of overlap in their tumour localisation pattern and a significant correlation in their interpatient comparison. In addition, in the retrospective study of patients treated by ARCO(N), both GLUT1 and CAIX overexpression were significantly associated with worse cause-specific and overall survivals but not local control.

Validation of proteins such as CAIX or GLUT1 as intrinsic markers of cellular hypoxia is clearly important to facilitate the routine study of tumour hypoxia in clinical trials and ultimately to use this to direct clinical practice outside trials. Several studies have attempted to achieve this goal. Airley et al (2003) undertook a direct comparison of all three markers in cervix cancer specimens. The study concluded that there were similar staining patterns for all three markers and that they were significantly correlated with each other, although this comparison was made on a semiquantitative scoring system. Other studies have shown a weak but significant correlation between GLUT1 and pO2 values in cervix cancer (Airley et al, 2001), a strong correlation between CAIX and pO2 values in cervix cancer (Loncaster et al, 2001) and a weak correlation between CAIX and pimonidazole in H&N cancer (Kaanders et al, 2002). The general consensus from these studies is that expression of both CAIX and GLUT1 might be potential surrogates for hypoxia. Interestingly, CAIX was predictive of both disease-free and metastasis-free control but not local control, while GLUT1 only had a significant association with metastasis-free survival in similar cervix cancer patients (Airley et al, 2001; Loncaster et al, 2001). CAIX was without clinical significance in H&N cancer in contrast to pimonidazole binding that predicted for locoregional control and disease-free survival (Kaanders et al, 2002).

In bladder cancer, we have previously shown concordance between CAIX and pimonidazole binding (Wykoff et al, 2000). Other studies have demonstrated that GLUT1 expression is not seen in normal bladder mucosa, but is present in malignant bladder mucosa with greater expression seen in muscle-invasive tumours compared to superficial bladder cancer (Chang et al, 2000). In this study, there was no correlation between recurrence and GLUT1 expression. CAIX expression was noted to be greater in superficial disease compared to invasive disease (Turner et al, 2002) and to show significant overlap with VEGF mRNA expression; CAIX did not predict outcome in superficial disease. In contrast, another study of patients treated by cystectomy for muscle-invasive bladder cancer found a significantly worse overall survival if they had >10% GLUT1-stained fraction compared to those with less staining (Younes et al, 2001).

This study demonstrates that bladder cancer is a tumour with an average hypoxic fraction of around 10% and we have now shown that intrinsic markers of hypoxia, both GLUT1 and CAIX, can be used to predict survival. Accordingly, bladder cancer can be seen alongside cervical cancer, H&N cancer and soft-tissue sarcomas as a tumour in which hypoxia is a predictive factor for survival. No correlation was seen, however, between hypoxia and local control or metastases-free survival. This finding may be explained by the fact that all tumours in this group were treated using a hypoxia-
modifying regimen that included the administration of carbogen and nicotinamide (Hoskin et al., 1997; Hoskin et al., 1999). As a consequence, any negative influence of hypoxia on the outcome from radiotherapy may have been overcome. In this series, there was a weak correlation between the Ki-67 index and local control. Other studies that have used Ki-67 as a marker of proliferation in transitional cell bladder cancer have reported a significantly lower progression-free and disease-specific survival rates in patients with higher Ki-67 indices (Popov et al., 1997; Lara et al., 1998; Moonen et al., 1998a; Rodel et al., 2000). Clinical data relating the effect of treatment duration on bladder outcome are equivocal (Maciejewski and Majewski, 1991; De Neve et al., 1995; Moonen et al., 1998b).

The data from this study establish that muscle-invasive bladder cancer is a relatively rapidly proliferating, hypoxic tumour where a rationale for alternative treatments such as ARCON is justified. To maximise the benefit of ARCON, it may be possible to consider individualisation of treatment on the basis of intrinsic immunohistochemical markers of hypoxia and proliferation, which can be performed on routine paraffin-embedded biopsy material. Validation of such an approach requires evaluation in a randomised cohort of patients that is currently being undertaken in the multicentre BCON trial of carbogen and nicotinamide in bladder cancer.

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