Carbonic anhydrase IX (CA IX) expression and outcome after radiotherapy for muscle-invasive bladder cancer.


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Running title: CA IX expression in muscle-invasive bladder cancer.
ABSTRACT

Aims: Carbonic anhydrase IX (CA IX) expression has been described as an endogenous marker of hypoxia in solid neoplasms. Furthermore, CA IX expression has been associated with an aggressive phenotype and resistance to radiotherapy (RT). Here we assess the prognostic significance of CA IX expression in patients with muscle-invasive bladder cancer treated with RT.

Methods and Materials: A standard immunohistochemistry technique was used to demonstrate CA IX expression in 110 muscle-invasive bladder tumours treated with RT. Clinico-pathological data was obtained from medical casenotes.

Results: CA IX immunostaining was detected in 89 (~81%) patients. Staining was predominantly membranous, with areas of concurrent cytoplasmic and nuclear staining and was abundant in luminal and perinecrotic areas. No significant correlation was demonstrated between overall CA IX status and initial response to RT, five-year bladder cancer-specific survival or time to local recurrence.

Conclusions: The distribution of CA IX expression in paraffin-embedded tissue sections seen in this series is consistent with previous studies in bladder cancer but does not provide significant prognostic information with respect to response to RT at 3 months and disease-specific survival following radical RT.

Keywords: bladder cancer, carbonic anhydrase IX, hypoxia, radiotherapy
INTRODUCTION

Approximately 30% of bladder tumours are muscle-invasive at presentation and are therefore associated with a significant risk of metastasis and a poor prognosis. Radical radiotherapy (RT) is the cornerstone of treatment regimens aimed at bladder preservation; however, complete local response is seen in only 50% of cases. Predictive information regarding the likely response of a bladder tumour to RT would be of enormous benefit in enhancing patient selection for bladder preservation.

An association between tumour hypoxia and resistance to treatment with ionising radiation has long been recognised. In other solid tumours, polarographic needle measurements of hypoxia correlate with increased metastatic potential, resistance to RT and an adverse prognosis [1-3]. However, bladder carcinomas are not readily accessible to microelectrodes and alternative strategies aimed at hypoxia measurement need to be assessed.

The transcriptional complex hypoxia-inducible factor-1 (HIF-1) is recognised as a key mediator of gene expression in hypoxic tumours. Hypoxic induction of the carbonic anhydrase genes CA9, CA12 and corresponding proteins (CA IX, CA XII), has been shown to be HIF-1-dependent [4]. In tumour cells, these enzymes are pivotal in maintaining the intracellular pH at physiological levels. The overall effect of CA activity is the relative acidification of the extracellular space. This has important ramifications in promoting further tumour growth and invasion. CA IX expression has been reported as an endogenous surrogate marker of hypoxia in solid neoplasms. In cervical carcinoma, for example, CA IX expression correlates well with
polarographic measurements of tumour oxygen tension [5]. More recently, CA IX expression has been associated with poor prognosis in non-small cell lung cancer [6] and has been associated with poor response to chemoradiotherapy in head and neck cancer [7].

In bladder cancer, CA IX immunostaining is attractive as a marker of hypoxia, since it is non-invasive and does not require systemic administration, compared with polarographic needle measurements and pimonidazole, respectively. Significant correlations have been observed between CA IX expression and pimonidazole levels in bladder cancer [4]; however, there is no clear consensus as to the prognostic value of CA IX immunostaining in this disease. Turner et al. [8] studied the distribution of vascular endothelial growth factor (VEGF) mRNA (by in situ hybridisation) and CA IX expression in 22 bladder cancers of varied pathological stages. Co-localisation of VEGF and CA IX expression was observed, with both being expressed predominantly on luminal surfaces and in perinecrotic areas. Expression of both factors was greater in superficial compared with invasive disease. The authors went further, to study the relationship between expression of CA IX and clinical outcome in 49 patients with superficial bladder cancer. CA IX expression was not predictive of clinical outcome. Hoskin et al. [9] investigated GLUT1 and CA IX as endogenous markers of hypoxia and their relationship to outcome in a retrospective series of 64 bladder cancer patients treated with radical RT with carbogen and nicotinamide (ARCON). GLUT1 and CA IX staining were found to be independent predictors for overall and disease-specific survival, but not for local control or metastasis-free survival. A prospective study was also reported in which pimonidazole, GLUT1 and CA IX staining was compared in 21 patients with bladder cancer. A good correlation was reported
between CA IX/GLUT1 expression and pimonidazole staining. More recently, CA IX expression has been studied in 57 patients with superficial or invasive disease [10]. Again, significantly more superficial bladder cancers expressed CA IX strongly. However, no significant association between CA IX staining and survival was established in either superficial or invasive disease.

In the present study, we evaluate CA IX expression in invasive bladder cancer using standard immunohistochemistry. We determine the prognostic significance of tumour CA IX expression in patients treated with RT; the primary endpoints being initial response to radiotherapy and survival (bladder-cancer specific) and a secondary endpoint being local recurrence.

**METHODS AND MATERIALS**

*Study Population*

Ethical approval was obtained for the study of archival paraffin-embedded tissue sections from 110 patients with pathological stage T2-T3 bladder cancer. The exact same study population was used in a recent immunohistochemical study to demonstrate that epidermal growth factor receptor status predicts local response to radical radiotherapy in muscle-invasive bladder cancer [11]. Therefore, we are confident that the size of the study population of the present study has the power to detect any differences, to the extent of the previous study, should they exist.
All patients were treated by 6 or 8 Mv X-rays between 1992 and 1997. The most commonly used regimen (77 patients) was 60 Gy in 30 fractions given over 42 days. 25 patients were treated with 50-55 Gy in 20 fractions. Others received varying doses between 45-64 Gy in 20 to 32 fractions. Treatment was given to the bladder only, with a 1-1.5 cm margin. Most patients were planned using a cystogram and cystoscopic findings were taken into account in deciding the treatment volume. In the last 2 years of the study, patients were planned on computed tomographic images. Of the specimens, 91 (82.7%) were from male and 19 (17.3%) were from female patients. Staging was based on biopsy reports from the initial transurethral resection of the bladder tumour. The clinico-pathological data is summarised in Table 1. Hospital notes were reviewed to determine the following clinical outcomes; initial response to RT, local and distant tumour recurrence rates and survival. Initial response to RT was determined at 3 months by check cystoscopy/histology and defined as follows: ‘complete’ (no evidence of tumour in bladder), ‘partial’ (tumour of a lower histological grade/stage than at diagnosis, present in the bladder) or ‘none’ (tumour of same or higher stage and grade as at diagnosis, present in the bladder). ‘Complete’ or ‘partial’ responses were considered positive and ‘none’ responders considered negative.

**Materials**

The tumour specimens evaluated were routinely processed, formalin-fixed and paraffin-embedded. All the TURBT sections were reviewed by a pathology SpR at Leicester General Hospital and a block was selected for analysis that was representative of invasive tumour. Tissue sections of 4μm thickness were cut onto
glass slides that were previously treated with 2% 3-aminopropylethoxysilane (in methanol) and dried overnight at 37°C to promote section-to-slide adhesion.

The murine monoclonal antibody M75, recognising the N-terminal domain of MN/CA IX protein, has been previously reported by Pastorekova et al [12]. The specificity of the monoclonal antibody M75 for CA IX has been previously reported using Western blots and immunostaining of COS-7 cells transfected with CA IX cDNA [13]. The secondary antibody was polymer-conjugated rabbit antimouse from the Envision kit (Dako, Ely, UK).

**Immunohistochemistry**

No antigen retrieval was required. Slides were dewaxed in xylene before rehydration by passage through graded alcohols. Endogenous peroxidase was blocked by applying 0.03% hydrogen peroxide containing sodium azide from the Envision kit (Dako) for 10 minutes. Non-specific staining was prevented by the application of 100μl of 10% human serum for 15 minutes. The working solution of M75 (stock solution diluted 1:50 (vol/vol) in 5% human serum) was added for 30 minutes. Polymer-conjugated rabbit anti-mouse secondary antibody from the Envision kit (Dako) was then added for 30 minutes. Diaminobenzidine substrate (DAB, applied for 8 minutes) was used to visualise CA IX. Sections were washed in Tris-buffered saline for 5 minutes following the sequential incubations with M75 and secondary antibody. After DAB staining, slides were immersed in running tap water for 5 minutes and counterstained with haematoxylin. Slides were dehydrated by reverse passage through graded alcohols and mounted using DPX (BDH Chemicals Ltd.).
**Interpretation of CA IX staining**

Tissue sections from a Non-Small Cell Lung tumour were used as positive controls. Negative controls consisted of bladder tumour sections processed without the use of the primary antibody. Tissue sections were evaluated blind using light microscopy by two independent observers. Consensus was then reached using a conference microscope. Sections were classified as positive or negative (<5%) for CA IX expression. In addition, the type of staining (i.e. membranous, cytoplasmic or nuclear) and the presence or absence of necrosis was documented.

**Statistics**

The SPSS software system (SPSS for Windows, version 12.0; SPSS Inc., Chicago, IL) was used to perform statistical analysis. The analysis comparing CA IX status with initial RT response were performed using either Chi-squared or Fisher’s exact tests. Survival curves were plotted using the Kaplan-Meier method and statistical significance was assessed using the log-rank test. Univariate analyses, in preparation for subsequent multivariate analysis, were performed using Kaplan-Meier analysis and multivariate analysis was performed using Cox’s regression to determine any potential independent prognostic factors for diminished bladder-cancer specific survival. The level of statistical significance was taken to be p< 0.05.

**RESULTS**

**CA IX Tumour Cell Expression**
Concordance between the two observers with respect to positivity of immunostaining in this series was 97%. Tumour cell CA IX immunostaining was detected in 89 (80.9%) patients. Staining was predominantly membranous, with areas of concurrent cytoplasmic staining (n = 42, Figure 1). CA IX expression was abundant in luminal and perinecrotic areas (Figures 1 & 2). In a small number of cases (n = 25), areas of nuclear staining (Figure 3) were observed along with membranous/cytoplasmic patterns.

CA IX Expression and Initial Response to RT

87 patients (79.1%) underwent check cystoscopy at 3 months post RT, to determine response to treatment. Cystoscopy was not performed if the patient had developed metastatic disease or was considered unfit for the procedure, i.e. an active decision was taken by the responsible clinician not to perform the procedure. 60 patients (69%) had a positive response (53 complete, 7 partial) and 27 (31%) had a negative response to RT. No significant correlation (Chi-squared and Fisher’s exact tests) was demonstrated between either overall CA IX status or nuclear CA IX staining and initial response to RT (\( p = 0.561 \) and \( p = 0.22 \) respectively).

CA IX Expression and Survival Following RT

Overall 5-year survival was 23.6% in this series. 5-year bladder cancer-specific survival was 40.9%. No significant association (log-rank test) was observed between either overall CA IX status, or nuclear CA IX staining and bladder cancer-specific survival following RT treatment (\( p = 0.9014 \) and \( p = 0.6381 \), respectively, Figures 4a & 4b)(overall CA IX-negative patients: median survival 29.46 months (IQR: 5.26-
CA IX Expression and Local Recurrence

29 (48%) patients who exhibited a positive response to RT subsequently suffered local recurrence of their bladder cancer. No significant association (log-rank test) between overall CA IX status and time (months) to local recurrence was demonstrated ($p = 0.4679$) (overall CA IX-negative patients: median time to local recurrence 16.9 months (IQR: 12.50-16.73); overall CA IX-positive patients: median time to local recurrence 14.85 months (IQR: 6.80-17.6).

Analysis of the effect of nuclear CA IX staining on tumour recurrence rates following positive response to RT revealed patients with positive nuclear CA IX staining to be significantly more likely to experience a more rapid local recurrence after an initial positive response to RT ($p = 0.0005$). However, the small number of patients exhibiting both nuclear CA IX staining and tumour recurrence following a positive response to RT, plus the high numbers of censored events, limited the statistical validity of this analysis (data not shown for this reason).

Multivariate analysis of the impact of CA IX expression on response to RT

In preparation for multivariate analysis, the effect of several potential independent prognostic factors was assessed using univariate (Kaplan-Meier) analysis (see Table 2). Only factors which significantly predicted altered bladder-cancer specific survival at a significance level of $p$–0.10 were entered into the final model. Thus CA IX status, nuclear CA IX staining, tumour grade and evidence of previous superficial
TCC were not retained for further multivariate analysis. Following stepwise conditional log rank analysis, whereby each factor is removed from the model in a stepwise manner to assess the effect on the model, the following 4 factors retained independent prognostic significance for reduced bladder cancer-specific survival; pre-treatment ureteric obstruction, lack of response to RT at 3-month cystoscopy, local recurrence and metastatic spread ($p = 0.005$, $p = 0.036$, $p = 0.03$ and $p = 0.002$ respectively, Table 3).

**DISCUSSION**

CA IX immunostaining has attracted widespread attention as a surrogate marker of hypoxia in many tumour models. However, previous studies of CA IX as a marker of hypoxia in bladder cancer have been undertaken using relatively small numbers of tumours, which are often heterogeneous with respect to tumour stage and treatment modality. Collectively, they provide no clear consensus as to the prognostic value, or clinical utility of CA IX immunostaining in major patient groups. No previous studies have examined the relationship between CA IX expression and outcome after radical RT (as monotherapy), which is still widely used in the treatment of muscle-invasive disease. There is a well-recognised need for predictive information regarding tumour radiosensitivity, to improve patient selection for RT in treating this disease. The present study, involving a large series of 110 patients, provides an assessment of the prognostic value of CA IX immunostaining in this important group of patients.
The abundance of CA IX immunostaining in luminal and perinecrotic areas of the tumour is consistent with previous studies in bladder cancer [8-10] and supports the notion that CA IX localises to areas of tumour hypoxia. The observation that CA IX immunostaining localised to the nucleus in 25 cases is interesting and not reported elsewhere.

In our series, overall CA IX immunostaining was not found to be of prognostic significance with respect to response to RT in patients with muscle-invasive bladder cancer. In addition, no association between overall CA IX status and local recurrence was observed. Patients with positive nuclear CA IX staining were found to be significantly more likely to experience more rapid local recurrence after a positive initial response to RT. However, the numbers of patients involved in this subgroup analysis were small, and the number of censored events relatively high, thereby casting doubt over the true significance of this observation (data not shown for this reason).

Using multivariate analysis, pre-treatment ureteric obstruction, lack of response to RT at 3-month cystoscopy, local recurrence and metastatic spread were all identified as adverse prognostic indicators. This is in keeping with previous studies [14-16] and provides validation of this series.

The lack of an association between CA IX expression and outcome after RT is to be contrasted with reports of other purported endogenous markers of hypoxia (for example GLUT-1 and HIF-1α) [5-7] in other tumours, where expression has been associated with adverse prognosis and radioresistance. This association has not been
observed universally, however. In a recent study of HIF-1α expression in locally advanced cervical cancer [17], no correlation was observed between HIF-1α expression and outcome after RT, indeed high HIF-1α expression tended to be associated with good outcome in larger tumours.

The present findings may reflect the importance of mechanisms other than hypoxia in dictating outcome after RT in this disease. For example, differences in the intrinsic radiosensitivity of human tumour cells are well recognised and these differences relate to clinical radiocurability [18]. One mechanism proposed to explain this variation is that the amount and distribution of DNA damage induced may vary between tumour cells of different radiosensitivity; a view that is supported by previous work with human bladder and breast cancer cell lines [19, 20]. Indeed, the importance of intrinsic radiosensitivity in determining radioresistance in bladder cancer, is highlighted by more recent studies in which alkaline comet assay measurements of damage formation and repair correlate with cell survival as determined by clonogenic analysis [21, 22]. Differences in tumour cell repopulation rates may represent a further confounding variable. In most centres, radiation therapy for bladder cancer (60-70 Gy) is usually delivered over a period of 6-8 weeks. Using Ki67 immunostaining, Lara et al. [23] observed that patients with very low proliferating tumours seemed to achieve better local control after fractionated RT compared with other patients.

Another explanation for the lack of association between CA IX expression and tumour response to RT may arise from mounting evidence that different intracellular pathways may modify HIF-1α expression and expression of its target proteins. This
may limit the specificity of CA IX expression as a surrogate marker of hypoxia. The phosphotidylinositol 3-kinase (PI3K), mitogen associated protein kinase (MAPK) [24, 25] and Smad pathways are known to influence HIF-1α expression independently of hypoxia and these pathways are overactive in a wide range of tumour models. In prostate cancer cell lines, epidermal growth factor receptor (EGFR) stimulation induces HIF-1α via the PI3K pathway independently and additively to hypoxia [26]. EGFR is a member of the c-erb family of tyrosine kinase receptors and is commonly over expressed in bladder cancer cells [27]. The effect of interactions between the hypoxia response pathway and other cell signalling pathways should be borne in mind when immunohistochemical studies using so-called surrogate markers of hypoxia are undertaken.

A further explanation that may contribute to a lack of association between CA IX expression and tumour response to RT arises from the observations of Sobhanifar et al. [28] that the half-life of CAIX was sufficiently long that, once formed, it remained for days in the absence of continued HIF-1 alpha expression; so it could be present in oxygenated tissue that had recently been hypoxic. Indeed, very recently Shin et al. [29] showed the CA IX levels fail to respond to manipulated changes in tumour oxygenation mediated by carbogen and hydralazine, treatments that respectively increased and decreased tumour oxygenation (carbogen was given 75 minutes and hydralazine 30 minutes before sacrifice). This indicates an inability of CA IX to reflect fluctuating (acute) hypoxia accurately and may contribute to the lack of association between CA IX expression and tumour response to RT observed in the present study.
This investigation may be hindered by the fact that bladder tumours are often extensive, multifocal and heterogeneous, containing numerous biologically different populations within a single neoplasm. This can give rise to tumour heterogeneity with respect to cellular morphology, karyotype, ploidy, cell cycle and cell proliferation kinetics, clonogenic potential, receptor expression and metastatic and tumorigenic properties [30, 31]. Biological heterogeneity has been shown to cause differing radiosensitivity in human bladder cancer cell lines \textit{in vitro} [32] and could therefore be considered as a cause of differing clinical response to radiation treatment for invasive bladder cancer. Finally, the range of radiotherapy doses and fractionation schemes used may to some extent confound the results.

\section*{CONCLUSIONS}

The distribution of CA IX expression in paraffin-embedded tissue sections seen in this series is consistent with previous studies in bladder cancer. However, CA IX immunostaining in tissue sections from patients with muscle-invasive bladder cancer does not provide significant prognostic information with respect to local control and bladder-cancer specific survival following radical RT. The clinical utility of CA IX immunostaining with respect to the treatment of invasive bladder cancer is therefore limited.

\section*{ACKNOWLEDGEMENTS}

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Royal College of Surgeons of England for the financial support of AJC and Professor J Louise Jones for her helpful advice in slide interpretation. This work was supported by a Cancer Research UK project grant (C13560/A4661) awarded to GDDJ.

REFERENCES

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**FIGURE LEGENDS**

**Figure 1**: Typical CA IX staining distribution. CA IX expression is predominantly membranous (M) with areas of concurrent cytoplasmic staining (C). Expression was strongest in tumour cells near the luminal surface (L) and increased with distance from the tumour microvasculature (V).

**Figure 2**: Prominent CA IX expression adjacent to a large area of necrosis (N).

**Figure 3**: Nuclear CA IX staining (n = 25).

**Figure 4**: Kaplan-Meier survival curve for bladder cancer-specific survival (Y axes are cumulative survival fraction) following treatment with RT, stratified by a) overall CA IX status and b) nuclear CA IX status (CA IXn). No significant differences are demonstrable ($p = 0.9014$ and $p = 0.9589$ by log-rank, respectively).
Figure 4a: Overall CA IX status vs. disease specific survival ($p = 0.9014$)

![Graph showing overall CA IX status vs. disease specific survival](image)

- CA IX Negative ($n = 21$)
- CA IX Positive ($n = 89$)

Figure 4b: CA IX nuclear staining (CA IXn) vs. disease specific survival ($p = 0.6381$)

![Graph showing CA IX nuclear staining vs. disease specific survival](image)
Time from RT to death in months

- CA IXn Negative (n = 85)
- CA IXn Positive (n = 25)
Table 1: Summary of clinicopathological data

<table>
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<th>Characteristic</th>
<th>n</th>
<th>%</th>
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<td><strong>No. of patients</strong></td>
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<td>100</td>
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<tr>
<td>50-59</td>
<td>8</td>
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<tr>
<td>60-69</td>
<td>25</td>
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<tr>
<td>80-89</td>
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</tr>
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<td>3</td>
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<td><strong>Radiation therapy</strong></td>
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<td>Mean total dose (range, SD)</td>
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<td>Modal fraction size (range, SD)</td>
<td>2 (2.0-2.5 ± 0.2 Gy)</td>
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<td>Modal fraction number (range, SD)</td>
<td>30 (20-32 ± 3.5)</td>
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<td>Mean duration of treatment (range, SD)</td>
<td>45.1 (27-87 ± 9 days)</td>
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**Table 2:** Potential prognostic variables associated with adverse disease-specific survival after RT

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<th>Factor</th>
<th>Log rank p value (Kaplan-Meier analysis)</th>
<th>Retained for final multivariate model</th>
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<tr>
<td>CA IX status (positive vs. negative)</td>
<td>0.9014</td>
<td>X</td>
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<tr>
<td>CA IX nuclear staining (positive vs. negative)</td>
<td>0.6381</td>
<td>X</td>
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<td>Tumour grade (1-2 vs. 3)</td>
<td>0.4075</td>
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<td>Clinical stage (2 vs. 3-4)</td>
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<td>0.0073</td>
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<tr>
<td>Lack of response to radiotherapy at 3 months (yes vs. no)</td>
<td>0.0001</td>
<td>✓</td>
</tr>
<tr>
<td>Local recurrence (yes vs. no)</td>
<td>0.0001</td>
<td>✓</td>
</tr>
<tr>
<td>Metastatic spread (yes vs. no)</td>
<td>0.0001</td>
<td>✓</td>
</tr>
<tr>
<td>Previous superficial TCC (yes vs. no)</td>
<td>0.9522</td>
<td>X</td>
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Table 3: Independent prognostic factors predicting diminished bladder cancer-specific survival following RT, determined by multivariate analysis.

<table>
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<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
<th>p value</th>
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<td>Ureteric obstruction</td>
<td>2.98</td>
<td>1.40-6.33</td>
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<td>Lack of response to radiotherapy at 3 months</td>
<td>2.86</td>
<td>1.06-7.69</td>
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<td>Local recurrence</td>
<td>3.31</td>
<td>1.13-9.72</td>
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