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Original Research Article

Glut-1 expression in small cervical biopsies is prognostic in cervical cancers treated with chemoradiation

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ABSTRACT

Background/purpose: Chemoradiation (CRT) is standard therapy for locally advanced cervical cancer (LACC). However, there is a lack of biomarkers to identify patients at high relapse-risk. We examine metabolic (glucose transporter-1 [Glut-1]), hypoxic (hypoxia inducible factor [HIF-1α]; carbonic anhydrase [CA-9]) and proliferative (Ki-67) markers for prognostic utility in LACC.

Materials/methods: 60 LACC patients treated with CRT had pre-treatment biopsies. Immunohistochemistry was performed for Glut-1, HIF-1a and CA-9, to generate a histoscore from intensity and percentage staining; and Ki-67 scored by percentage of positive cells. For each biomarker, treatment response and survival was compared between low and high-staining groups by logrank testing and multivariate analyses.

Results: High Glut-1 expression was associated with inferior progression-free survival (PFS), (hazard ratio [HR] 2.8, $p = 0.049$) and overall survival (OS), (HR 5.0, $p = 0.011$) on multifactor analysis adjusting for stage, node positivity, tumour volume and uterine corpus invasion. High Glut-1 correlated with increased risk of distant failure (HR 14.6, $p = 0.001$) but not local failure. Low Glut-1 was associated with higher complete metabolic response rate on post-therapy positron emission tomography scan (odds ratio 3.4, $p = 0.048$). Ki-67 was significantly associated with PFS only (HR 1.19 per 10 units increase, $p = 0.033$). Biomarkers for hypoxia were not associated with outcome.

Conclusions: High Glut-1 in LACC is associated with poor outcome post CRT. If prospectively validated, Glut-1 may help select patients for more intensive treatment regimens.

Introduction

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in women worldwide [1]. Standard treatment in locally advanced disease is concurrent chemoradiation [2]. Clinical prognostic factors include tumour stage and nodal involvement [3], although metabolic response after chemoradiation has been shown to have even stronger prognostic value [4–7]. Additionally, post-therapy ^{18}F-fluoro-deoxyglucose positron emission tomography (FDG-PET) response can help direct potentially curative salvage interventions in patients who fail primary therapy. However, post-therapy FDG-PET is unable to inform the design of investigations or therapeutic interventions before or during treatment.

There have been numerous putative pathobiological prognostic factors studied, including factors involving the angiogenesis, hypoxia, epidermal growth factor receptor and COX-2 pathways [8]. We set out to assess the prognostic significance of biomarkers for metabolism (glucose transporter-1 [Glut-1]), proliferation (Ki-67) and hypoxia (hypoxia-inducible factor-1α [Hif-1α] and carbonic anhydrase IX [CA-9]) in cervical cancer patients treated with chemoradiation. We hypothesised that these pre-treatment

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biomarkers were predictive of metabolic response on post-therapy FDG-PET, and correlate with progression-free and overall survival.

Materials and methods

Patient accrual and treatment

This is a retrospective analysis of a prospective tumour registry of 105 patients with locally advanced cervical cancer treated from January 2002 to June 2007 with chemoradiation at the Peter MacCallum Cancer Centre (PMCC), Melbourne, Australia, and its affiliated satellite sites. Patient characteristics, staging, treatment, toxicities and follow-up details were prospectively recorded into our institutional database. Human ethics approval for the study was received from the PMCC institutional ethics board. This study is reported conforming to the REporting recommendations for tumour MARKer prognostic studies (REMARK) criteria [9]. This is a set of guidelines on assessing validity of tumour markers.

Irradiation techniques have been previously described [6]. In brief, conventionally fractionated external beam radiotherapy was planned to between 40 and 45 grays (Gy) to the pelvis with a nodal boost to 50–50.4 Gy as required, with concurrent cisplatin chemotherapy at a dose of 40 mg/m^2 weekly for 4–6 cycles. Within 10 days of completion, a high-dose rate intracavitary brachytherapy boost was delivered twice weekly to a dose of 28 Gy in 4 fractions (or equivalent, to a total tumour dose of 80 Gy. All patients had histologically confirmed carcinoma of the uterine cervix, International Federation of Gynecology and Obstetrics (FIGO) stage Ib to IVa and ECOG performance status 2 or less.

A single post-therapy FDG-PET/CT was performed using the methods/techniques reported previously [6], between 3 and 6 months after completion of chemoradiation therapy, in accordance with The National Comprehensive Care Network (NCCN) 2016 guidelines. Metabolic changes post-therapy were scored as complete metabolic response (CMR) where there is no tracer uptake or background level of FDG-activity within the treated disease, partial metabolic response (PMR) where there is residual FDG-activity within the treated disease, and progressive metabolic disease (PMD) where there is increased intensity or distribution of FDG-avid disease, as previously published by our institution [10]. Clinical follow-up of patients including medical history and physical examination was performed at 4 weeks post-therapy, 3 monthly until 2 years post-therapy, 6 monthly in years 3, 4 and 5, then yearly thereafter.

Immunohistochemistry

Immunohistochemistry was performed on cut sections from formalin fixed paraffin embedded (FFPE) tumour tissue. 4 μm tissue sections were cut and de-waxed through histolene, and graded alcohols then into water. Antigen retrieval was performed using Dako high pH Target Retrieval Solution (Dako) for 3 min at 125 °C for Glut-1, HIF-1 α and CA-9. Slides were then loaded onto a Dako autostainer (Dako) for the following incubations: primary antibody for Glut-1 (1:200, Dako), HIF-1α (1:50, Novus Biologicals) or CA-9 (1:4000, Novus Biologicals) for 60 min at room temperature; antibody detection with Envison+ (Dako) rabbit (Glut-1 and CA-9) or mouse (Hif-1α) antibody for 60 min at room temperature; colour reaction with 3,3′-Diaminobenzidine (DAB) for 10 min at room temperature. Staining for Ki-67 was performed on a Ventana BenchMark Ultra (Roche Diagnostics, USA). Antigen retrieval was performed in a high pH Ultra cell conditioning solution (CC1, Roche Diagnostics) for 52 min followed by incubation with the Ki-67 antibody (SP6, Cell Marque, diluted at 1/50), at 36 °C for 32 min. Amplification kit (amplifiers A and B, Roche Diagnostics) and UltraView Universal DAB detection kit (Roche Diagnostics) were used in accordance with the manufacturer's instructions for on-board detection. Slides were then removed from the autostainer, counterstained with hematoxylin, mounted and coverslipped.

Scoring criteria and cut-offs

Scoring of membranous staining for Glut-1 and CA-9, or nuclear staining for HIF-1α was performed according to a previously used semi-quantitative system [11–15]. A histoscore (0–12) was generated by multiplying intensity (score 0–3) by a categorical percentage score (0 for No staining, 1 for <25%, 2 for 25–29%, 3 for 50–74% and 4 for ⩾75% of cells). This method of assigning histoscore based on a combination of percentage and intensity of staining is a commonly utilized and accepted methodology of immunohistochemical scoring, including being used for Glut-1 in the melanoma setting [16]. Cohorts were dichotomised into low and high staining groups in equal proportion for statistical analysis, consistent with other similar studies [17,18]. This resulted in the following cut off values: for Glut-1 low is defined as score 0–3, high is 4–12; CA-9 low is defined as 0–2 and high is 3–12; for HIF-1α low is 0–2 and high is 3–12. Ki-67 scoring was performed by counting 1000 representative tumour cells and calculating the percentage of positive cells.

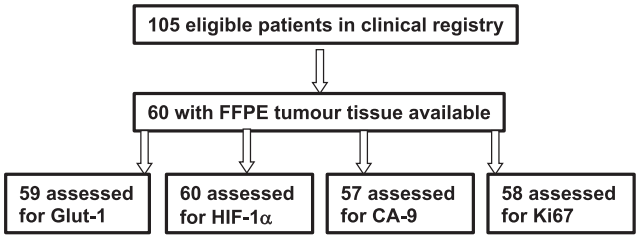
Statistical analysis

The primary objective was to evaluate the association between pre-treatment biomarkers and post-therapy PET metabolic response. The secondary objectives were to evaluate the association of these biomarkers with progression-free survival and overall survival. All time to event analyses were calculated from the date of commencement of radiotherapy to the date of the event. Death was a censoring event for time to local, time to nodal and time to distant failure. The impact of biomarkers on time to event outcomes were assessed using likelihood ratio test (Ki-67) and logrank test (all other biomarkers) on univariate analysis. Cox proportional hazards model was used to assess the impact of the biomarkers adjusting for possible confounders using known pre-treatment prognostic factors namely, tumour volume on magnetic resonance imaging (MRI), FIGO stage, node positivity and uterine corpus invasion. Our centre has previously published our findings that uterine corpus invasion in cervical cancer is correlated with overall survival [19], an observation collaborated by another group [20]. Time-to-event curves were described using Kaplan–Meier methods. The association between metabolic response and expression of biomarkers as dichotomous variables was performed using Barnard's test. The association between Ki-67 as a continuous variable and metabolic response was examined by the Wilcoxon rank sum test.

Results

Immunohistochemistry

Sixty of 105 cases had tumour blocks available for immunohistochemical (IHC) biomarker analysis (Fig. 1). Baseline characteristics of the IHC study population are shown in Table 1. IHC results were obtained in 57 patients for CA-9, 59 for Glut-1 and 60 for HIF-1α staining. Fifty-eight cases were assessed for Ki-67 proliferation index and there were 57 cases where staining for all four markers was available. Supplementary Fig. 1 shows representative staining for each of the tested biomarkers. For these biomarkers, staining, when present, was generally diffuse. The variation between cases was mainly on intensity of staining rather than percentage staining.



FFPE: formalin fixed paraffin embedded

Fig. 1. Flow of cases through the study according to REMARK criteria.

Table 1
Patient characteristics.

Characteristic	Category	Number	%
FIGO stage	Ib	19	31.7%
	II	27	45.0%
	III	14	23.3%
Age	Median (range)	54 (26–84)	
Uterine corpus invasion	Yes	39	65%
	No	21	35%
Tumour volume (cc)	Median	35.9	
	Interquartile range	16–71	
Nodal status	Node positive	24	40%
	Node negative	36	60%
No. of lymph nodes involved	1	8	13.3%
	2	7	11.7%
	3	5	8.3%
	4+	4	6.7%
Histology	Squamous	58	96.7%
	Adenosquamous	1	1.6%
	Clear cell	1	1.6%

There were no significant number of cases with small percentage but intense level of staining. We did not choose a specific cut-off such as >50% staining of 2+ or more for high level of staining; because there is currently no biological or clinical rationale supporting a specific cutoff. Furthermore, it has been common practice for other studies examining these biomarkers to assign high and low expression groups by the median value [17,18].

There were 29/57 patients (51%) with high membrane staining for CA-9, 29/60 patients (48%) with high nuclear staining for HIF-1α and 28/59 patients (47%) with high membrane staining for Glut-1. The median Ki-67 proliferation index was 40% (range 3–95%).

Biomarkers as predictors of metabolic response

Of the 60 patients, 44 had CMR, 7 had PMR and 9 had PMD. The association between metabolic response (CMR vs non CMR) and expression of CA-9, HIF-1α and Glut-1 is described in Table 2. High Glut-1 expression was associated with a lower CMR rate, (odds ratio [OR] 0.30 [95% CI 0.08–0.97], $p = 0.048$). There was no evidence that the CMR rate is associated with CA-9 ($p = 0.86$), Hif-1α ($p = 0.12$) or Ki-67 ($p = 0.08$).

Biomarkers and survival outcomes

The median follow-up for the 60 patients was 5.2 years (range 1.4–8.0 years). Glut-1 was significantly associated with progression-free survival [PFS] (HR 2.8 [95% CI 1.0–7.9], $p = 0.049$) and OS (HR 5.0 [95% CI 1.3–19.2], $p = 0.011$) (Figs. 2 and 3), on multifactor analysis adjusted for possible potential confounding factors namely, MRI volume, FIGO stage, node positivity and uterine corpus invasion (Table 3). On multifactor analysis, the HR of Ki67 (per 10 units increase) for PFS was 1.19 [95% CI 1.01–1.41], $p = 0.033$). Ki-67 was not significantly associated with OS. Hif-1α and CA-9 were not associated with survival.

Patterns of failure

The estimated five-year local failure-free rate in all patients was 84% [95% CI (74–94%)], five-year nodal failure-free was 70% [95% CI (59–83%)], and five-year distant failure-free rates were 78% [95% CI (67–89%)]. None of the investigated biomarkers were associated with the risk of local failure, However, Glut-1 was significantly associated with risk of distant failure (HR 14.6 [95% CI 1.9–112.9], $p = 0.001$).

Discussion

Glut-1, a glucose transporter protein, has been implicated as a mechanism for increased glycolytic metabolism of tumours. Its overexpression is a poor prognostic marker in a variety of tumours including non-small cell lung [21], colorectal [22], gastric [23] and oral squamous cell carcinoma [24]. With regards to cervical cancer, Airley et al. [25] found negative Glut-1 staining to be significantly associated with increased metastasis-free survival ($p = 0.022$), after adjustment for the effect of tumour stage, grade and patient age. This is in keeping with our observation of a significant association between high Glut-1 staining and risk of distant disease failure ($p = 0.001$).

Additionally, we found high Glut-1's association with inferior progression-free ($p = 0.049$) and overall survival ($p = 0.011$). In Airley's study however, Glut-1 staining was not significantly associated with disease-free survival. The two studies are similar with respect to the patient population of cervical squamous cell carcinoma treated with radiotherapy, although radiosensitising chemotherapy was also used in ours but not Airley's study; and both had five years follow-up period. However, Airley et al. reported results based on absent versus present Glut-1 staining, whereas we dichotomised the cohort into low and high staining groups of approximately equal

Table 2
Metabolic response according to CA-9, HIF-1 α, Glut-1 and Ki-67 expression levels.

Biomarker	Group	CMR	Non-CMR	OR (95% CI)	*p*-Value
CA-9	Low	21 (75%)	7 (25%)	1	0.86
	High	21 (72%)	8 (28%)	0.88 (0.26–2.87)	
HIF-1α	Low	20 (65%)	11 (35%)	1	0.12
	High	24 (83%)	5 (17%)	2.64 (0.82–9.57)	
Glut1	Low	26 (84%)	5 (16%)	1	0.048
	High	17 (61%)	11 (39%)	0.30 (0.08–0.97)	
Ki-67 (×10 units)	Median difference* (95% CI)	−1.5 (−3.5 to 0.0)		0.82 (0.65–1.01)	0.081

CMR: complete metabolic response, OR: odds ratio.
* Negative number represents lower Ki67 on CMR.

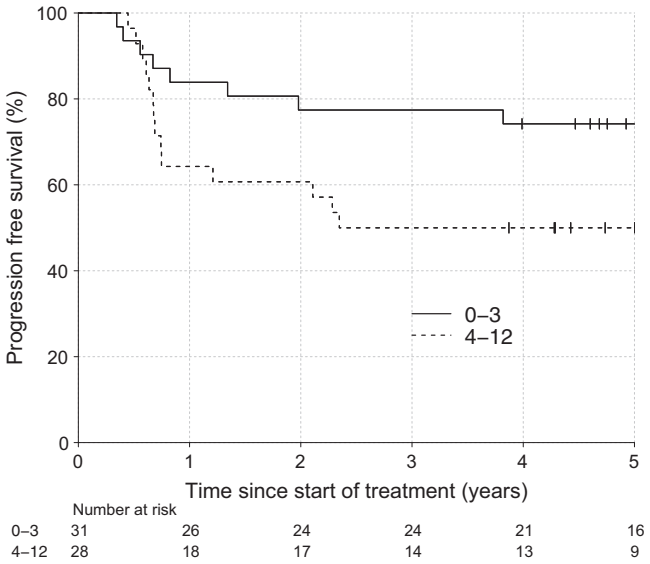


Fig. 2. Progression free survival according to Glut-1 expression (histoscore).

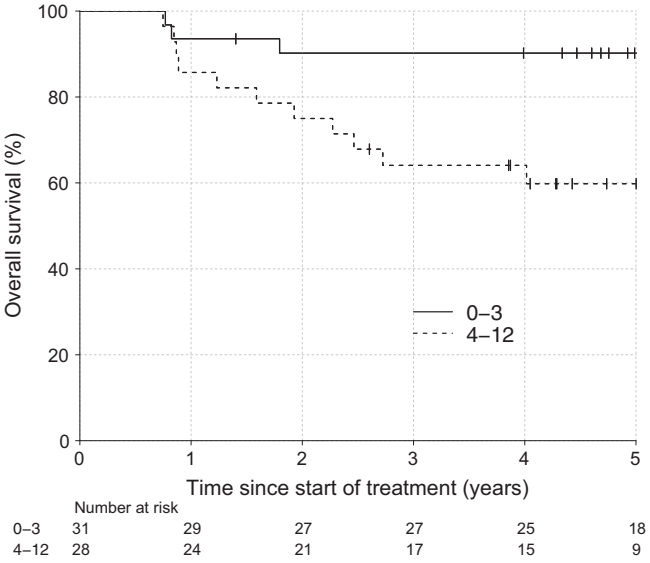


Fig. 3. Overall survival according to Glut-1 expression (histoscore).

case numbers. It may be that low and absent Glut-1 staining have a similar advantage in survival; therefore the association between Glut-1 and survival becomes diluted when examining only presence or absence of staining, as performed by Airley et al.

Post-therapy PET metabolic response has been shown to be a strong predictor of survival following chemoradiation for cervical cancer [5,6,26]. In this study, we have shown pre-treatment Glut-1 staining in cervical tumour biopsy correlates with CMR on PET post chemoradiation ($p = 0.048$). As previously reported, the presence of CMR was significantly associated with superior survival outcome [6]. Patients with a CMR had a 5-year OS of 95% [95% CI (89–100%)], compared with 21% [95% CI (8–57%)] in patients without a CMR ($p < 0.001$) [6]. Use of a pre-treatment biomarker is advantageous in informing treatment planning, and provides an opportunity for risk stratification of therapy.

Tumour hypoxia is recognized as an adverse prognostic factor in cervical cancer [27–29] treated with radiation alone and has been associated with resistance to radiation treatment [30,31]. In the setting of chemoradiation, a study in head and neck squamous cell carcinoma found significantly higher locoregional failure in patients with baseline tumour hypoxia as measured using ^{18}F-Misonidazole PET [32]. Hypoxia inducible factor 1-alpha (HIF-1α) protein levels are increased in response to decreased cellular oxygen concentration [33], and it has been examined as a surrogate for tumour hypoxia. There are studies supportive of HIF-1α as a prognostic factor for cervical cancer treated with radiotherapy; showing high HIF-1α expression to be significantly associated with progression-free [34,35] and cancer-specific survival [35] and risk of distant metastases [34]. Other studies, including ours, did not find HIF-1α expression to be prognostic for survival in similar populations [36,37]. Potential explanations for these differences include tumour heterogeneity in the level of oxygenation and hence HIF-1α expression; and HIF-1α's rapid degradation with restoration of normoxia, making HIF-1α more reflective of acute rather than chronic tumour hypoxia [36]. Oxygen probe studies have found only weak ($r = 0.40$) [37] to moderate ($r = -0.26$) [36] association between tumour HIF-1α expression and oxygenation. Additionally, HIF-1α has a short half-life and is therefore more transiently expressed, compared with CA-9 and Glut-1, resulting in increased potential false-negative results from HIF-1α staining. It is also possible that HIF-1α is up-regulated by factors other than hypoxia [38]. Another observation is that tumour size may modulate the prognostic implication of HIF-1α [36].

High levels of CA-9 expression have been demonstrated to predict for tumour hypoxia in cervical cancer by direct needle probe oxygenation measurements in Longcaster's study [18] studies. They

Table 3
Progression-free survival (PFS) and overall survival (OS) according to biomarkers.

Endpoint	Biomarker	Group	5 years estimate [95% CI]	Univariate analysis HR [95% CI]	*p*-Value	Multivariate analysis* HR [95% CI]	*p*-Value
PFS	CA-9	Low	61 [45–82]	1	0.83	1	0.80
		High	66 [50–85]	0.9 [0.4–2.1]		0.9 [0.4–2.2]	
	Hif-1α	Low	52 [37–73]	1	0.14	1	0.17
		High	72 [58–91]	0.5 [0.2–1.2]		0.5 [0.2–1.3]	
	Glut1	Low	74 [60–91]	1	0.08	1	0.049
		High	50 [35–72]	2.3 [0.9–5.4]		2.8 [1.0–7.9]	
	Ki-67 (×10)	Continuous		1.12 [0.96–1.30]	0.16	1.19 [1.01–1.41]	0.033
OS	CA-9	Low	82 [69–98]	1	0.58	1	0.26
		High	72 [58–91]	1.5 [0.5–4.6]		2.0 [0.6–6.4]	
	Hif-1α	Low	73 [59–91]	1	0.60	1	0.81
		High	79 [66–96]	0.7 [0.3–2.1]		0.9 [0.3–2.7]	
	Glut1	Low	90 [80–100]	1	0.01	1	0.011
		High	60 [44–82]	4.6 [1.3–16.4]		5.0 [1.3–19.2]	
	Ki-67 (×10)	Continuous		1.09 [0.90–1.30]	0.38	1.19 [0.98–1.46]	0.080

* Factors adjusted for included MRI volume, FIGO stage, node positivity, uterine corpus invasion.

found CA-9 expression to be prognostic for overall survival and risk of metastasis. Lee et al. also found CA9 to be associated with poorer disease-free survival, especially nodal spread [39]. In these studies, the prognostic significance of CA-9 has been most strongly demonstrated when comparing outcome between patients with absent versus any CA-9 staining; whereas in our study the comparison was between low and high staining.

Consistent with our results, Hedley et al. [17] did not find a significant association between CA-9 expression and patient survival, whether CA-9 was expressed as a continuous variable or dichotomised at the median. Potential explanations include variability in scoring criteria for staining between studies and intra-tumoural heterogeneity of CA-9 expression, leading to false-negative results when a single tumour biopsy was used per case. Furthermore, CA-9 staining did not correlate with needle probe oxygen (pO2) measurements in Hedley's study. The authors raised the possibility of CA-9 expression being influenced by other biological factors, rather than being a pure surrogate for presence of tissue hypoxia. Collectively, our findings raise caution on the reliability of CA-9 and HIF-1α as clinical biomarkers for tumour hypoxia in the setting of small tumour biopsies. This is not to dispute tumour hypoxia per se is predictive of chemoradiation response and/or prognostic for survival; but CA-9 and HIF-1α may not be the best or most reliable surrogate markers of the hypoxic state in cervical cancer.

Ki-67 protein expression is regarded as a surrogate for mitoses and proliferation in many tumour types. Its correlation with chemoradiation response and prognostic significance in cervical cancer has been examined by various studies with contrasting findings [40–47]. Several series reported a lack of association between Ki-67 and treatment response or survival [40,41,43,46,47]. In our study, high Ki-67 was associated with worse PFS ($p = 0.049$) but did not reached statistical significance with OS ($p = 0.08$), although the hazard ratios were the same for OS and PFS (HR 1.19). Conversely, there are studies which report low tumour expression of Ki-67 was significantly associated poorer survival [42,44,45]. The survival advantage of high Ki-67 tumours was attributed to increased radiosensitivity, as determined using serum squamous cell carcinoma antigen level as a surrogate in the study by Suzuki et al. [45]. However, when we measured radiosensitivity directly through metabolic response on post-therapy PET scan, there was no significant association with Ki-67 level. Potential reasons for discrepancies in the different study findings include inter-observer variability in Ki-67 reporting. For example, Vosmik et al. [46] had median value of Ki-67 staining was 80% (range 30–100%), compared with our study's median of 40% (range 3–95%). It is also possible intra-tumoural heterogeneity in tumour Ki-67 levels which may not be reflected through testing of a single cervical cancer biopsy specimen.

Limitations of our study include its relatively small sample size which may not allow detection of small differences in patient outcome between different biomarker levels that maybe present. We detected a statistically significant effect of Glut-1 on PFS and OS. However, given the small sample size and number of events, the confidence interval for the hazard ratio is wide, and we cannot estimate with adequate precision the effect size of Glut-1. A larger sample size and number of events are required to more precisely assess the degree of impact of Glut-1 on survival. The results of this retrospective study are hypothesis generating and should be confirmed on a prospective clinical trial of chemoradiation in cervical cancer [48].

In summary, we observed that high Glut-1 expression in pre-treatment cervical cancer biopsies is associated with worse survival and higher distant failure rate in patients undergoing chemoradiation. High Glut-1 was also significantly associated with shorter PFS and lower CMR rate on post-therapy PET. Our findings support Glut-1 as a promising pre-treatment biomarker of metastatic–relapse risk in advanced cervical cancer treated with chemoradiation.

Disclosure/Conflict of interest

Nil to declare by all authors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ctro.2017.01.003.

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