The Journal of Laryngology & Otology (2010), ***124***, *650–658*.

doi:10.1017/S0022215110000228

Main Article

Use of multiple biological markers in radiotherapy-treated head and neck cancer

P Silva*†‡, N J Slevin**, P Sloan§, H Valentine*, D Ryder#, P Price*, C M L West*, J J Homer*†‡

Abstract

Objective: Management of patients with head and neck squamous cell carcinoma is often based on clinical parameters, with little appreciation of the underlying tumour biology. Single biological marker studies fail to acknowledge the complexity of these tumours. Our aim was to define a profile of biological markers associated with outcome.

Design: This retrospective study involved consecutive patients with oropharyngeal squamous cell carcinoma treated with primary radiotherapy between 1996 and 2001. Pre-treatment biopsies were used to study the immunohistochemical expression of nine biological markers. Markers were chosen to reflect biologically relevant pathways.

Results: Following analysis of nine markers, a profile of two markers was derived (carbonic anhydrase 9 and major vault protein), the co-expression of which conferred a significantly poor probability of locoregional control. The prognostic effect of these biomarkers in combination was greater than their effect individually.

Conclusion: Biomarker profiles can be established which highlight large differences in locoregional control. Identifying tumours that express both carbonic anhydrase 9 and major vault protein may facilitate patient selection for more aggressive treatment.

Key words: Head and Neck Neoplasms; Tumour Markers; Squamous Carcinoma

Introduction

More than 500 000 new cases of head and neck squamous cell carcinoma are identified yearly, with approximately 40 per cent of patients having locally advanced disease at presentation.[1] Radiotherapy plays an important role in their management. However, despite advances in treatment options, the prognosis is poor, with three-year cure rates rarely exceeding 50–60 per cent.[2] The main focus of surgeons and oncologists involved in patient management is locoregional control, which also represents the dominant form of treatment failure.

In many new cases of head and neck squamous cell carcinoma, the fundamental treatment decision lies between treatment based upon primary radiotherapy and that based upon primary surgery. This is more relevant now than ever before. Advances in conservation surgery, for example using transoral laser excision as well as modern reconstructive techniques, have made surgery an option for early to intermediate stage disease, rather than radiotherapy. Equally, advances in radiotherapy (for example, concurrent chemoradiotherapy alone or following induction chemotherapy) have made this an organ-preserving option for advanced disease, instead of primary surgery with post-operative radiotherapy.[3,4]

The most basic form of treatment individualisation using biological markers would therefore ideally include markers that predict response to radiotherapy. The importance of identifying patients who would not respond to radiotherapy (i.e. who would be radiotherapy failures) is significant, as the functional and oncological outcomes of surgical salvage are poorer than those of primary surgery.[5] Conversely, the use of surgery on a tumour that may have been cured with radiotherapy exposes the patient unnecessarily to the potential risks of surgery and organ sacrifice.

With the increasing availability of antibodies to a wide variety of molecular markers, much work has focussed on examining protein expression in tumours using immunohistochemistry – a technique suitable for routine clinical use. It is increasingly recognised that the response of a tumour to radiotherapy is unlikely to be dependent on a single biological parameter in isolation (such as hypoxia, proliferation or intrinsic radiosensitivity) but rather

From the *School of Cancer & Enabling Sciences, The University of Manchester, †Departments of Surgery, **Clinical Oncology, #Medical Statistics, Christie Hospital NHS Trust, and §Departments of Pathology and ‡Otolaryngology-Head and Neck Surgery, Manchester, UK.
Presented at the 7th International Conference on Head and Neck Cancer, 22 July 2008, San Francisco, California, USA.
Accepted for publication: 13 November 2009. First published online 14 April 2010.

on a myriad of biological factors all interlinked together. The molecular complexity of tumours, combined with the availability of a large number of antibodies and new statistical approaches for cluster analysis, provide the rationale for investigation of molecular marker profiles.

The potential of this approach has been highlighted in recent papers which defined profiles associated with response to radiotherapy in patients with head and neck squamous cell carcinoma.[6,7] Buffa *et al.* assessed the expression of proteins p53, Ki67, Bcl-2 (B cell leukaemia-2), cyclin D1 and CD31 (cluster of differentiation 31) glycoprotein, and used cluster analysis to identify marker profiles associated with a good response to conventional radiotherapy or to continuous hyperfractionated accelerated radiotherapy.[6] One profile was associated with approximately 69 per cent locoregional control following hyperfractionated accelerated radiotherapy but only approximately 33 per cent control following conventional radiotherapy. Similarly, Eriksen *et al.* assessed a cohort of patients from the Danish head and neck cancer group trials, and defined a profile which identified patients who did not benefit from a reduction in the overall treatment time.[7] This profile was characterised by wild-type p53, low expression of E-cadherin and B cell leukaemia-2, and moderate expression of Ki67 and EGFR (epidermal growth factor receptor).

With this in mind, we examined the expression of nine biological markers. The aim of the study was to define a biological marker profile associated with the response to radiotherapy of oropharyngeal carcinomas. Marker proteins were selected to reflect a range of biologically diverse yet relevant pathways, as follows: for hypoxia, hypoxia inducible factor-1 α (HIF-1α), carbonic anhydrase 9 (CA9) and glucose transporter 1 (Glut-1); for proliferation Ki67 and EGFR; for radiosensitivity, radiation response and chemoresistance, posphorylated v-akt murine thymoma viral oncogene homolog 1 (pAkt) and major vault protein (MVP) and Bcl-2; and for metastasis and invasion, fragile histidine triad gene (Fhit).

The hypoxia-related markers HIF-1α, CA9 and Glut-1 have all been shown to be important in response to radiotherapy. CA9 and HIF-1α may also be dependent on other oncogenic pathways independent of hypoxia, whilst glucose transporter 1 expression and its prognostic effect may be closely related to glucose availability, which is fundamental to cell survival and apoptosis.[8,9] Tumour proliferation is recognised as an important biological factor determining the outcome of fractionated radiotherapy.[10] Immunohistochemical investigation of Ki67 in biopsy specimens is widely used to assess tumour cell proliferation. Epidermal growth factor receptor signalling is frequently increased in cancer, particularly in head and neck tumours.[11] Over-expression of epidermal growth factor receptor is associated with increased tumour cell proliferation, angiogenesis, loss of differentiation and reduced apoptosis.[12,13] Phosphorylated Akt has been linked with radioresistance in many cancers and may be involved in cell survival regulation; it may also play a role in the early stages of malignant transformation.[14] The role of major vault protein in chemoresistance has been demonstrated, and is thought to be secondary to its role in intracellular transport.[15,16] B cell leukaemia-2 is an anti-apoptotic protein involved in cell cycle regulation.[17] The fragile histidine triad tumour suppressor gene has been shown to be down-regulated early in carcinogenesis.[18]

Methods

Between 1996 and 2001, a consecutive series of 60 patients were identified as having received primary radiotherapy at the Christie Hospital, Manchester, for a histologically proven squamous cell carcinoma of the oropharynx (i.e. tonsil and posterior third of the tongue). The medical records of all these patients were reviewed.

Primary tumour sites were irradiated, and patients with positive neck nodes underwent a prior neck dissection. Megavoltage radiation was delivered by means of a 4 MV linear accelerator, in daily fractions, five times a week for three weeks. The radiation dose prescribed at the time followed strict guidelines, with posterior tongue tumours receiving lateral parallel pair radiation (50 Gy in 16 fractions), while tonsillar tumours received ipsilateral therapy of 52.50 Gy in 16 fractions. This represented the standard curative treatment used by our centre at the time, i.e. a hypofractionated accelerated regimen. This fractionation approach is biologically equivalent to 71 Gy in eight weeks for local control in patients with tonsil cancer.[19] The regimen provides excellent tumour control in other head and neck sites such as the larynx and oral cavity.[20,21]

Tumour blocks were obtained from referring hospital biopsies. From each tissue block, haematoxylin and eosin slides were used to confirm the presence of sufficient amounts of tumour. Following deparaffinisation of the sections, the antigen-binding sites (epitopes) were unmasked, using various epitope retrieval steps which involved heat, chemicals or enzymes. To avoid non-specific binding of the primary antibody, blocking proteins were added. In addition, endogenous peroxidase activity within the tissue was quenched using hydrogen peroxide. After binding of the primary antibody to the antigen under investigation, a secondary biotinylated antibody was added. The secondary antibody was species-specific and directed against immunoglobulins from whatever species the primary antibody was derived (e.g. rabbit or goat). After removal by washing of unbound secondary antibody, horseradish peroxidase streptavidin complex was added, which bound to the biotin on the secondary antibody. The horseradish peroxidase acted on the chromagen substrate diaminobenzidine, which was subsequently added. This causes a brown precipitate to be deposited where there was bound horseradish peroxidase (and therefore also the antigen under investigation). Finally, the tissue sections were counterstained to visualise the cells and tissue architecture. All of the steps described were based on established laboratory protocols, but were optimised according to the antigen under investigation (Table I).

https://doi.org/10.1017/S0022215110000228 Published online by Cambridge University Press

TABLE I

SUMMARY OF IMMUNOHISTOCHEMICAL METHODS USED

Marker	Ag retrieval	Primary Ab	Dilution	Supplier	Control Ab	Dilution	Incubation time	Secondary Ab	Detection method
HIF-1α	MW*	Mouse monoclonal	1:100 TNB	BD Biosciences 610958	Mouse Dako X0931	1:40 TNB	ON 4°C	RAMBO Biotin Dako EO413	Streptavadin-HRP (TSA Biotin system)
Ca9	None	Mouse monoclonal	1:50 TBS	Gift‡	Mouse Dako 0944	1:250 TBS	30 min RT	EnVision Polymer HRP	DAB EnVision
Glut1	None	Rabbit polyclonal	1:100 TBS	Alpha Diagnostic International GT 12-A	Rabbit Dako XO903	1:2000 TBS	1 hr 37°C	–	DAB EnVision
Ki67	MW†	Mouse monoclonal	1:100 TBS	DakoCytomation M7240	Mouse Dako X0931	1:125 TBS	ON 4°C	–	DAB EnVision
EGFR	MW†	Mouse monoclonal	1:30 TBS	Nova Castra NCL	Mouse IgG2a Dako XO943	1:30 TBS	ON 4°C	RAMBO Biotin Dako EO413	DAB + Dako K3467
Bcl-2	MW†	Mouse monoclonal	1:100 TBS	DakoCytomation MO887	Mouse Dako XO931	1:33 TBS	ON 4°C	Polymer HRP EnVision	DAB EnVision
MVP	MW*	Mouse monoclonal	1:200 TBS	AbCam Ab2376	Mouse Dako XO931	1:200 TBS	1 hr RT	GAMBO Biotin Dako EO433	DAB EnVision
pAkt	MW*	Rabbit polyclonal	1:50 TBS	Cell Signalling #9277	TBS	–	ON 4°C	GARBO Biotin Dako XO432	DAB EnVision
Fhit	MW*	Rabbit polyclonal	1:100 TBS	Zymed Laboratories	TBS	–	–	Polymer HRP EnVision	DAB EnVision

MW* = microwaved in citrate buffer for 25 min; MW† = microwaved in ethylene diamine triacetic acid (EDTA) (0.05Tris–HCL/1 mM EDTA) for 25 min. ‡A gift from Profs. S. Pastorekova and J. Pastorek, Slovak Academy of Sciences, Bratislava, Slovak Republic; microwaved in 10 mM Tris with 1 mM ethylene diamine triacetic acid (EDTA) and 0.05% TWEEN 20 for 25 min. Ag = antigen; Ab = antibody; HIF-1α = hypoxia-inducible factor-1; Ca9 = carbonic anhydrase 9; Glut1 = glucose transporter 1; Ki67 = Ki67; EGFR = epidermal growth factor receptor; Bcl-2 = B cell leukaemia-2; MVP = major vault protein; pAkt = phosphorylated Akt; Fhit = fragile histidine triad gene; TNB = 0.1 M Tris-hydrocholoric acid; TBS = tris buffered saline; – = not included in the analysis; ON = overnight; RT = room temperature; min = minutes; hr = hours; RAMBO = rabbit antimouse biotinylated antibody; HRP = horse radish peroxidase; GAMBO = goat antimouse biotinylated antibody; GARBO = goat antirabbit biotinylated antibody; TSA = tyramide signal amplification; DAB = diaminobenzidine

All the markers studied in our patient series, except for glucose transporter 1, were scored using the following, semiquantitative system: 0 = no staining; 1 = <10 per cent staining; 2 = 10–29 per cent staining; and 3 = >30 per cent staining. Glucose transporter 1 was scored as either present or absent. Scoring was performed by two independent, experienced oral pathologists; conflicts in scoring were resolved by discussion and consensus. Scorers were blinded to outcome. The markers were studied individually and within a multivariate analysis. Multivariate analysis was performed using the Cox regression model for locoregional control and cancer-specific survival. The analysis included the clinical factors of site (tonsil or tongue), stage (I/II, III or IV) and haemoglobin level (<13.2 g/dL or ≥13.2 g/dL). The approach adopted for selection of marker terms was based on the guidelines published by Collett.[22]

Results

The characteristics of the 60 patients are summarised in Table II. The median duration of follow up for the surviving patients was 5.2 years (range 1.8–7.5). There were 33 patients with locoregional failure. Distant metastases were recorded in seven patients. The five-year locoregional control, cancer-specific survival and overall survival rates for the 60 patients were 38.2, 45.7 and 21.3 per cent, respectively. Univariate Cox regression analysis identified the following clinicopathological factors to be associated with poor locoregional control: low pre-treatment haemoglobin level ($p = 0.05$), and advancing tumour ($p = 0.006$), node ($p = 0.04$) and disease ($p = 0.005$) stage.

TABLE II

PATIENT CHARACTERISTICS

Parameter	No. of Pts
Gender	
Male	43 (72)*
Female	17 (28)
Age	
Median (range (yrs))	53.4 (31.3–85.4)
Tobacco use?	
Yes	40 (66)
No	12 (21)
Unknown	8 (13)
Alcohol	
None	6 (10)
Low	18 (30)
Moderate	9 (15)
High	15 (25)
Unknown	12 (20)
Stage	
I or II	15 (25)
III or IV	45 (75)
Site	
Tongue	33 (55)
Tonsil	27 (45)
Follow up	
Median (range (yrs))	5.2 (1.8–7.5)

*percentage unless indicated otherwise? Pts = patients; yrs = years

Examples of immunostaining are shown in Figure 1. The expression of carbonic anhydrase 9 was mainly on the plasma membrane, with no significant variation in intensity between tumours. Glucose transporter 1 protein was expressed diffusely throughout the tumour, with either intense or near-absent staining. Although occasional cytoplasmic staining of the tumour cells was observed for epidermal growth factor receptor, only staining of the tumour cell membranes was considered to be specific. B cell leukaemia-2 staining was observed in both the membrane and cytoplasm. Phosphorylated Akt immunohistochemical analysis demonstrated homogeneous staining of the cytoplasm and nucleus. Immunohistochemical analysis for Ki67 revealed a typical nuclear staining pattern. Major vault protein staining was predominantly cytoplasmic. HIF-1α staining was predominantly nuclear, with variable cytoplasmic expression. Immunohistochemical staining for fragile histidine triad gene revealed strong cytoplasmic and moderate nuclear staining.

The overall results for immunohistochemical and univariate analyses are shown in Table III. Expressions of carbonic anhydrase 9, HIF-1α and major vault protein were highly significant prognostic factors for both locoregional control and cancer-specific survival. In addition, low Ki67 expression was associated with improved cancer-specific survival.

Multivariate analysis

After examining the various markers individually, multivariate analysis of all the markers was conducted. Complete data for the nine markers were available for all 60 patients. Analysis of outcome relationships showed that the markers appeared to cluster into two groups (i.e. 0/1 versus 2/3); consequently, for the multivariate analyses, patients were stratified into two similarly sized groups. Multivariate analysis was performed using the Cox regression model for locoregional control and cancer-specific survival. The analysis included the clinical factors of site (tonsil or tongue), stage (I or II, or III or IV) and haemoglobin level (<13.2 g/dL or ≥13.2 g/dL). The approach adopted for selection of marker terms was based on guidelines published by Collette.[22] A stepwise model was used to derive a panel of biological markers (Table IV).

Step one showed that HIF-1α, carbonic anhydrase 9 and major vault protein were statistically significant prognostic factors for locoregional control, in a univariate analysis. Step two used all the terms significant from step one, and the clinical parameters of site, stage and haemoglobin level, in a multivariate analysis. This was then assessed by dropping each of the constituent terms from the model. The only remaining term which retained independent significance was major vault protein. In step three, each of the terms was added, one at a time, to the model. This resulted in both carbonic anhydrase 9 and HIF-1α regaining significance. This behaviour was explained by the fact that both carbonic

https://doi.org/10.1017/S0022215110000228 Published online by Cambridge University Press

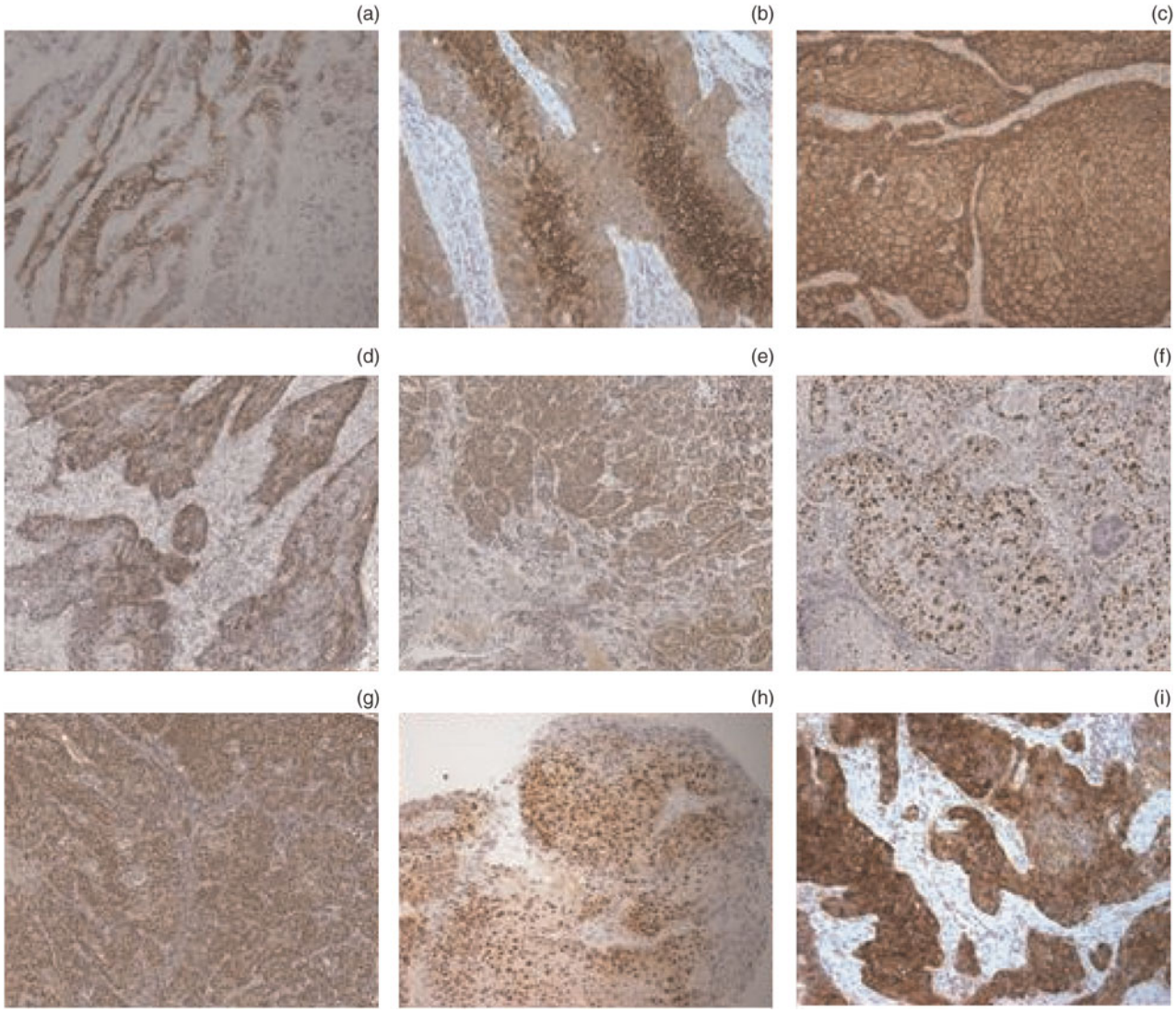

FIG. 1

Photomicrographs showing examples of high levels of tumour expression of (a) carbonic anhydrase 9, (b) glucose transporter 1, (c) epidermal growth factor receptor, (d) B cell leukaemia-2, (e) phosphorylated Akt, (f) ki67, (f) fragile histidine triad gene, (h) HIF-1α, and (i) major vault protein. (×100)

anhydrase 9 and HIF-1α were important on their own but not together, as the two variables carried virtually the same information. This was demonstrated by cross-tabulation of the cases (Table V).

The difference between the prognostic significance of carbonic anhydrase 9 and HIF-1α was small. However, carbonic anhydrase 9 was slightly more significant ($p = 0.00001$), and was therefore chosen over HIF-1α ($p = 0.00003$). This resulted in a model with only two markers that retained independent significance, namely major vault protein and carbonic anhydrase 9. A test for interaction between these two variables failed to reveal any significant relationship ($p = 0.69$). Table VI outlines the hazard ratios from the multivariate analysis.

From the fitted model, it is clear that, after adjustment for site, stage and haemoglobin level, raised major vault protein and carbonic anhydrase 9 expressions were adverse prognostic features for locoregional control, with carbonic anhydrase 9 having the larger effect. Table VII illustrates the ordering of the prognostic groups for locoregional control.

Applying this model to our dataset illustrated the prognostic categories.

It could be seen that the groups divided into four distinct survival patterns, whereby those patients with a combination of high major vault protein expression and high carbonic anhydrase 9 expression had the poorest probability of locoregional control (Figure 2). In terms of cancer-specific survival, major vault protein was not formally significant ($p = 0.13$) after adjustment for all the factors; however, the Kaplan–Meier curve (Figure 3) illustrated a trend for poorer outcome when both markers were combined.

Discussion

The use of individual biological markers in the investigation of head and neck squamous cell carcinoma often fails to address the complex biology of these

https://doi.org/10.1017/S0022215110000228 Published online by Cambridge University Press

TABLE III

UNIVARIATE OUTCOME ANALYSES FOR NINE BIOLOGICAL MARKERS STUDIED

Marker	Grp	LRC		CSS	
		HR (95% CI)	*p*	HR (95% CI)	*p*
pAkt	0/1				
	2/3	0.81 (0.41–1.63)	0.56	1.17 (0.61–2.23)	0.64
Bcl-2	0/1				
	2/3	0.76 (0.37–1.55)	0.44	0.74 (0.36–1.52)	0.40
Fhit	0/1				
	2/3	0.83 (0.42–1.65)	0.60	0.83 (0.41–1.64)	0.58
Ki67	0/1				
	2/3	1.84 (0.97–3.50)	0.06	2.03 (1.07–3.88)	0.03
EGFR	0/1				
	2/3	1.52 (0.76–3.05)	0.23	1.80 (0.91–3.56)	0.08
Glut1	0				
	1	0.64 (0.33–1.26)	0.20	0.69 (0.36–1.32)	0.25
Ca9	0/1				
	2/3	5.21 (2.48–10.90)	<0.0001	5.08 (2.53–10.20)	<0.0001
HIF-1α	0/1				
	2/3	4.96 (2.40–10.30)	<0.0001	4.85 (2.44–9.65)	<0.0001
MVP	0/1				
	2/3	4.14 (2.16–7.94)	<0.0001	3.56 (1.88–6.73)	0.0002

Grp = stratifying group; LRC = locoregional control; CSS = cancer-specific survival; HR = hazard ratio; CI = confidence interval; pAkt = phosphorylated Akt; Bcl-2 = B cell leukaemia-2; Fhit = fragile histidine triad gene; Ki67 = Ki67; EGFR = epidermal growth factor receptor; Glut1 = glucose transporter 1; Ca9 = carbonic anhydrase 9; HIF-1α = hypoxia-inducible factor-1; MVP = major vault protein

tumours. We chose a total of nine markers, in order to reflect a range of different biological pathways, as follows: Ki67, epidermal growth factor receptor, carbonic anhydrase 9, glucose transporter 1, fragile histidine triad gene, B cell leukaemia-2, phosphorylated Akt, HIF-1α and major vault protein. Our approach of using pre-treatment biopsy material and standard immunohistochemistry techniques provided an inexpensive, clinically applicable test that could be easily employed in routine practice. We studied a relatively homogeneous patient and tumour group, with one site and one principal treatment modality. The study group was consecutive, eliminating any selection bias.

Three markers were found to be prognostic for locoregional control and cancer-specific survival: carbonic anhydrase 9, HIF-1α and major vault protein. In a multivariate analysis, the results of the individual markers were used to derive a profile of two markers (carbonic anhydrase 9 and major vault protein), the high co-expression of which conferred a significantly poorer probability of locoregional control within the oropharynx. The prognostic effect of these markers in combination was greater than their effects individually.

The role of carbonic anhydrase 9 in the transcriptional response to hypoxia is well recognised. Our study showed increased tumour carbonic anhydrase 9 expression to be highly predictive of locoregional control and cancer-specific survival. In the current literature, the prognostic role of this protein is unclear. Two studies, both by the same group, found increased tumour carbonic anhydrase 9 expression to be significantly associated with poor locoregional control.[23,24] Of six other studies, two revealed a non-significant relationship with poor local control, one found the reverse, and the remainder found no association.[25–31]

TABLE IV

COLLETT STEPWISE MODEL USED TO DERIVE BIOLOGICAL MARKER PANEL

Marker	Locoregional control				Cancer-specific survival		
	Step 1	Step 2	Step 3	Step 4	Step 1	Step 2	Step 3
HIF-1α	0.00003	0.66	0.0005	0.62	<0.0001	0.75	0.50
MVP	0.001	0.04	0.001	0.02	0.001	0.18	0.13
EGFR	0.62	–	0.80	0.14	0.44	–	0.30
Bcl-2	0.12	–	0.81	0.27	0.26	–	0.35
pAkt	0.65	–	0.40	0.33	0.53	–	0.70
Glut1	0.13	–	0.40	0.34	0.40	–	0.51
Ca9	0.00001	0.13	0.0001	0.0001	<0.0001	0.08	<0.0001
Ki67	0.09	0.92	0.63	0.82	0.10	0.73	0.51
Fhit	0.46	–	0.09	0.31	0.77	–	0.77

Data represent p-values. HIF-1α = hypoxia-inducible factor-1; MVP = major vault protein; EGFR = epidermal growth factor receptor; Bcl-2 = B cell leukaemia-2; pAkt = phosphorylated Akt; Glut1 = glucose transporter 1; Ca9 = carbonic anhydrase 9; Ki67 = Ki67; Fhit = fragile histidine triad gene; – = not included in the analysis

https://doi.org/10.1017/S0022215110000228 Published online by Cambridge University Press

TABLE V

CROSS-TABULATION OF HIF-1α AND CA9 CASES

HIF-1α grp	Ca9 grp	
	0/1	2/3
0/1	26	2
2/3	2	30

HIF-1α = hypoxia-inducible factor-1; Ca9 = carbonic anhydrase 9; grp = outcome group

It is known that carbonic anhydrases participate in a variety of biological processes, including pH regulation, respiration and calcification, and it is known that factors other than hypoxia may influence the expression of carbonic anhydrase 9.[8] These differences may be a reflection of this, or indeed the heterogeneity of the subsites, with only one study concentrating on a single subsite.[27] Interestingly, in cervical carcinomas, high carbonic anhydrase 9 expression was not found to be associated with poor locoregional control, but with a low probability of metastasis-free survival, suggesting it may not measure hypoxic radioresistance.[32] Expression of carbonic anhydrase 9 in these tumours may reflect the activity of other oncogenic pathways independent of hypoxia.

HIF-1α has been studied extensively as a potential indirect assessor of tumour hypoxia, and most studies have found high expression to be an adverse prognostic factor in patients undergoing radiotherapy.[24,27,33,34] We found both carbonic anhydrase 9 and HIF-1α to be significant for locoregional control on multivariate analysis. Their relationship with each other shows that they are measures of the same entity (i.e. tumour response to hypoxia), and it was only for statistical reasons that HIF-1α lost its significance in favour of carbonic anhydrase 9.

Our previous study of major vault protein expression revealed it to be strongly prognostic of treatment outcome following primary radiotherapy.[35] Various theories have been postulated to explain this protein's role in mediating drug resistance, including its subcellular localisation serving to bind drugs and transport them to locations remote from their cellular drug target; however, its role in radioresistance remains to be elucidated.[36]

It is evident that tumour biology cannot wholly be described using one single marker. This may explain the heterogeneity in results across various studies. The use of a panel of biological markers to identify a profile associated with outcome has been described in two previous studies.[6,7] Both these studies used cluster analyses to identify groups of patients who would benefit from accelerated (as opposed to conventional) schedules. Grouping our patients undergoing radiotherapy according to their tumours' carbonic anhydrase 9 and major vault protein expression appeared to have a powerful predictive value; for example, the hazard ratio in patients with high tumour major vault protein and carbonic anhydrase 9 expression was over 15 for locoregional control. This knowledge could be used to produce predictive classifiers enabling stratification of patients to specific, individualised therapies.

Such strategies might include synchronous chemoradiotherapy with or without induction chemotherapy, hypoxia modification strategies, the use of accelerated regimens (such as continuous hyperfractionated accelerated radiotherapy) or the use of bioreductive drugs such as tirapazamine.[37,38] Another alternative would be the selection of primary surgery for patients with poorer predicted response to radiotherapy. Clearly, the response from these various regimens is heterogeneous, and therefore the aim would be to identify those patients who would benefit most from such treatments.

Now that chemoradiotherapy (i.e. synchronous chemotherapy, induction chemotherapy or both) is standard for this patient group, it may be possible to identify which patients may be candidates for less toxic treatment, for example without cytotoxic chemotherapy. These patients would have a biomarker-predicted good response to radiotherapy, and hence a good prognosis.

Our approach of using pre-treatment biopsy material and standard immunohistochemistry techniques offers an inexpensive, clinically applicable test which could be easily incorporated into routine practice. The training required to attain good clinical laboratory practice is minimal, and is financially less

TABLE VI

MULTIVARIATE ANALYSES

Factor	Variant	Locoregional control			Cancer-specific survival		
		HR	95% CI	*p*	HR	95% CI	*p*
Site	Tonsil						
	Tongue	1.84	0.86–3.96	0.01	3.19	1.42–7.15	0.005
Hb	<13.2 g/dL						
	≥13.2 g/dL	0.72	0.31–1.65	0.43	0.85	0.38–1.91	0.35
Stage	I or II						
	III or IV	1.30	0.44–3.86	0.06	3.54	1.03–12.14	0.08
Ca9	0/1 grp						
	2/3 grp	5.82	2.24–15.07	<0.0001	10.66	4.18–27.18	<0.0001
MVP	0/1 grp						
	2/3 grp	2.61	1.18–5.78	0.02	NS	NS	NS

HR = hazard ratio; CI = confidence interval; Hb = haemoglobin; Ca9 = carbonic anhydrase 9; MVP = major vault protein; grp = outcome group; NS = not significant

https://doi.org/10.1017/S0022215110000228 Published online by Cambridge University Press

TABLE VII

PROGNOSTIC GROUPS FOR LOCOREGIONAL CONTROL

Outcome grp		Prognostic grouping	HR (95% CI)
MVP	Ca9		
0/1	0/1	Low MVP & low Ca9	Reference
2/3	0/1	High MVP & low Ca9	2.62 (1.18–5.78)
0/1	2/3	Low MVP & high Ca9	5.82 (2.24–15.07)
2/3	2/3	High MVP & high Ca9	15.18 (4.72–48.77)

Grp = stratifying group; HR = hazard ratio; CI = confidence interval; MVP = major vault protein; Ca9 = carbonic anhydrase 9

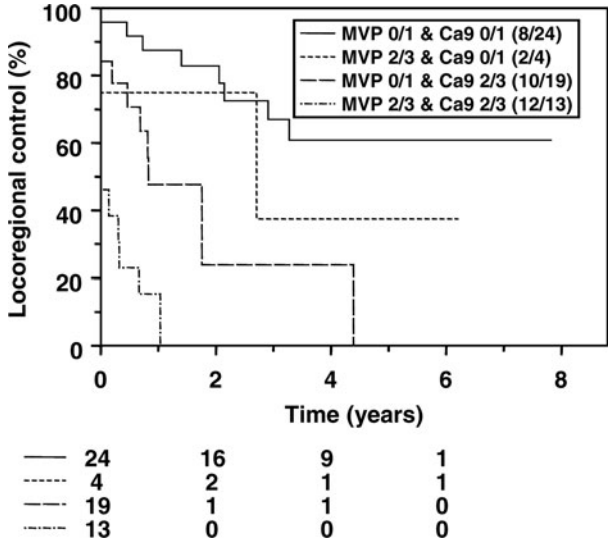

FIG. 2

Kaplan–Meier curve demonstrating locoregional control according to tumour expression of major vault protein (MVP) and carbonic anhydrase 9 (Ca9) ($p<0.0001$).

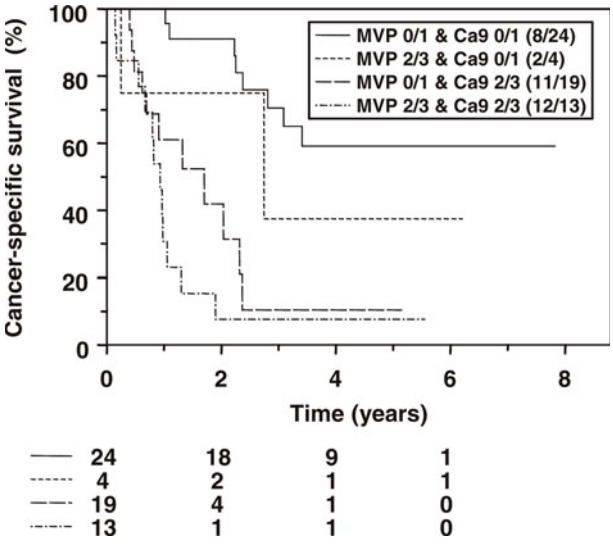

FIG. 3

Kaplan–Meier curve demonstrating cancer-specific survival according to tumour expression of major vault protein (MVP) and carbonic anhydrase 9 (Ca9) ($p = 0.13$).

constraining than other techniques such as microarrays or cytogenetics.

Clearly, further study is required to reproduce these findings, and to determine whether tumour expression of both major vault protein and carbonic anhydrase 9 can act as a marker of tumour aggression or indeed of radioresistance. Additionally, the underlying genetic pathways that define these markers needs to be further elucidated, facilitating the application of this knowledge to enable treatment modification and improved radiotherapy response.

In conclusion, biomarker profiles can be established which highlight large differences in locoregional control in patients with head and neck squamous cell carcinoma undergoing radiotherapy.

- **Head and neck squamous cell carcinomas involve diverse subsites, treatments and underlying tumour biology**
- **Management decisions are largely based on clinical parameters independent of tumour biology**
- **Single biological marker studies fail to acknowledge the complexity and heterogeneity of this disease**
- **This study identified a panel of biological markers the combined prognostic effect of which was greater than their individual effects alone**
- **This biological marker panel may be useful to select appropriate patients for more aggressive therapy**

Acknowledgements

This work was supported by Cancer Research UK and the National Translational Cancer Research Network of the UK.

References

1 Ferlay J, Bray F, Pisani P, Parkin DM. *GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide*, vol **5**. Lyon: IARC Press, 2004
2 Bernier J, Bentzen SM. Altered fractionation and combined radio-chemotherapy approaches: pioneering new opportunities in head and neck oncology. *Eur J Cancer* 2003;**39**:560–71
3 Lefebvre JL, Chevalier D, Luboinski B, Kirkpatrick A, Collette L, Sahmoud T. Larynx preservation in pyriform sinus surgery: preliminary results of a European Organisation for Research and Treatment of Cancer phase II trial. EORTC Head And Neck Cancer Coop Group. *J Natl Cancer Inst* 1996;**88**:890–9
4 Pignon JP, Bourhis J, Domenge C, Designe L. Chemotherapy added to locoregional treatment head and neck squamous for cell carcinoma: three meta-analyses of updated individual data. *Lancet* 2000;**355**:949–55
5 Clark JR, de Almeida J, Gilbert R, Irish J, Nelligan P, Gullane PJ. Primary and salvage (hypo)pharyngectomy: analysis and outcome. *Head Neck* 2006;**28**:671–7
6 Buffa FM, Bentzen SM, Daley FM, Dische S, Saunders MI, Richman PI, Wilson GD. Molecular marker profiles predict locoregional control of head and neck squamous cell carcinoma in a randomised trial of continuous hyperfractionated acclerated radiotherapy. *Clin Cancer Res* 2004;**10**:3745–54

https://doi.org/10.1017/S0022215110000228 Published online by Cambridge University Press

7 Eriksen JG, Buffa FM, Alsner J, Steiniche T, Bentzen SM, Overgaard J. Molecular profiles as predictive markers for the effect of overall treatment time of radiotherapy in supraglottic larynx squamous cell carcinomas. *Radiother Oncol* 2004;**72**:275–82
8 Sly WS, Hu PY. Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annu Rev Biochem* 1995;**64**: 375–401
9 Hammerman PS, Fox CJ. Beginnings of a signal induction pathway for bioenergetic control of cell survival. *Trends Biochem Sci* 2004;**29**:586–92
10 Fowler JF. How worthwhile are short schedules in radiotherapy? A series of exploratory calculations. *Radiat Oncol* 1990;**18**:165–81
11 Grandis J, Tweardy D. Elevating levels of TGF-alpha and EGFR are early markers of carcinogenesis in head and neck cancer. *Cancer Res* 1993;**53**:3579–84
12 Shin DM, Ro JY, Hong WK, Hittelman WN. Dysregulation of EGFR expression in premalignant lesions during head and neck tumourigenesis. *Cancer Res* 1994;**54**:3153–9
13 Christensen ME. The EGF receptor system in head and neck carcinomas and normal tissue. Immunohistochemical and quantitative studies. *Dan Med Bull* 1998;**45**:121–34
14 Panigrahi AR, Pinder SE, Chan SY, Paish EC, Robertson JF, Ellis IO. The role of PTEN and its signalling pathways, including AKT, in breast cancer; an assessment of relationships with other prognostic factors and with outcome. *J Pathol* 2004;**204**:93–100
15 Kickhoefer VA, Siva AC, Kedersha NL, Inman EM, Ruland C, Streuli M. The 193-kD vault protein, VPARP, is a novel poly(ADP-ribose) polymerase. *J Cell Biol* 1999;**146**:917–28
16 Schroeijers AB, Scheffer GL, Flens MJ, Meijer GA, Izquierdo MA, van der Valk P *et al*. Immunohistochemical detection of the human major vault protein LRP with two monoclonal antibodies in formalin-fixed, paraffin-embedded tissues. *Am J Pathol* 1998;**152**:373–80
17 Wilson GD, Saunders MI, Dische S, Richman PI, Daley FM, Bentzen SM. bcl-2 expression in head and neck cancer; an enigmatic prognostic marker. *Int J Radiat Oncol Biol Phys* 2001;**49**:435–41
18 Sozzi G, Pastorino U, Moiraghi L, Tagliabue E, Pezzella F, Ghirelli C *et al*. Loss of FHIT function in lung cancer and preinvasive bronchial lesions. *Cancer Res* 1998;**58**:5032–7
19 Withers HR, Peters LJ, Taylor JM, Owen JB, Morrison WH, Schultheiss TE *et al*. Local control of carcinoma of the tonsil by radiation therapy: an analysis of patterns of fractionation in nine institutions. *Int J Radiat Biol* 1995; **33**:549–62
20 Gowda RV, Henk JM, Mais KL, Sykes AJ, Swindell R, Slevin NJ. Three weeks radiotherapy for T1 glottic cancer: the Christie and Royal Marsden experience. *Radiother Oncol* 2003;**68**:105–11
21 Turner SL, Slevin NJ, Gupta NK, Swindell R. Radical external beam radiotherapy for 333 squamous carcinomas of the oral cavity – evaluation of late morbidity and a watch policy for the clinically negative neck. *Radiother Oncol* 1996;**41**:21–9
22 Collette D. *Modelling Survival Data in Medical Research*, 2nd edn. London: Chapman & Hall, 2004
23 Koukourakis MI, Giatromanolaki A, Sivridis E, Simopoulos K, Pastorek J, Wykoff CC *et al*. Hypoxia regulated carbonic anhydrase-9 relates to poor vascularisation and resistance of squamous cell head and neck cancer to chemoradiotherapy. *Clin Cancer Res* 2001;**7**:3399–403
24 Koukourakis MI, Bentzen SM, Giatromanolaki A, Wilson GD, Daley FM, Saunders MI *et al*. Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited into the CHART randomised trial. *J Clin Oncol* 2006;**24**:727–35
25 Beasley NJ, Wykoff CC, Watson PH, Leek R, Turley H, Gatter K *et al*. Carbonic anhydrase IX, an endogenous hypoxia marker, expression in head and neck squamous cell carcinoma and its relationship to hypoxia, necrosis and microvessel density. *Cancer Res* 2001;**61**:5262–7
26 Kaanders JH, Wijffels KI, Marres HA, Ljungkvist AS, Pop LA, van den Hoogen FJ *et al*. Pimonidazole binding and tumour vascularity predict for treatment outcome in head and neck cancer. *Cancer Res* 2002;**62**:7066–74
27 Hui EP, Chan AT, Pezzella F, Turley H, To KF, Poon TC *et al*. Coexpression of hypoxia inducible factors 1 alpha and 2 alpha, carbonic anhydrase IX and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival. *Clin Cancer Res* 2002;**8**:2595–604
28 Hoogsteen IJ, Marres HA, Wijffels KI, Rijken PF, Peters JP, van den Hoogen FJ *et al*. Colocalisation of carbonic anhydrase 9 expression and cell proliferation in head and neck squamous cell carcinoma. *Clin Cancer Res* 2005;**11**:97–106
29 De Schutter H, Landuyt W, Verbeken E, Goethals L, Hermans R, Nuyts S. The prognostic value of the hypoxia markers CA9 and GLUT1 and the cytokines VEGF and IL6 in head and neck squamous cell carcinoma treated by radiotherapy +/- chemotherapy. *BMC Cancer* 2005;**5**:42
30 Jonathan RA, Wijffels KI, Peeters W, de Wilde PC, Marres HA, Merkx MA *et al*. The prognostic value of endogenous hypoxia related markers for head and neck squamous cell carcinomas treated with ARCON. *Radiother Oncol* 2006; **79**:288–97
31 Bache M, Reddemann R, Said HM, Holzhausen HJ, Taubert H, Becker A *et al*. Immunohistochemical detection of osteopontin in advanced head and neck cancer: prognostic role and correlation with oxygen electrode measurements, hypoxia inducible factor-1-alpha related markers and hemoglobin levels. *Int J Radiat Oncol Biol Phys* 2006;**66**:1481–7
32 Loncaster JA, Harris AL, Davidson SE, Logue JP, Hunter RD, Wycoff CC *et al*. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. *Cancer Res* 2001;**61**:6394–9
33 Aebersold DM, Burri P, Beer KT, Laissue J, Djonov V, Greiner RH *et al*. Expression of hypoxia-inducible factor-1 alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. *Cancer Res* 2001; **61**:2911–16
34 Koukourakis MI, Giatromanolaki A, Liberis V, Sivridis E. Hypoxia inducible factor 1 alpha and 2 alpha expression is independent of anemia in patients with stage I endometrial cancer. *Anticancer Res* 2002;**22**:4137–40
35 Silva P, West CML, Slevin N, Valentine H, Ryder WD, Hampson L *et al*. Tumor expression of major vault protein is an adverse prognostic factor for radiotherapy outcome in oropharyngeal carcinoma. *Int J Radiat Oncol Biol Phys* 2007;**69**:133–40
36 Chugani DC, Rome LH, Kedersha NL. Evidence that vault ribonucleoprotein particles localise to the nuclear pore complex. *J Cell Sci* 1993;**106**:23–9
37 Gandara DR, Lara PNJ, Goldberg Z, Le QT, Mack PC, Lau DH *et al*. Tirapazamine: prototype for a novel class of therapeutic agents targeting tumour hypoxia. *Semin Oncol* 2002;**29**:102–9
38 Henk JM, Bishop K, Shepherd SF. Treatment of head and neck cancer with CHART and nimorazole: phase II study. *Radiother Oncol* 2003;**66**:65–70

Address for correspondence:
Prof Catharine West,
Translational Radiobiology Group,
School of Cancer and Enabling Sciences,
The University of Manchester, Christie Hospital,
Manchester M20 4BX, UK.

Fax: +44 161 446 8111
E-mail: catharine.west@manchester.ac.uk

Mr Jarrod Homer and Prof Catharine West takes responsibility for the integrity of the content of the paper.
Competing interests: None declared