Molecular staging of surgical margins in oral squamous cell carcinoma using promoter methylation of p16^{INK4A}, cytoglobin, E-cadherin and *TMEFF2*

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Running Head: Methylation biomarker staging of OSCC margins

Synopsis: Prognostic molecular staging of surgical margins in a series of prospectively-collected, fresh frozen tissue from oral squamous cell carcinoma using DNA methylation biomarkers, determined by quantitative methylation-specific PCR. Comparison with more usual, pathologically derived, indicators of recurrence.

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Abstract

Background: Local recurrence in oral squamous cell carcinoma (OSCC) despite clear surgical margins may indicate the presence of residual, sub-microscopic disease. Molecular assessment of surgical margins may provide a greater prognostic sensitivity compared to histopathology. We aim to determine whether promoter methylation in deep and mucosal resection margins can predict recurrence in OSCC.

Methods: 48 consecutive OSCC cases were recruited and a $5mm^3$ tumour sample plus 5 deep and 5 mucosal margin samples snap frozen. Clinical, pathological, adjuvant therapy and outcome data were recorded. Tumours were informative if >5% promoter methylation was found for ≥1 of 4 genes using qMSP. Margins were declared molecularly positive if >1% promoter methylation was found in any margin.

Results: 30/48 (63%) cases were methylation-informative. Mucosal margin samples were largely positive for methylation (26/30; 87%) indicating the presence of field cancerisation. Methylation at ≥1 gene promoters in ≥1 deep margin correlated with the presence of close/involved mucosal margins (P=0.027) and increased pT status (P=0.027) but not the status of deep margins, recurrence or survival.

Conclusions: The current gene panel did not add prognostic information to histopathological reporting of resection margins. Future efforts should concentrate on improving gene selection, informativity and assay performance in the patient group with intermediate indications for adjuvant therapy.

Introduction

The principal aim of surgical ablation for malignancy is to achieve clear resection margins. These are routinely defined by histopathology , where an additional 5mm of 'normal' tissue beyond the tumour should be identified in three dimensions [1]. This requirement is based on the assumption that histopathologically invisible cancer cells exist within this margin and might explain the common finding of local recurrence despite histopathologically defined clear margins [2]. In head and neck cancer (HNSCC), the temptation to increase excision margins to minimise local recurrence must be tempered against the concern of unjustified and irreversible loss of function. For this reason, the novel staging methodologies, such as molecular staging, have been explored to a greater extent in head and neck surgery than other surgical disciplines [3] with a view to providing a greater sensitivity for the detection of local recurrence.

The technical and theoretical hurdles to be overcome are, however, substantial. Firstly, the assay should be robust and clinically applicable. Ideally, the molecular aberration should be observed with high specificity, i.e. in the tumour but not in normal tissues, and informativity, i.e. in all or a high percentage of cases. Additionally, an assay needs to be developed that identifies this aberration with high sensitivity, and the technical platform should be reliable, reproducible, inexpensive and applicable in routine clinical practice. Secondly, it must be clear that an appropriate and effective intervention is available for cases with molecularly involved but histologically clear margins. It is known that involved and close margins are associated with other markers of biological aggression [4] such as T stage, N stage and extra-capsular spread (ECS) in regional lymph nodes [5, 6], and it is a matter for debate if increasing the surgical margin could

prevent tumour recurrence in such cases. Thirdly, the extent to which the entire surgical margin can be assessed by any technique (conventional or molecular) requires assessment. It follows that the reliability of any decision made on the basis of margins only, rather than an overall decision made on multiple markers of recurrence, might be questionable.

Molecular margin analysis has been investigated using a number of techniques. Given their high frequency in HNSCC, p53 mutations have traditionally been used [7, 8] but there are many sites where mutation has been shown to occur in this large gene with multiple exons. As such, the potentially attractive, sensitive PCR assay for any individual mutation offers very poor informativity, and the complex plaque phage functional assays for p53 are expensive and difficult to implement clinically. The common sites for chromosomal allelic loss in HNSCC have been explored as alternative molecular biomarkers of margin involvement [9, 10], but here the potential for highly sensitive assays is limiting. In contrast promoter hypermethylation is common [11], exists in a homogenous form [12] that can be detected by a single assay when present, and can be detected with high sensitivity [13, 14]. Previous analyses of promoter hypermethylation in HSNCC [15-19], as well as resection of liver [20] and lung [21] malignancies, are characterised by small cohort size, analysis of a limited number of margin samples, and a lack of distinction between mucosal and deep margins. Importantly there have been a variety of methylation detection assays used with little consensus or justification for the cut-off at which a margin might be declared reliably positive.

The aims of this study are to evaluate DNA hypermethylation in the analysis of surgical margins using a consecutive cohort of oral squamous cell carcinoma (OSCC) patients treated by primary surgery. We will focus on quantitative methylation analysis using real time quantitative methylation-specific PCR (qMSP) assays in DNA derived from multiple fresh-frozen margin samples from each case. The overall informativity of a panel of gene promoter methylation markers, as well as their individual contribution will be defined. The ability of methylation status of deep margins, and separately, mucosal margins, in predicting recurrence will be defined and compared with the histopathologically reported margin status and other clinicopathological features.

Methods

Clinical

53 consecutive OSCC patients were selected for inclusion in this study over the period 1st April 2007 – 30th April 2008, and all gave informed consent under a specific institutional ethical approval (REC 07/Q1505/15). No power calculation was undertaken as this was designed as a pilot study. Inclusion criteria were histologically confirmed stage T2-T4 OSCC with a treatment decision for primary surgery. FiveT1 tumours presenting over this time period were excluded from the study in order to enrich for cases likely to show either involved margins or local recurrence, leaving 48 tumours for analysis. Following surgical resection, thorough irrigation of the tumour bed was carried out with 1000ml of 0.9% NaCl applied though a pressurised giving set. Subsequently, five mucosal (peripheral) and five deep margin samples, $5mm^3$, were randomly selected and surgically excised prior to reconstruction. Intra-operative frozen sections were not taken, as is the norm for UK practice. These samples were placed in 10 separately pre-labeled containers and immediately frozen and stored at -

80C until DNA preparation. An additional frozen sample was taken from the primary tumour and stored similarly. Detailed histopathological analysis was recorded for each surgical resection according to standardized protocols, together with details of adjuvant therapy, and the clinical outcome for each patient was recorded for a minimum of 24 months.

Laboratory

DNA was extracted from 2mm^3 of each tissue sample using a DNeasy tissue kit (Qiagen Ltd, UK). DNA concentration was measured by spectrophotometry and subsequently adjusted to 40ng/ml. Bisulphite treatment of 1µg of each sample was undertaken using the EZ DNA Methylation Kit (Zymo Research Corporation, Orange, CA, USA) and the converted DNA eluted in 30µl of 0.1 TE buffer. Human genomic DNA (4µg) was artificially methylated as a positive control using SssI (CpG) Methylase (New England Biolabs, UK).

qMSP assays were used to determine DNA methylation in the promoters of p16^{INK4A} (*CDKN2A*), cytoglobin (*CYGB*), E-cadherin (*CDH1*) and *TMEFF2*. The incidence of promoter methylation at these genes in a similar OSCC cohort had been previously shown to exceed 25% [12, 22, Risk et al, unpublished data]. qMSP assays were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) (primer and probe sequences and PCR conditions used available on request). A total reaction volume of 25µl contained Taqman Universal Master Mix II (Applied Biosystems), 500nM of each primer, 250nM of probe and 100ng of bisulphite-treated DNA. A separate assay utilising a methylation-independent primer/probe set specific for the ß-actin gene (*ACTB*) was used to normalise for the DNA input in each sample. Real-time PCR

reactions were performed on an Applied Biosystems 7500 FAST system. Dilutions (5%, 1%, 0.5%, 0.1% and 0%) of in vitro methylated (SssI) human lymphocyte DNA made in untreated lymphocyte DNA were used as a reference. ΔΔCT values were generated for each target after normalisation by *ACTB* values. The RQ values were subsequently calculated ($2^{-\Delta\Delta CT}$) referenced to the artificially methylated samples for statistical analysis. All analysed data were the mean of duplicate reactions.

For tumour specimens, a threshold of 5% methylation was used to define a sample that was methylated at that particular gene promoter, and hence the case was deemed to be informative for that marker. This threshold was based on our previous methylation data using a variety of techniques and HNSCC tumour types [12, 23] and has been used in other similar studies [17]. Both 1% and 0.1% methylation were considered as possible thresholds for a positive margin, irrespective of whether deep or mucosal in origin. A threshold of 1% was chosen for the analyses presented in this paper as this was the lowest value for which reproducible assignment of methylation positivity could be obtained in the present series of samples containing variable numbers of tumour cells diluted with 'normal' cells.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS, v 18, Chicago) was used to undertake statistical analysis, including Chi-square test for categorical data and Kaplan-Meier survival analysis.

Results

Clinical characteristics of the cohort

Of the 48 OSCC patients used in this study, 5 were lost to follow up and 6 had incomplete pathological information. The cohort was compared clinically and demographically with a larger HNSCC population (n=489) from the same geographic location in order to confirm that they were representative of this larger cohort [5]. The only significant differences between the two populations were an increased incidence of higher pathological stage (p=0.022), the use of post-operative, adjuvant treatment (p=0.008) and presence of neck dissection (p=0015) in the cohort for the current study. These are a direct consequence of excluding T1 tumors in this cohort.

Promoter methylation

Thirty of 48 tumours (63%) demonstrated ≥5% promoter methylation at ≥1 gene. Thirteen tumours (26%) were methylated at *TMEFF2*, 11 (22%) at p16, 9 (18%) at *CDH1* and 8 (16%) at *CYGB*. The 'promoter positive' cohort had a younger profile than the 'promoter negative' cohort (p=0.048), while the 'promoter negative' cohort contained more large (p=0.018), well differentiated (p=0.043) tumours than the 'promoter positive' cohort (Table 1). No other statistically significant differences in clinical characteristics or demographic data were observed between these two groups, although the 'promoter positive' cohort showed a trend towards improved 2 year survival (3/26 [12%] vs 5/17 [29%]DOD; Table 1).

At least one mucosal margin from 26 of the 30 informative tumours (87%) showed promoter methylation at ≥1 gene, while in 19 /30 (63%) at least one deep margin showed methylation at ≥1 gene (Supplementary table). As gene promoter methylation

at mucosal margins did not appear to be discriminatory, this data was not included for further analysis.

CDH1 promoter methylation was observed in ≥1 mucosal margin in all the positive tumours (9/9) and in ≥1deep margin from 8/9 positive tumours and was thus deemed to be not discriminatory and removed from further analyses.
Thus, 26 tumours remained for correlation of promoter methylation at 3 genes in deep margin samples with clinicopathological data.

Correlation of margin methylation with clinicopathological features
Methylation at ≥1 out of 3 gene promoters in ≥1 deep margin correlated with the presence of close/involved mucosal margins (P=0.027), an absence of dysplasia at the surgical margin (P=0.024) and with tumour stage (P=0.027), most notably an increase in pT4 tumours (Table 2). There was no correlation with the histopathologically documented presence of deep margin involvement, pattern of invasion, nodal involvement, ECS, recurrence, pattern of recurrence or survival. Indeed, close or involved pathological margins were superior to methylation of ≥1 gene promoters at predicting recurrence in methylation positive tumours (5/7 recurrences were in patients with close/involved surgical margins vs 3/7 recurrences in patients with methylation positive deep surgical margins). Interestingly, histopathological assessment of margins was not such a good prognostic indicator for methylation negative tumours, where only 3/9 recurrences were in patients with close/involved margins (not significant: Supplementary table).

Of the 11 tumours with p16 promoter methylation, 7 showed concordant methylation in ≥1 deep margin tissues. This correlated with the presence of a non-cohesive invasive tumour front (P=0.015) and showed some association with the presence of histopathologically close or involved deep margins (Table 2). Although the numbers are small, there was some indication that patients with p16 positive margins presented with recurrence earlier than those with p16 negative margins and had a shorter survival period after recurrence (not significant: patients 3329,3338, 3371 & 3363, Supplementary table).

Discussion

In this study, we investigated the surgical margins in OSCC for promoter methylation as a predictor of clinical outcome. 30/48 tumours showed promoter methylation at ≥1 of 4 genes, with the incidence of individual gene promoter methylation comparable to those previously described using similar, quantitative assays [19]. Mucosal margin samples were largely positive for methylation (26/30; 87%) supporting a concept of field cancerisation at this anatomical site. As only 27% of methylation positive tumours recurred, it seems unlikely that the gene panel investigated in this study would have clinical value at mucosal margins. However, only 19/30 (63%) of deep margins were positive for methylation so their discriminatory effect was determined for single genes and for combinations of genes.

Using the three gene combination of p16, *TMEFF2* and *CYGB*, promoter methylation in deep margins correlated with tumour stage, indicating a greater risk of residual disease remaining at the deep margin. However, this did not directly correlate with

pathologically involved deep surgical margins or recurrence. The lack of association with recurrence may have been related to the small numbers of recurrences seen in the methylation positive tumours (7/26, 27%) compared to the methylation negative tumours (9/17, 53%). This suggests either that new methylation markers need to be identified, or it may confirm previous observations that tumours showing methylation at these specific gene promoters are inherently less aggressive [19, 22].

Analysis of data for individual genes provided some insight as to why observations using data from all three genes was not a good prognostic indicator. p16 promoter methylation at deep margins was observed to be associated with pattern of invasion and possibly with close deep pathological margins and early recurrence. Three of the four recurrences from tumours demonstrating p16 methylation also showed p16 promoter methylation in the deep margins. However, four additional patients with methylation of this gene promoter in deep margin tissue did not recur. Conversely, *TMEFF2* promoter methylation in deep margins showed an association with smaller tumour size. These data suggest a possible role of p16 downregulation in tumour recurrence, while *TMEFF2* may be a bystander event.

The advantages of the present study over many previous reports [15-19] are that we have obtained snap frozen tissue with detailed pathology and at least 2 year follow-up. Furthermore, we have used a quantitative, real time MSP methodology with a pre-determined cutoff. We have also investigated mucosal and deep margins separately, finding mucosal margins show lack of specificity with extensive methylation in presumed field change.

Limitations of the present study include lack of informativity (30/48 tumours positive for promoter methylation at ≥1 genes) and the choice of biomarkers. This was unexpected given our previously determined incidence of informativity for these genes, but may reflect the different methodologies employed [12, 22]. However, previous published reports reflect even fewer informative cases - a cumulative total of only 70 informative tumours have been previously published amongst five previous series using methylation assays in surgical margins [15-19]

Given the intensive nature of sample collection and analysis of 11 samples per tumour involved in this study, some conclusions regarding sample selection/pooling should be drawn from our data before embarking on a larger series. We have found it difficult to use DNA methylation biomarkers to distinguish field change or premalignant lesions [24] at the mucosal margin from residual tumour. Further, it may be more appropriate to sample the deep margins at several sites, but to then pool the DNA to create a single sample for prognostic purposes. Similarly, the selection of the panel of biomarkers used in the present study was unfortunate in that one marker (*CDH1*) was largely uninformative, while the sensitivity of one other marker (*CYGB*) appeared to be lower than the other two genes, as shown by the low number of positive margins associated with *CYGB* positive tumours. The identification of further markers with suitable sensitivity for inclusion in a methylation biomarker panel is required and candidates may yet emerge with improving genome-wide array techniques.

Lastly, it is worthwhile to reflect on the clinical context for which molecular margin analysis may be of greatest therapeutic value. In those cases defined as the intermediate risk group [25] where the role for adjuvant therapy remains unproven, the value of

molecular margin analysis might be best highlighted. This may be particularly the case for those resections with close margins as a sole adverse prognostic feature. Concentrating future efforts on improving informativity and the utility of assay performance in this group seems logical. This would clarify if these, as yet unproven, techniques can be translated into clinical practice.

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Table 1 Clinical and demographic characteristics of the patient cohort used in this study

	All tumours (n=48)	me+ tumour[a] (n=30: 63%)	me– tumours[b] (n=18: 38%)
Gender:			
Male	33 (72%)	20 (71%)	13 (72%)
Female	13 (28%)	8 (29%)	5 (28%)
Age:			
< 55	13 (28%)	12 (41%)	1 (6%)
55-64	21 (45%)	10 (34%)	11 (61%)
65-74	8 (17%)	5 (17%)	3 (17%)
75+	5 (11%)	2 (7%)	3 (17%)*
Tumour site:			
Buccal	5 (11%)	2 (7%)	3 (17%)
Lower gum	7 (15%)	5 (17%)	2 (11%)
Tongue (ant 2/3)	16 (34%)	9 (31%)	7 (39%)
Floor of mouth	11 (23%)	6 (21%)	5 (28%)
Other	8 (17%)	7 (24%)	1 (6%)
Tumour differentiation:			
Poor	2 (5%)	1 (4%)	1 (6%)
Moderate	28 (67%)	21 (81%	7 (44%)
Well	12 (29%)	4 (15%)	8 (50%)*
Invasive front[c]:			
Cohesive	11 (24%)	9 (31%)	2 (12%)
Non-cohesive	35 (76%)	20 (69%)	15 (88%)

Mucosal margins:			
Clear ≥ 5mm	24 (51%)	14 (48%)	10 (56%)
Close < 5mm	16 (34%)	9 (31%)	7 (39%)
Involved	7 (15%)	6 (21%)	1 (6%)
Deep margins:			
Clear ≥ 5mm	22 (51%)	11 (42%)	11 (65%)
Close < 5mm	17 (40%)	12 (46%)	5 (29%)
Involved	4 (9%)	3 (12%)	1 (6%)
pT: T1/T2	24 (51%)	15 (52%)	9 (50%)
T3/T4	23 (49%)	14 (48%)	9 (50%)
pN: 0	24 (52%)	18 (62%)	6 (35%)
1	6 (13%)	3 (10%)	3 (18%)
2-3	16 (35%)	8 (28%)	8 (47%)
p stage: 2	11 (24%)	8 (28%)	3 (18%)
3	7 (15%)	4 (14%)	3 (18%)
4	28 (61%)	17 (59%)	11 (65%)
Nodal status:			
N0	24 (52%)	18 (62%)	6 (35%)
N+ ECS –	10 (22%)	6 (21%)	4 (24%)
N+ ECS +	12 (26%)	5 (17%)	7 (41%)
Adjuvant radiotherapy			
Yes	26 (60%)	17 (65%)	9 (53%)
No	17 (40%)	9 (35%)	8 (47%)
Recurrence:			
Yes	16 (37%)	7 (27%)	9 (53%)

No	27 (63%)	19 (73%)	8 (47%)
2 yr Survival:			
Disease free	32 (74%)	21 (81%)	11 (65%)
DOD	8 (19%)	3 (12%)	5 (29%)
Died (other)	3 (7%)	2 (8%)	1 (6%)

[a] me+ tumours: tumours showing ≥5% methylation at ≥ 1 gene promoter

[b] me- tumours: tumours showing <5% methylation at all gene promoters

[c] invasive front classified into cohesive and non-cohesive patterns

ECS: Extracapsular spread; DOD: Died of disease

[*] p=0.05

Table 2 Correlation of methylation at deep margins with clinicopathological parameters

	Gene Promoter Methylation at:			
	p16 and/or *TMEFF2* and/or *CYGB*	P16 alone	*TMEFF* alone	*CYGB* alone
Close/involved deep margins	ns	0.071	ns	ns
Close/involved mucosal margins	0.027	ns	ns	ns
Invasive front[a]	ns	0.015	ns	ns
Depth	ns	ns	ns	ns
Nodes/ECS[b]	ns	ns	ns	ns
ECS	ns	ns	ns	ns
Nodes	ns	ns	ns	ns
Stage	0.074	ns	ns	ns
pT (pT2 v pT3 v pT4)	0.027	ns	0.043	ns
(pT2/3 v pT4)	0.035	ns	ns	ns
Anneroth score	ns	ns	0.032	ns
Recurrence	ns	ns	ns	ns
DSS[c]	ns	ns	ns	ns
OS[d]	ns	ns	ns	ns

[a] invasive front classified into cohesive and non-cohesive

[b] ECS: extracapsular spread

[c] DSS: Disease-specific survival

[d] OS: Overall survival