Tetraspanins CD9 and CD151, epidermal growth factor receptor and cyclooxegenase-2 expression predict malignant progression in oral epithelial dysplasia

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Abstract

Background

Prognostic biomarkers aim to improve on the current inadequate method of identifying patients with oral epithelial dysplasia at greatest risk of malignant transformation, namely histological assessment. We aimed to assess the prognostic ability of 6 protein biomarkers linked to the EGFR and associated tetraspanin pathway, along with clinical parameters, in a large multicentre oral dysplasia cohort.

Methods

148 cases with varying degrees of epithelial dysplasia underwent immunohistochemistry. The markers assessed were CD9, CD151 and CD82, EGFR, Her-2, and Cyclooxygenase-2 (COX-2). Scoring was performed independently by two observers. Univariate analyses using both logistic and Cox regression models and a multivariate regression were performed.

Results

Malignant progression was significantly greater in those cases with decreased expression of CD9 (p=0.02), and increased expression of CD151 (p=0.02), EGFR (p=0.04) and COX-2 (p=0.003). Histological grade (p=0.0002), and morphology (p=0.03) were also prognostic, whilst smoking and alcohol were not. The optimal combination by backward variable selection was histological grade (hazard ratio 1.64; 95% CI 1.12, 2.40), COX-2 over-expression (HR 1.12; 1.02, 1.24) and CD9 under-expression (HR 0.88; 0.80, 0.97). CD82 and Her-2 demonstrated no prognostic ability.

Conclusions

This is the first study of the expression and prognostic potential of the tetraspanins in oral dysplasia. A combination of certain biomarkers with clinical factors appeared to improve the accuracy of determining the risk of malignancy in individuals with oral dysplasia. These findings may also offer potential new therapeutic approaches for this condition.

Introduction

Cancers of the oral cavity arise through a combination of progressive genomic alteration and exposure to environmental carcinogens[2]. Many OSCCs arise in areas of genomic and histological abnormality, termed oral epithelial dysplasia (OED). The degree of cytological and architectural abnormality seen on histological examination is used to assign a grade of severity to OED[3,4]. Quantifying the risk of transformation of an individual OED lesion to cancer is complex, due to both a lack of knowledge of the natural history of OED and because of the wide variability in reported transformation rates in the published literature (5% to 36%)[5,6]. A recent meta-analysis estimated the malignant transformation of OED to be 12% (95% CI 8-18%)[7]. Furthermore, while dysplasia grade assessed by histological examination is currently the best predictor of future malignant behaviour, it has significant limitations. Despite more severe grades of dysplasia being associated with higher transformation rates, cases with mild dysplasia may still progress to cancer, while a significant proportion with severe dysplasia do not transform, irrespective of environmental factors[1,3,4,7,8]. In addition, histological grading of OED is known to be largely subjective, resulting in significant inter and intra-rater variability[9-12]. This results in histological grading having only a moderate prognostic ability at best. However, it remains the gold standard on which treatment decisions are based[13].

The differential expression of biomarkers in cancer, potentially malignant lesions and normal mucosa offers the possibility of better identification of those lesions with the highest risk of malignant progression. To date, many biomarkers have been described, yet due to low sample size and methodological limitations, few have been validated and none have as yet been incorporated into routine clinical use. The search for effective prognostic biomarkers for this indication continues.

The Epidermal Growth Factor receptor (EGFR) family has been extensively studied in relation to cancer biology. Strong evidence exists for their role in carcinogenesis in many solid tumours, including those arising in the breast, ovary, colon and lung[14]. Overexpression of EGFR occurs in around 80% - 90% of head and neck cancers and in some studies has been shown to be correlated with worse survival outcomes[15-17]. Another of the EGF family, Her2 is also upregulated in oral dysplasia and cancer[5,6,18]. This pathway is also of interest in as there are already several molecular therapies targeted against EGFR; including small molecule tyrosine kinase inhibitors (e.g. gefitinib) and monoclonal antibodies (e.g. cetuximab), which may potentially be of benefit for the treatment of OED.

Our aim was to examine the prognostic potential of EGFR and associated biomarkers known to regulate this pathway, along with clinical factors in one of the largest cohorts of OED reported in the literature.

Methods

This study has been reported using the REporting recommendations for tumour MARKer prognostic studies (REMARK) guidelines[19]. Ethical approval was granted from the Coventry research ethics committee (06/Q2802/79) and the Human Biomaterials Resource Centre at the University of Birmingham (10-008).

Patient selection

This was a retrospective cohort study. Consecutive cases were selected after systematic searching of the pathology archives from 5 institutions: University Hospital Coventry and Warwickshire, University Hospital Birmingham, Birmingham Dental Hospital, George Eliot Hospital Nuneaton, and the University Of Leeds. Searching was performed using the Systematized Nomenclature of Medicine Clinical Terms (SNOMED) and free field text, to include any biopsies taken between 1996 and 2008. Inclusion in the cohort required patients to be over 18 years of age at time of biopsy (no upper age limit was set), have a confirmed diagnosis of OED using the WHO classification system, and have a minimum follow-up for non-transformed cases of 12 months, or transformation to cancer after 3 months of diagnosis of OED. Where several biopsies were available from a single patient, the first diagnostic biopsy was used. Where the first diagnostic biopsy was not available, the next oldest biopsy was used. Cases were excluded if positive for candida on diastase-resistant periodic acid schiff (dPAS) staining, along with diagnoses of lichenoid inflammation with atypia (histological changes are likely a result of inflammation and therefore represents a different process to true neoplastic change) and proliferative verrucous leukoplakia. Any patient with OED that had a previous diagnosis of head and neck cancer (identified either through the pathology database or a search of the clinical records) was excluded, as this population of patients are known to already be at increased

risk of developing a second malignancy, and previous treatment may have affected the behaviour of the lesion under investigation. Clinical information on the exposure to known or suspected risk factors such as age, sex, anatomical site, lesion morphology and smoking/alcohol history were collected.

Immunohistochemistry

All samples were taken from formalin-fixed, paraffin-embedded tissue. 4μm sections were taken either from donor blocks or a tissue microarray containing some of the cases. We have previously demonstrated near perfect agreement in immunohistochemical scoring between tissue microarrays and slides using these biomarkers in OED[7,20]. After deparaffinsation in xylene, sections were rehydrated in distilled water. Unmasking of the epitopes was performed using a PickCell antigen retrieval unit, exposing the samples to both heat and pressure while in Tris-ethylenediaminetetraacetic acid (EDTA) buffer concentrate at pH7.8, or Citrate buffer pH6 (determined by prior optimisation and validation). The Novocastra™ Polymer Detection System was used for this study. Endogenous peroxidase and protein was blocked with 3% hydrogen peroxide and 0.4% Casein in phosphate-buffered saline respectively. Slides were then incubated at 4°C with monoclonal antibodies at optimal concentrations (supplementary table 1). After 30 minute incubations with post primary block and polymer, 3,3'-Diaminobenzidine (DAB) working solution was applied for five minutes. Application of Mayer's haematoxylin for 1 minute provided counterstaining. All positive controls stained correctly and no negative controls demonstrated any staining during the procedure.

Immunohistochemical scoring

Two individual raters, with different levels of experience in immunohistochemistry assessment, independently scored each case. Raters were blinded to the clinical details of the case. It has been suggested that when scoring immunoreactivity in small specimens (such as OED specimens examined here) only the area with maximal staining should be interpreted[21]. This approach was applied here. The sections were presented in random order to the raters with cases of disagreement undergoing consensus scoring. Antibody expression was determined by assessing the intensity and proportion of cells stained. Staining intensity was scored from 0 to 3: 0 = negative (No staining); 1 = weak staining; 2 = moderately strong staining; and 3 = strong staining. Proportion was also scored on a 4-point scale: 1 (<25% of cells stained); 2 (25-50% of cells stained); 3 (51-75% of cells stained); 4 (>75% cells stained). An overall score for each case was generated by the sum of the intensity and proportion scores, resulting in a range of scores from 0 to 12.

Statistical analysis

Scoring agreement between raters was calculated using a kappa statistic (κ) and intraclass correlation coefficients (ICCC). The latter measure is felt to be superior when correlating immunohistochemistry scores between raters, as it is calculated using the whole range of data, thereby not being influenced on how the data is categorised, as is the case with kappas[22]. However, many studies quote kappa scores and hence they were also included here to allow comparison. Kappa scores were interpreted using a scale proposed by Landis and Koch, with scores of 0 – 0.2 representing slight, 0.2 – 0.4 fair, 0.4 – 06 moderate, 0.6 – 0.8 substantial and 0.8 – 1.0 near perfect agreement[23]. An ICCC of < 0.40 was regarded as poor, 0.4-0.59 as fair, 0.6 – 0.74 as good, >0.74 as excellent and 1 perfect correlation[24]. Consensus

scores between the two raters were used for subsequent analyses. The capability of each biomarker or clinical factor to predict progression was initially calculated using univariate logistic regression. Consideration of the additional effect of time on the prognostic ability was assessed using a Cox regression analysis, with significance defined as $p<0.05$. Clinical factors were analysed as categorical variables as shown in table 1. Missing data was handled using listwise deletion, where any cases with missing clinical data were excluded from the analysis of that particular variable. Multivariate analysis with backwards variable selection was performed to examine which factors remained independent indicators of transformation. This method negates one of the disadvantages of forward variable selection, whereby addition of each new variable to the model may make a previously significant variable, non-significant.

To further explore the scoring thresholds that predict progression, a logistic regression was performed on the continuous immunohistochemistry scores. Where a linear effect was not seen, scores were then converted to categorical variables to examine whether prognostic ability differed between these categories. Categorisation was as follows: score of 0 = 0 (truly negative), scores of 1-4 = 1 (weakly positive), 5-8 = 2 (moderately positive), >9 = 3 (strongly positive). Finally, Pearson chi-squared analysis was used to identify the optimal binary scoring threshold to group cases into the most and least likely to transform. Oral cancer free survival was calculated for these different groups using Kaplan Meier survival curves. Differences between the resulting curves were calculated using a Log-rank (Mantel-Cox) test. Calculations were performed using SPSS version 19.0 for Mac; SPSS Inc., Chicago, IL, USA and SAS version 9.2.

Results

Characteristics of patients in this cohort

The 148 patients included in this cohort of patients with OED were almost equally male (76) and female (72). The mean age was 61 years (SD 13.6) with a range from 19 to 90. Other demographic data including OED dysplasia grade is summarised in table 1. 39 cases out of 148 progressed from dysplasia to cancer (26%) with a median time to transformation for these cases of 26 months.

Inter-rater scoring reliability

There was strong agreement between the two raters for all biomarkers used in this study. Kappa scores ranged from 0.66 to 1.0, demonstrating substantial agreement. This finding was confirmed with intraclass correlation coefficients ranging from 0.82 – 1.0 (supplementary table 2). The most significant disagreement was seen on scoring the intensity of COX2 staining (k 0.66; ICCC 0.85) and proportion of EGFR cells stained (k 0.74; ICCC 0.82). This still represents substantial agreement.

Prognostic ability of clinical factors

Using a univariate logistic regression, higher grades of dysplasia were seen to significantly predict malignant transformation in this cohort (p=0.0002). This remained significant when time to transformation was analysed using a Cox regression model (p=0.001). The morphology of the individual lesions was also associated with progression (p=0.03). In ascending order, the proportion of progressors for each morphological type was: leukoplakia (17/94, 18%), ulcerated lesions (7/22, 32%), speckled lesions (5/13, 38%), mass lesions (2/4, 50%) and erythroplakia (8/15, 53%).

However, morphology did not remain independently significant once added to grade in a multivariate analysis. Anatomical site, smoking and alcohol consumption were not prognostic (p=0.73, 0.29 and 0.61 respectively). Gender did not independently predict progression, yet showed a trend towards significance when added with histological grade into the multivariate model, with females more at risk than males (p=0.05).

Prognostic ability of biomarkers

Immunohistochemical expression of each of the biomarkers is summarised in supplementary table 3. The pattern of staining was predictable, with CD9, CD151, CD82 and EGFR localising to the cell membrane, and COX2 to the cytoplasm (figures 1 and 2). Only 8 out of the 148 cases demonstrated any Her2 staining, with all of these being membranous in location. Nearly 80% of cases had very weak or no CD82 staining (scores ≤3). Both raters agreed that scoring was not possible in 4 out of 888 slides (0.5%) because of inadequate tissue.

Univariate logistic regression demonstrated a significantly increased risk of progression to cancer in cases with under-expression of CD9 (p=0.02) or over-expression of CD151 (p=0.02), EGFR (p=0.04) or COX2 (p=0.003). When also considering time to transformation, CD9 (p=0.02), EGFR (p=0.04) and COX2 (p=0.008) were still able to significantly predict progression (table 2). On multivariate analysis, CD9 (p=0.009) and COX2 (p=0.008) remained significant independent predictors of transformation to oral cancer. EGFR was not independently significantly associated with transformation on multivariate analysis.

Logistic regression was performed in an attempt to more accurately define relevant scoring thresholds for the biomarkers with prognostic potential. COX2 was the only

marker to demonstrate a clear linear effect, with increasing scores associated with increasing risk of malignant progression (p=0.002). No linear effect was seen with the other markers, even after the continuous scores (0-12) were converted to categorical variables. Pearson chi-squared analysis identified the optimal scoring thresholds to divide cases into those most and least likely to undergo malignant transformation. For CD9 and CD151, the threshold was between those cases scoring 0 or 1 versus the rest (2-12) (p<0.0001and 0.0002 respectively), and 0-2 versus the rest (3-12) for EGFR (p=0.006) (figure 3).

Because CD9 has been postulated to have an action via direct effects on EGFR expression, any association between these markers was explored. Yet, the correlation was low (0.04), with no evidence of an association between them in cases undergoing progression or not.

Prognostic ability of clinical factors and biomarkers

In combining both the clinical factors and biomarkers, the overall best combination by backwards variable selection was high dysplasia grade (hazard ratio 1.64; 95% CI 1.12, 2.40, p=0.01), COX2 over expression (HR 1.12; 95% CI 1.02, 1.24, p=0.02) and under expression of CD9 (HR 0.88, 95% CI 0.80, 0.97, p=0.01) (table 3).

Discussion

This is the first study to examine the expression of members of the tetraspanin family in OED and the first to demonstrate a prognostic ability of CD9, CD151, COX2 and EGFR in a retrospective longitudinal oral epithelial dysplasia cohort. Decreased expression of CD9 was associated with a significantly increased risk of malignancy, especially when expression was almost completely absent (scores of 0 or 1; $p<0.0001$). Increased expression of CD151, EGFR and COX2 were similarly associated with malignant transformation. Immunohistochemical scores of greater than 2 and 3 for CD151 and EGFR respectively were significant ($p=0.0002$ and 0.006 respectively). COX2 demonstrated a much more linear effect, as increasing expression correlated with increasing risk of cancer. Her2 and CD82 had no prognostic ability in this cohort and indeed demonstrated little expression overall in dysplastic tissues. CD9 and COX2 remained independently prognostic when accounting for the effect of other variables on multivariate analysis ($p=0.009$ and 0.007 respectively). When both clinical factors and biomarkers were included in multivariate analysis, the best combination for predicting malignant progression was high dysplasia grade (hazard ratio 1.64) strong COX2 staining (HR 1.12) and weak CD9 staining ($p=0.01$).

As might have been expected, increasing severity of dysplasia and erythroplakic lesions had higher malignant transformation rates on univariate logistic regression ($p=0.0002$ and 0.03 respectively). The anatomical site within the oral cavity was not prognostic in this cohort, which may in part be explained by the slightly low numbers of known high-risk floor of mouth lesions (13%), along with the grouping of all tongue lesions together (ventral tongue lesions are known to higher rates of transformation than others). Alcohol and smoking consumption were similarly not prognostic in this cohort. This is in keeping with other studies, where these habits have been demonstrated to increase the likelihood of developing potentially malignant lesions

but not their subsequent malignant transformation{Napier:2008gz, Liu:2010ea}. Gender was also not independently prognostic, however females were more at risk when included in a multivariate analysis in combination with histological grade.

The tetraspanins are a family of 33 proteins that form web complexes on the cell surface. When joined by gangliosides and cholesterol these aggregations are termed tetraspanin enriched microdomains[25]. Through these domains the tetraspanins are able to organise other transmembrane molecules including growth factor receptors[9-12,26], integrins[13,27], and G-protein coupled receptors[14,25,28]. Because of the strong association with integrins and growth factor receptors, tumorigenic processes such as cell adhesion, motility, invasion and angiogenesis may be modulated and controlled. There have been relatively few studies examining the role tetraspanins play with specific regards to head and neck cancer, and none examining them in OED. However, the findings of these studies support our results. Decreased immunohistochemical expression of CD9 was detected in 42% of 129 oral cancer samples, with these cases significantly associated with regional nodal metastases ($p=0.017$) and a reduced overall and disease-free 5-year survival ($p=0.071$, $p=0.01$ respectively)[15-17,29]. In the same study, 80% of cases had reduced or absent CD82 staining, however no correlation with disease-free or overall survival was observed[29]. A study of 34 patients with head and neck cancer identified the same prolongation of overall and disease free survival ($p=0.02$; 0.004) with lower recurrence rates and stage of regional lymphadenopathy ($p=0.02$; 0.04) in cases with increased CD9 expression[30]. Decreased CD9 expression was also seen in lymphatic vessels of tumour samples compared to normal tissue, which along with the increased stage of lymphadenopathy in cases with reduced CD9 expression suggests a role for this tetraspanin in preventing lymphatic spread. A third study of 78 oral cancers again confirmed the increased metastatic potential of tumours with lower CD9 expression, with higher incidence of cervical lymphadenopathy and poorer outcome[31]. Loss of

CD9 expression at the invasive front of the tumours was noted in these cases suggesting a role for CD9 in cell adhesion and invasion.

CD9 has also been shown to exert an effect on EGFR, with complexes of CD9, EGFR and β1 integrin co-localised in areas of cell-cell interaction. Through EGF induced EGFR receptor internalisation, CD9 has also been shown to attenuate EGFR signalling by reducing cell surface EGFR expression[26]. Additional indirect effects on the EGF receptor occur through its receptor ligands. CD9 not only binds to Transforming growth factor α (TGF-α), but also affects its maturation, cell-surface presentation and cell-surface distribution. CD9 stabilises membrane bound TGF-α preventing its cleavage to produce free ligand that may circulate and stimulate EGF receptors at distant sites, instead stimulating juxtacrine EGFR activation. This alteration in EGF receptor stimulation leads ultimately to differences in the effect of receptor activation. In the same series of experiments, co-expression of CD9 and TGFα were found to increase cellular adhesion and decrease migratory potential, compared to cells in which only one or other were expressed[32,33]. These results taken together might suggest that the consequences of decreased CD9 expression in OED are not driven through a direct effect, but through the alteration in balance of EGFR activation. This would be in keeping with the finding from the experiments conducted here, demonstrating increased EGFR expression as a prognostic variable on univariate analysis, despite no obvious direct correlation seen between the expression patterns of the two markers.

Results from studies assessing CD151 are more contradictory. Increased expression of CD151 conferred a significantly poorer prognosis in 73 gingival squamous cell carcinomas[34]. However, a recent publication found no prognostic significance of CD151 expression in 83 oral cancer cases, despite the widespread expression of the protein[35]. This difference may be due to inconsistent methodologies between the two studies. Interestingly, the authors of the latter study did detect a significant

association between CD151 and EGFR, both of which were also found at the invasive front along with the α3β1 integrin (which is also known to form complexes with CD151). The suggestion from this study was that CD151 acts to modulate and coordinate an interaction between EGFR and α3β1 integrin. This would be consistent with the findings here of upregulation of both CD151 and EGFR conferring a worse prognosis in cases of OED.

Increased COX-2 expression is known to occur in premalignant tissues in many sites, including the colon, bladder and stomach[36-38]. Similar upregulation occurs in premalignant lesions and cancers of the head and neck. Cross sectional studies have all demonstrated an increased expression of COX-2 in premalignant tissue compared to normal mucosa[39]. This finding has been replicated in other studies, along with a significant increase in COX-2 expression with increasing severity of dysplasia[40,41]. Despite the interest in COX enzymes as biomarkers in carcinogenesis, until now, no longitudinal studies have examined their role as predictors of malignant transformation of OED in the head and neck. We have demonstrated not only that COX2 has a significant prognostic potential, but also that the risk of malignant transformation appears to escalate with increasing COX2 expression.

EGFR over expression is known to occur in oral premalignant lesions[18]. In contrast to the results presented here, Benchekroun et al examining an cohort of oral premalignant lesions failed to show a statistically significant risk of progression to oral squamous cell cancer in patients with elevated EGFR immunoreactivity, despite high EGFR expression occurring in 71% of the patients[42]. This disparity may be explained by over two thirds of that particular cohort having a histological diagnosis of hyperplasia only without dysplasia. The prognostic potential of EGFR on univariate analysis in this cohort would support the hypothesis of treating these high-risk lesions with EGFR antagonists. Furthermore, evidence is beginning to emerge about the interaction between EGFR and COX2, CD9 and CD151. This also raises the

possibility of multimodal approaches to chemoprevention in the management of oral premalignant lesions.

Limitations of the study

Although 4 of the biomarkers were prognostic in this study, the thresholds identified to differentiate between cases likely or not to progress (CD9, CD151 and EGFR) are data driven, and therefore possibly unique to this dataset. In this respect, the results must be viewed with caution and are perhaps best considered as representing a hypothesis-generating group. A validation cohort would be required to test these thresholds. Furthermore, it was not possible to construct a prognostic classifier based on the numbers in this study and so any validation cohort would need to be larger to enable this.

Despite being one of the largest cohorts of oral dysplasia used to date in assessing the prognostic ability of biomarkers, there remain the same limitations such as inadequate data collection and variability in the treatment of similar lesions from individuals at different institutions that affect all retrospectively collected cohorts. As an example of this, while some studies have reported higher transformation rates of oral leukoplakia in females and from particular anatomical areas (lateral border of tongue and floor of mouth) other recent large cohort studies have similarly to here, failed to demonstrate this[8,43,44]. It is possible that this difference may be explained in part because of difference in cohorts (e.g. in this study all cases were OED, whereas in others leukoplakia without dysplasia were also included). Poor clinical recording did not allow a sub site analysis of lesions of the tongue to be performed. This meant all cases affecting the tongue (the largest site numerically) were analysed together, potentially obscuring a significant effect of anatomical site. These limitations may only be improved by the prospective enrollment of patients with OED into clinical trials.

Conclusions

This study, using one of the largest multicenter cohorts of OED in the literature, demonstrates 4 biomarkers (EGFR, CD151, CD9 and COX2) with a prognostic ability. It is also the first study to examine both the expression and prognostic ability of the tetraspanins in OED. If validated, these results may help improve identification of those patients at highest risk of malignant transformation and also suggests other avenues for chemoprevention and chemotherapeutics in the treatment of this condition.

Table 1: Clinical characteristics of cohort (number and percent) with prognostic ability by univariate logistic regression (p value)

Histological grade		**0.0002**
Mild	69 (47)	
Moderate	50 (24)	
Severe	27 (18)	
CIS	2 (1)	
Gender		**0.14**
Male	76 (51)	
Female	72 (49)	
Site of lesion		**0.73**
Tongue	69 (47)	
Floor of mouth	20 (13)	
Palate	18 (12)	
Buccal	38 (26)	
Retromolar	3 (2)	
Morphology of lesion		**0.03**
White patch	94 (63)	
Red patch	15 (10)	
Speckled patch	13 (9)	
Ulcer	22 (15)	
Lump	4 (3)	
Alcohol consumption		**0.61**
>21 U/week	23 (15)	
<21 U/week	58 (40)	
None	44 (30)	
Unknown	23 (15)	
Smoking status		**0.29**
Current	69 (47)	
Ex	9 (6)	
Non	47 (32)	
Unknown	23 (15)	

Table 2: Prognostic ability of individual biomarkers on univariate analysis using logistic and Cox regression (p values)

Biomarker	Univariate analysis (Logistic)	Univariate analysis (Cox)
COX2	0.003	0.008
CD9	0.02	0.02
CD151	0.02	0.33
EGFR	0.04	0.04
CD82	0.62	0.69
Her2	0.73	0.50

Table 3: Multivariate analysis demonstrating hazard ratios for the best combination of clinical factors and biomarkers in predicting malignant progression by backwards-variable selection

Variable	Hazard ratio (95% CI)	p value	Higher risk group
Grade	1.64 (1.12, 2.40)	0.01	High grade
COX2	1.12 (1.02, 1.24)	0.02	High score
CD9	0.88 (0.80, 0.97)	0.01	Low score

Figure 1: Representative tetraspanin immunohistochemistry. Tiles a-c (CD151), d-f (CD82) and g-i (CD9), demonstrate increasing expression from scores of 2 (top row) to 6 (middle row) to 12 (bottom row). All 3 tetraspanin biomarkers exhibit membranous staining.

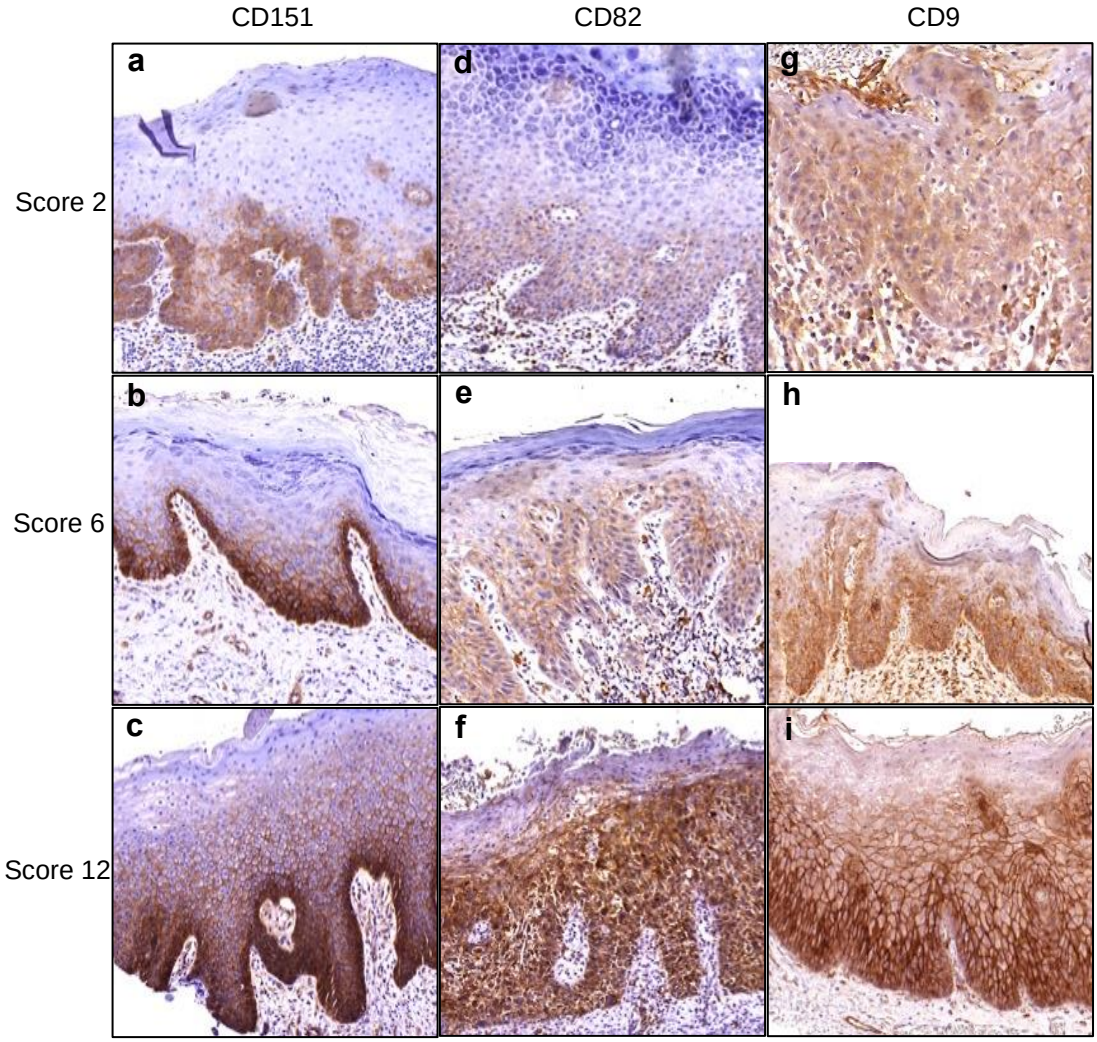


Figure 2: Representative immunohistochemistry. Tiles a-c (COX2) and d-f (EGFR) display increasing expression from scores of 2 (top row), to 6 (middle row) to 12 (bottom row). COX2 demonstrates predominantly cytoplasmic staining, while EGFR is strongly membranous. The strongest her2 staining was scored at 3/12 (i). Some cases demonstrated both cytoplasmic and membranous staining, (h) and were considered positive. Where only cytoplasmic staining occurred (g) this was considered negative and given a score of 0.

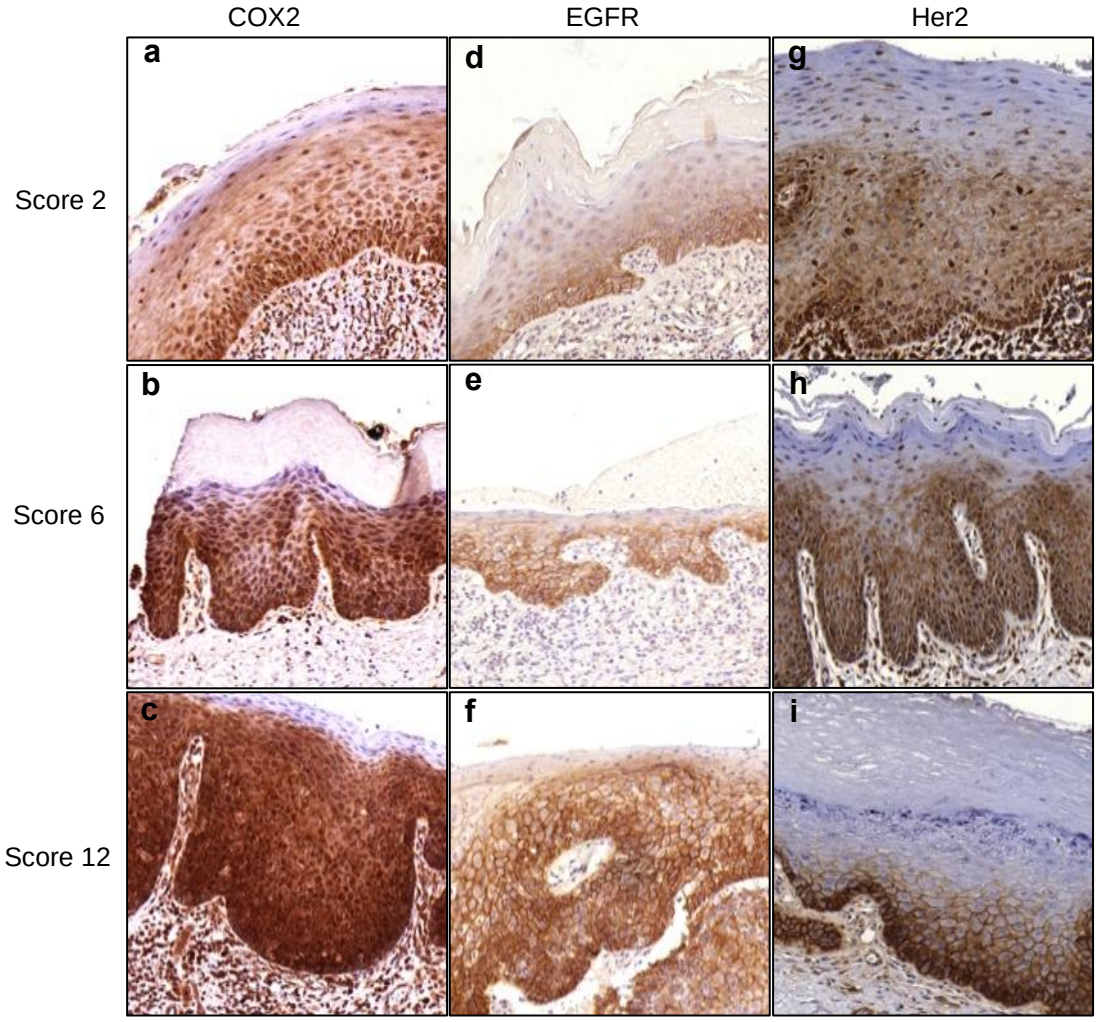
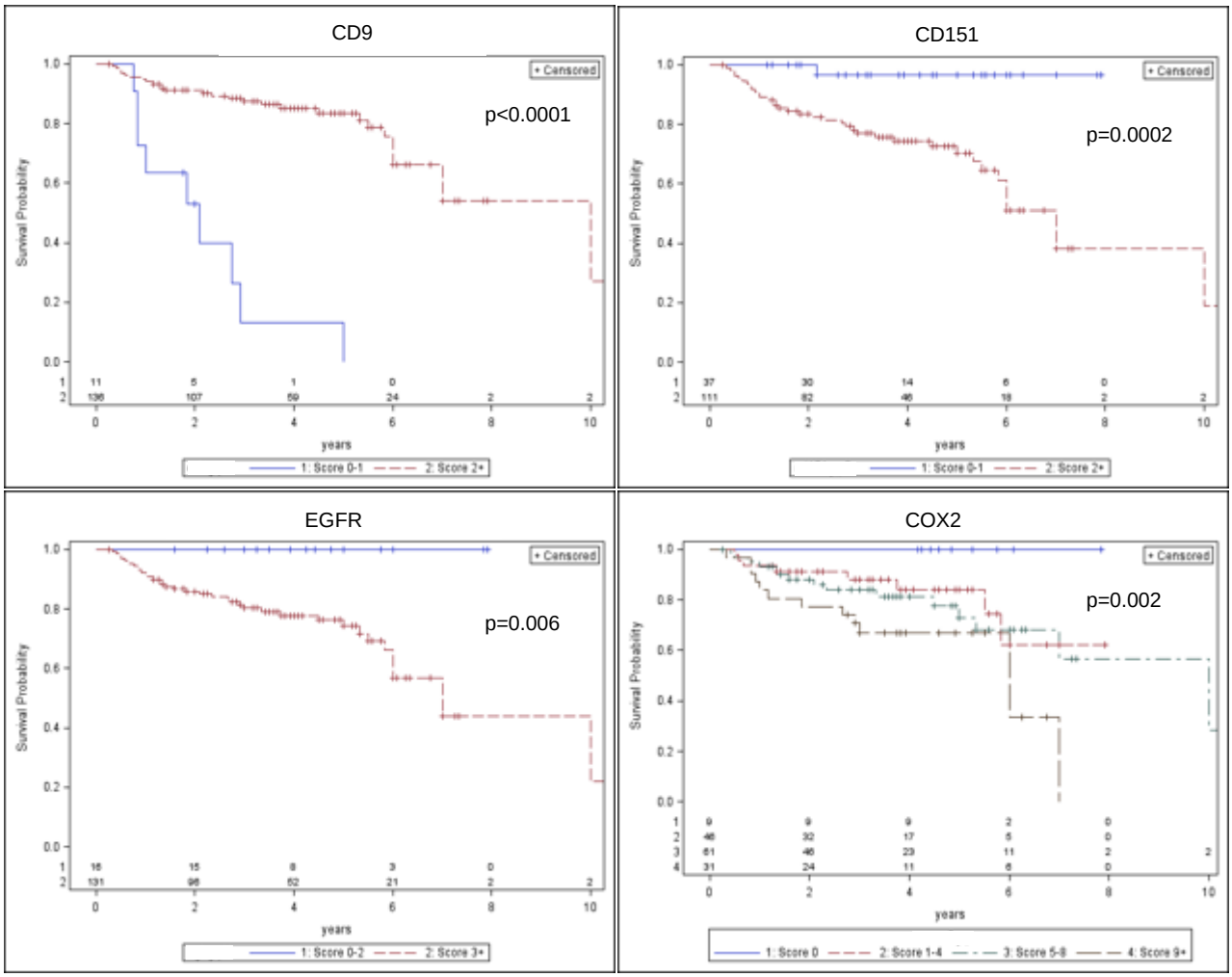


Figure 3: Oral cancer free survival utilising different ordered scoring thresholds for CD9, CD151, EGFR and COX2



Supplementary table 1: Monoclonal antibodies and optimisations used in this study

Marker	Antibody dilution	pH	Antibody clone
EGFR	1:50	6.0	Cell signalling rabbit monoclonal antibody clone: D38B1 Cell signaling technologies®, New England Biolabs (UK) Ltd, UK
COX-2	1:3250	7.8	Novocastra™ mouse monoclonal antibody clone: 4H12, Leica Biosystems Newcastle Ltd, Newcastle, UK
CD9	1:600	7.8	Novocastra™ mouse monoclonal antibody clone: 72F6 Leica Biosystems Newcastle Ltd, Newcastle, UK
CD151	1:900	7.8	Novocastra™ mouse monoclonal antibody clone: RLM30 Leica Biosystems Newcastle Ltd, Newcastle, UK
CD82	1:25	6.0	Novocastra™ mouse monoclonal antibody clone: 5B5 Leica Biosystems Newcastle Ltd, Newcastle, UK
Her2	1:50	6.0	Novocastra™ mouse monoclonal antibody clone: NCL-CB11 Leica Biosystems Newcastle Ltd, Newcastle, UK

Supplementary table 2: Agreement between raters when scoring intensity and proportion of each immunohistochemical marker

	Kappa		Intraclass Correlation Coefficient	
	Intensity	Proportion	Intensity	Proportion
COX2	0.66	0.85	0.85	0.90
CD9	0.72	0.79	0.87	0.88
CD151	0.73	0.78	0.86	0.85
CD82	0.91	0.84	0.96	0.89
EGFR	0.77	0.74	0.87	0.82
Her2	1.0	1.0	1.0	1.0

Supplementary table 3: Mean consensus immunohistochemical scores for each marker by histological grade and progression status

		Mean immunohistochemical score (standard deviation)					
OED grade	**Progressor (no. of cases)**	**COX2**	**CD9**	**CD151**	**CD82**	**EGFR**	**Her2**
Mild	No (62)	5.8 (3.4)	7.0 (3.6)	3.8 (3.1)	2.6 (2.9)	6.3 (3.6)	0.2 (0.9)
	Yes (7)	6.9 (2.5)	3.0 (3.5)	5.0 (3.1)	1.6 (1.3)	6.3 (2.9)	0.0
	Total (69)	5.9 (3.3)	6.6 (3.7)	4.0 (3.1)	2.5 (2.8)	6.3 (3.5)	0.2 (0.9)
Moderate	No (32)	5.1 (3.4)	6.9 (4.0)	3.8 (3.4)	2.6 (2.9)	6.8 (3.4)	0.2 (0.6)
	Yes (18)	7.0 (3.1)	5.5 (3.4)	5.8 (2.2)	2.8 (2.8)	7.3 (3.3)	0.3 (1.1)
	Total (50)	5.8 (3.4)	6.4 (3.9)	4.5 (3.2)	2.6 (2.9)	7.0 (3.4)	0.2 (0.8)
Severe/ CIS	No (15)	5.8 (3.1)	5.9 (3.0)	4.8 (4.3)	1.9 (2.0)	7.1 (3.8)	0.0
	Yes (13)	8.8 (3.1)	5.7 (5.0)	4.9 (3.0)	1.9 2.3)	9.8 (3.1)	0.1 (0.5)
	Total (28)	7.2 (3.4)	5.8 (4.0)	4.9 (3.7)	1.9 (2.1)	8.4 (3.7)	0.1 (0.4)
Total	No (109)	5.6 (3.3)	6.8 (3.6)	4.0 (3.3)	2.5 (2.8)	6.6 (3.5)	0.2 (0.8)
	Yes (39)	7.6 (3.1)	5.1 (4.1)	5.3 (2.6)	2.3 (2.4)	8.0 (3.4)	0.2 (0.8)

References

1. Ferlay J, Shin H-R, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010 Dec 15;127(12):2893–917.

2. Califano J, der Riet van P, Westra W, Nawroz H, Clayman G, Piantadosi S, et al. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res. 1996 Jun 1;56(11):2488–92.

3. Barnes L, Organisation WH, Cancer TIAFRO. Pathology and Genetics of Head and Neck Tumours. 1st ed. Barnes L, Eveson JW, Reichart P, Sidransky D, editors. World Health Organization; 2005.

4. Warnakulasuriya S, Reibel J, Bouquot J, Dabelsteen E. Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement. J. Oral Pathol. Med. 2008. pp. 127–33.

5. Hsue SS, Wang WC, Chen CH, Lin CC, Chen YK, Lin LM. Malignant transformation in 1458 patients with potentially malignant oral mucosal disorders: a follow-up study based in a Taiwanese hospital. J Oral Pathol Med. 2006(null) ed. 2007 Jan;36(1):25–9.

6. Silverman S, Gorsky M, Lozada F. Oral leukoplakia and malignant transformation. A follow-up study of 257 patients. Cancer. 1984 Feb 1;53(3):563–8.

7. Mehanna HM, Rattay T, Smith J, McConkey CC. Treatment and follow-up of oral dysplasia - a systematic review and meta-analysis. Head Neck. 2009 Dec;31(12):1600–9.

8. Liu W, Bao Z-X, Shi L-J, Tang G-Y, Zhou Z-T. Malignant transformation of oral epithelial dysplasia: clinicopathological risk factors and outcome analysis in a retrospective cohort of 138 cases. Histopathology. 2011 Oct;59(4):733–40.

9. Abbey LM, Kaugars GE, Gunsolley JC, Burns JC, Page DG, Svirsky JA, et al. Intraexaminer and interexaminer reliability in the diagnosis of oral epithelial dysplasia. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1995 Aug 1;80(2):188–91.

10. Fischer DJ, Epstein JB, Morton TH, Schwartz SM. Interobserver reliability in the histopathologic diagnosis of oral pre-malignant and malignant lesions. J Oral Pathol Med. 2004 Feb;33(2):65–70.

11. Karabulut A, Reibel J, Therkildsen MH, Prætorius F, Nielsen HW, Dabelsteen E. Observer variability in the histologic assessment of oral premalignant lesions. J Oral Pathol Med. 1995 May;24(5):198–200.

12. Tabor MP, Braakhuis BJM, van der Wal JE, van Diest PJ, Leemans CR, Brakenhoff RH, et al. Comparative molecular and histological grading of epithelial dysplasia of the oral cavity and the oropharynx. J Pathol. 2003

Mar;199(3):354–60.

13. Nankivell P, Mehanna H. Oral Dysplasia: Biomarkers, Treatment, and Follow-up. Curr Oncol Rep. 2011 Apr;13(2):145–52.

14. Normanno N, Bianco C, Luca A, Strizzi L, Gallo M, Mancino M, et al. Expression and prognostic significance of the EGFR in solid tumors. EGFR Signaling Networks in Cancer Therapy. 2008;:210–23.

15. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res. 1993 Aug 1;53(15):3579–84.

16. Etienne MC, Pivot X, Formento JL, Bensadoun RJ, Formento P, Dassonville O, et al. A multifactorial approach including tumoural epidermal growth factor receptor, p53, thymidylate synthase and dihydropyrimidine dehydrogenase to predict treatment outcome in head and neck cancer patients receiving 5-fluorouracil. Br J Cancer. 1999 Apr;79(11-12):1864–9.

17. Ang KK, Berkey BA, Tu X, Zhang H-Z, Katz R, Hammond EH, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. Cancer Res. 2002 Dec 15;62(24):7350–6.

18. Rautava J, Jee KJ, Miettinen PJ, Nagy B, Myllykangas S, Odell EW, et al. ERBB receptors in developing, dysplastic and malignant oral epithelia. Oral Oncol. 2008 Mar 1;44(3):227–35.

19. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. REporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer. 2005 Aug 22;93(4):387–91.

20. Nankivell PC, Williams H, Bartlett JMS, Mehanna H. Validation of tissue microarrays in oral epithelial dysplasia using a novel virtual-array technique. J Clin Pathol. 2012 Dec;65(12):1084–7.

21. Goldstein NS, Bosler D. An approach to interpreting immunohistochemical stains of adenocarcinoma in small needle core biopsy specimens: the impact of limited specimen size. American Journal of Clinical Pathology. 2007 Feb;127(2):273–81.

22. Kirkegaard T, Edwards J, Tovey S, McGlynn LM, Krishna SN, Mukherjee R, et al. Observer variation in immunohistochemical analysis of protein expression, time for a change? Histopathology. 2006 Jun;48(7):787–94.

23. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977(null) ed. 1977 Mar;33(1):159–74.

24. Carrasco JL, Jover L. Estimating the generalized concordance correlation coefficient through variance components. Biometrics. 2003 Dec;59(4):849–58.

25. Zöller M. Tetraspanins: push and pull in suppressing and promoting metastasis. Nat Rev Cancer. 2009;9(1):40–55.

26. Murayama Y, Shinomura Y, Oritani K, Miyagawa J-I, Yoshida H, Nishida M, et al. The tetraspanin CD9 modulates epidermal growth factor receptor signaling in cancer cells. J Cell Physiol. 2008 Jul 1;216(1):135–43.

27. Berditchevski F. Complexes of tetraspanins with integrins: more than meets the eye. J Cell Sci. 2001 Dec 1;114(Pt 23):4143–51.

28. Little KD, Hemler ME, Stipp CS. Dynamic regulation of a GPCR-tetraspanin-G protein complex on intact cells: central role of CD81 in facilitating GPR56-Galpha q/11 association. Mol. Biol. Cell. 2004 May;15(5):2375–87.

29. Buim MEC, Lourenço SV, Carvalho KC, Cardim R, Pereira C, Carvalho AL, et al. Downregulation of CD9 protein expression is associated with aggressive behavior of oral squamous cell carcinoma. Oral Oncol. 2010 Mar 1;46(3):166–71.

30. Erovic BM, Pammer J, Hollemann D, Woegerbauer M, Geleff S, Fischer MB, et al. Motility-related protein-1/CD9 expression in head and neck squamous cell carcinoma. Head Neck. 2003 Oct 1;25(10):848–57.

31. Kusukawa J, Ryu F, Kameyama T, Mekada E. Reduced expression of CD9 in oral squamous cell carcinoma: CD9 expression inversely related to high prevalence of lymph node metastasis. J Oral Pathol Med. 2001 Feb 1;30(2):73–9.

32. Shi W, Fan H, Shum L, Derynck R. The tetraspanin CD9 associates with transmembrane TGF-alpha and regulates TGF-alpha-induced EGF receptor activation and cell proliferation. J Cell Biol. 2000 Feb 7;148(3):591–602.

33. Imhof I, Gasper WJ, Derynck R. Association of tetraspanin CD9 with transmembrane TGF{alpha} confers alterations in cell-surface presentation of TGF{alpha} and cytoskeletal organization. J Cell Sci. 2008 Jul 1;121(Pt 13):2265–74.

34. Hirano C, Nagata M, Noman AA, Kitamura N, Ohnishi M, Ohyama T, et al. Tetraspanin gene expression levels as potential biomarkers for malignancy of gingival squamous cell carcinoma. Int J Cancer. 2009 Jun 15;124(12):2911–6.

35. Romanska HM, Potemski P, Collins SI, Williams H, Parmar S, Berditchevski F. Loss of CD151/Tspan24 from the complex with integrin Î±3Î²1 in invasive front of the tumour is a negative predictor of disease-free survival in oral squamous cell carcinoma. Oral Oncol. Elsevier Ltd; 2012 Oct 22;:1–6.

36. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology. 1994(null) ed. 1994 Oct;107(4):1183–8.

37. Shirahama T. Cyclooxygenase-2 expression is up-regulated in transitional cell carcinoma and its preneoplastic lesions in the human urinary bladder. Clin Cancer Res. 2000 Jun;6(6):2424–30.

38. Sung JJ, Leung WK, Go MY, To KF, Cheng AS, Ng EK, et al. Cyclooxygenase-2 expression in Helicobacter pylori-associated premalignant and malignant gastric lesions. Am J Pathol. 2000(null) ed. 2000

Sep;157(3):729–35.

39. Shibata M, Kodani I, Osaki M, Araki K, Adachi H, Ryoke K, et al. Cyclo-oxygenase-1 and -2 expression in human oral mucosa, dysplasias and squamous cell carcinomas and their pathological significance. Oral Oncol. 2005 Mar 1;41(3):304–12.

40. Nathan CA, Leskov IL, Lin M, Abreo FW, Shi R, Hartman GH, et al. COX-2 expression in dysplasia of the head and neck: correlation with eIF4E. Cancer. 2001 Oct 1;92(7):1888–95.

41. Banerjee AG, Gopalakrishnan VK, Bhattacharya I, Vishwanatha JK. Deregulated cyclooxygenase-2 expression in oral premalignant tissues. Mol Cancer Ther. 2002 Dec 1;1(14):1265–71.

42. Taoudi Benchekroun M, Saintigny P, Thomas SM, El-Naggar AK, Papadimitrakopoulou V, Ren H, et al. Epidermal growth factor receptor expression and gene copy number in the risk of oral cancer. Cancer Prevention Research. 2010 Jul 1;3(7):800–9.

43. Napier SS, Speight PM. Natural history of potentially malignant oral lesions and conditions: an overview of the literature. J Oral Pathol Med. 2008 Jan;37(1):1–10.

44. Holmstrup P, Vedtofte P, Reibel J, Stoltze K. Long-term treatment outcome of oral premalignant lesions. Oral Oncol. 2006 May;42(5):461–74.