Nuclear F-actin Cytology in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma

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APPENDIX

Study Participants

The data used in this study originated from a 1053-patient study which aimed to assess the diagnostic accuracy of this cytology-on-a-chip system relative to scalpel biopsy and histopathology (Abram et al. 2016; Speight et al. 2015). The study was conducted through consecutive sampling at 4 sites: (1) the University of Texas Health Science Center (UTHSC) at Houston, (2) the University of Texas Health Science Center at San Antonio, (3) Bluestone Center for Clinical Research at New York University, and (4) Sheffield Teaching Hospitals National Health Service Foundation Trust, Sheffield, U.K. The study population consisted of three groups of subjects. Group 1: patients who present to any of the four clinic sites for a biopsy of a potentially malignant lesion. A potentially malignant oral lesion is defined as a) any unexplained ulcer of >3 weeks duration, b) any unexplained mass of >3 weeks duration, or c) any unexplained white or red patch that cannot be definitively diagnosed as benign by a non-expert clinician. Group 2: patients who have already been diagnosed with a malignant oral lesion by incisional scalpel biopsy and are presenting to any of the four clinic sites for follow-up care. The purpose of this group is to increase the number of malignant cases in the study population. The incisional scalpel biopsy must have been performed within 45 days of the study enrollment visit and the remaining lesion must be large enough to allow for brushing the lesion at least 5 mm away from the scalpel biopsy site. In addition, the area of the lesion available for brushing must be at least 5 mm in diameter. Group 3: patients with normal appearing oral mucosa who present to either of the clinic sites in Texas for oral health problems other than oral lesions. Subjects from three groups consented to enrollment of the original study. Of those 1053 subjects, 54 withdrew from the study and, thus, cytology measurements were not recorded. Of the 999 remaining enrolled subjects for the original study, 513 were not eligible for the current study due to the following reasons: partial cytology measurements (n=21); inadequate number of cells in the sample (n=47); samples were used for other purposes (n=2); samples were lost due to shipping errors and/or freezer failures

(n=44); cytology results were not measured due to funding constraints or missing (n=399). All the remaining 486 subjects with complete cytology data were included in the current analysis.

Clinical Protocol

The clinical protocol for this study was published previously (Speight et al. 2015) and is summarized as follows. Patients in Group 1 underwent brush sampling of the oral lesion and a brush sampling of the contralateral, clinically normal mucosa. The brush cytology sample was taken immediately before the same lesion underwent a scalpel biopsy. Patients in Group 2 underwent brush biopsy of the known cancerous lesion, as well as the contralateral, clinically normal mucosa. For healthy volunteers in Group 3, a brush biopsy of normal appearing tissue on the lateral or ventral surface of the tongue and a brush biopsy of normal appearing tissue on the left or right buccal mucosa were taken. Brush biopsy samples were taken using a soft Rovers Orcellex oral cytology brush (Rovers Medical Devices B.V., Oss, The Netherlands) according to the manufacturer's instructions. The brush was applied directly to the lesion or control oral mucosa using moderate pressure and rotated 360 degrees approximately 10-15 times in the same direction to obtain the cytologic sample. This procedure was standardized across all sites. Histopathological specimens were examined and coded as follows: (1) normal/no lesion, (2) benign, (3) mild dysplasia, (4) moderate dysplasia, (5) severe dysplasia or carcinoma *in situ*, and (6) malignant/OSCC. See **Appendix Table 1** for definition of diagnostic outcomes.

Cytology-on-a-Chip Protocol

The following methods have been published previously (Abram et al. 2016) and are summarized here. Immediately after brush cytology samples were collected, cells were harvested by vortexing the brush head in minimum essential medium (MEM) culture media, followed by a PBS wash, re-suspension in FBS containing 10% of the cryo-preservative dimethyl-sulfoxide (DMSO), frozen, and stored in a -80°C freezer. Prior to processing on the device, patient samples were thawed rapidly in a 37°C water bath, washed with PBS, and fixed for one hour in 0.5% formaldehyde prepared fresh from a 16% stock solution (Polysciences, Warrington, PA, #18814-20). After fixation, cells were washed twice in PBS, re-suspended in 150µL 0.1% PBS with 0.1% BSA (PBSA), and stored at 4°C until ready to process. Before sample delivery, the cell suspension was diluted in a 20% glycerol/0.1% PBSA solution to improve cell distribution across the membrane and to reduce cell clumping. Using a custom built manifold connecting external fluidic tubing to the inlet and outlet ports of the microfluidic device, the assembly was positioned on a

robotically controlled microscope stage (ProScan II, Prior Scientific, Cambridge, UK) and connected to a peristaltic pump (SciQ 400, Watson Marlow, Wilmington, MA) and manually controlled 6-position injector valve (Vici, Valco Instruments, Houston, TX). Antibody stock solutions were vortexed for 30 seconds and centrifuged at 14,000rpm for five minutes before preparing working dilutions to avoid precipitates. All assays contained Phalloidin and DAPI in the secondary antibody cocktail, but each was specific for a single molecular biomarker primary-secondary antibody pair. Working dilutions of antibodies were prepared in 0.1% PBSA with 0.1% Tween-20 (EMD Millipore, Billerica, MA, # 655206). Primary monoclonal antibodies were raised from either mouse (EGFR [Life Technologies, Carlsbad, CA, #MS-378-P, 10μg/mL]), rabbit (αvβ6 [Abcam, Cambridge, MA, #Ab124968, 6μg/mL], Ki67 [Abcam #Ab15580, 29μg/mL], and MCM2 [Abcam #Ab108935, 10μg/mL]), or goat (CD-147 [EMMPRIN] [R&D Systems, Minneapolis, MN, #AF972, 20μg/mL]. AlexaFluor-488 conjugated secondary antibodies were specific for F $(ab')_2$ fragments of mouse IgG (Life Technologies #A11017, 20μg/mL for EFGR), rabbit IgG (Life Technologies #A11070, 50μg/mL for αvβ6, 64μg/mL for Ki67, and 23.5μg/mL for MCM2), or goat IgG (Life Technologies #A11078, 40μg/mL for CD147). A working concentration of 0.33μM was used for Phalloidin-AlexaFluor-647 (Life Technologies #A22287) and 5μM for DAPI (Life Technologies #D3571).

In summary, the lab-on-a-chip sample processing was comprised of the following steps: 1) the device was primed with PBS at a flow rate of 735μL/min for two minutes, 2) the cell suspension in 20% glycerol/0.1% PBSA was delivered at 1.5mL/min for two minutes, 3) cells were washed with PBS at 1mL/min for 2.5min, 4) the primary antibody solution was delivered through a 0.2μm PVDF syringe filter at 250μL/min for 2.5min, 5) a wash step similar to step 3 was performed, 6) the secondary antibody solution was delivered under the same conditions as step 4, 7) a final wash step was performed, and 8) automated image capture was performed.

Sample Digitization

More complete details on cytology sample digitization and a complete list of intensity and morphological parameters can be found in our previous publication (Abram et al. 2016). Images were recorded with a motorized reflected fluorescence microscope (Olympus BX-RFAA) equipped with a CCD camera (Hamamatsu ORCA-03G) through a 10x objective (10x/0.30NA UPlanFl, Olympus). A total of 25 unique fields of view repeated for three different z-focal planes were automatically captured across a $20mm^2$ area using a robotic x-y-z microscope stage. Due to the complex three-dimensional morphology of oral squamous cells, multiple z-focal planes were

captured and subsequently combined into a single, enhanced depth-of-field image to simplify the multi-spectral detection of the three fluorescent labels using ImageJ “stack focuser”. Combinations of custom macros and the open-source image analysis tools ImageJ (Schneider et al. 2012) and CellProfiler (Carpenter et al. 2006) were developed to automatically detect individual cells and define their nuclear and cytoplasmic boundaries as individual regions of interest (ROI). These ROIs were used to obtain intensity measurements associated with the three spectral channels and were used to define morphometric parameters. The DAPI and Phalloidin molecular labels served primarily to assist in the automated segmentation of individual nuclei and cytoplasm, respectively.

Cell Identification Model Training and Validation

A cell type classification model was explored for its ability to discriminate and quantitate the frequency and distributions of four cell types: differentiated squamous epithelial (DSE) cells, small round (SR) cells, mononuclear leukocytes (ML), and lone nuclei (LN). An additional model further classified DSE cells according to nuclear phenotypes for cells with (NA+) and without (NA-) nuclear F-actin (i.e., cells with or without F-actin localized in or around the nucleus). Both cellular and nuclear algorithms were trained on a subset of 144 cellular/nuclear features from cytology, including morphological and biomarker intensity-based measurements. A training set was manually compiled by randomly selecting and labeling cells, resulting in approximately 100-200 single-cell objects for each of the cell types. All features were log-normalized and standardized for zero mean and unit variance. Principal component analysis was performed on the training set, and scatterplots of the principal components were generated to visualize the internal data structure and variance. A *k*-nearest neighbors (*k*-NN) classifier was trained on the standardized features using 10-fold cross-validation and configured to find the nearest seven neighbors in feature space (Euclidean distance). Cross-validated predicted responses by the *k*-NN classifier were recorded, and accuracy was reported for the overall cross-validation set and individually for each of the cell types. *k*-NN model responses with four or less out of seven similar neighbors were labelled “unknown” type, and cross-validated accuracy was reported for the overall training set after accounting for unknown object types. The classification models were retrained on the entire training dataset, and this final model was applied to the study population and averaged across each of the six molecular biomarker assays. Plots were generated to show the distributions of cell phenotypes across diagnostic categories as follows: 121 normal/non-neoplastic, 241 benign, 59 dysplasia, and 65 malignant. Median values of cell phenotypes were compared for all

lesion determinations using a two-sided Wilcoxon rank sum test at a significance level of $p = 0.05$. Cell phenotype frequencies and distributions for each subject were retained for use in clinical algorithm development.

Lasso Logistic Regression Models

The analysis of dichotomous outcomes with mutually exclusive levels is common in clinical diagnostics, and logistic regression is regarded as the standard method of analysis for these situations attributed to its probabilistic interpretation and ability to function as a dichotomous classifier. Clinical data are often challenged by high-dimensionality and highly correlated predictors that may generate model coefficients with high variance. For these situations, a size penalty as implemented by the lasso technique may be applied to shrink the effect sizes and reduce coefficient variability. Additionally, the lasso technique performs automatic parameter selection by eliminating predictors with less importance. In high-dimensional data sets, reducing the set of predictors often leads to better prediction performance and generalizability and has shown improvements over manual stepwise selection methods. This lasso logistic regression model is suited to our platform because it is inherently more intuitive than previous methods which consider hundreds of measurements from cytology that are difficult to interpret. A lasso logistic regression approach was used to prevent overfitting, reduce coefficient variability, and retain a sparse model with improved generalizability and interpretability. Diagnostic accuracy (area under the curve [AUC], sensitivity, and specificity) for several models was determined between various histopathology gradings with case *vs* non-case, as indicated by "|". Only subjects with evaluable data for all biomarker measurements and oral potentially malignant disorders (OPMD) were considered. The results from six molecular biomarker assays on the cytology-on-a-chip system were pooled to obtain final estimates. Non-zero lasso logistic regression coefficients were retained for the following predictors: NA+, NA-, SR, ML, age, sex, smoking pack years, lesion area, lichen planus, and lesion color. AUC, sensitivity, and specificity were reported as mean and 95% confidence interval values for the cross-validated test set.

Literature Review

We reviewed prior diagnostic studies on PubMed using the search terms "(nuclear OR nucleus) F-actin AND cancer" using the narrow filter. The search returned 17 studies with only one study investigating nuclear F-actin in cell lines (Hemstreet et al. 1996). There were no previous studies that evaluated nuclear F-actin as a clinical cancer diagnostic biomarker. We also reviewed the

literature for previous studies of oral cancer adjuncts (Huber 2018; Lingen et al. 2017; Rashid and Warnakulasuriya 2015). Many adjunct study designs were biased. For example, studies only performed matched gold-standard histopathology on a subset of subjects with a higher index of suspicion for malignancy, effectively ignoring lesions with a lower index of suspicion which are more regularly encountered in primary care settings (Poate et al. 2004; Sciubba 1999). One prominent adjunct frequently returned an ambiguous "atypical" result (Svirsky et al. 2002). Another study only evaluated control subjects without lesions and oral cancer subjects, leading to overly optimistic results by excluding subjects with dysplasia (Pereira et al. 2016). Further, most cytological tests were conducted at remote laboratories, resulting in significant delays between sample collection and test results.

Figures and Tables

Appendix Figure 1. The point-of-care (POC) oral cytology tool allows for the cellular analysis of minimally invasive brush biopsy samples. The cell suspension collected in this manner allows for the simultaneous quantification of cell morphometric data and expression of molecular biomarkers of malignant potential in an automated manner using refined image analysis algorithms based on pattern recognition techniques and advanced statistical methods. This novel approach turns around biopsy results in a matter of minutes as compared to days for traditional pathology methods, thereby making it amenable to POC settings. The POC testing is expected to have tremendous implications in the rapid management of patient disease by enabling dental practitioners and primary care physicians to circumvent the need for multiple referrals and consultations before obtaining assessment of molecular risk of OPMD.

Appendix Table 1. Definitions of diagnostic outcomes from histopathology

Diagnosis	**Histopathologic criteria**
Non-neoplastic / normal	Surface stratified squamous epithelium has normal thickness without hyperplasia or hyperkeratinization. The underlying lamina propria is devoid of chronic inflammatory cell infiltrate.
Benign	Surface stratified squamous epithelium may reveal hyperkeratosis and hyperplasia but without cellular atypia and disordered maturation (dysplasia). The underlying lamina propria may exhibit chronic inflammatory cell infiltrate: chronic mucositis. This category may encompass a range of benign lesions including benign hyperkeratosis and lichen planus.
Mild dysplasia	Surface stratified squamous epithelium reveals cellular atypia and disordered maturation (dysplasia) limited to the basal and parabasal layers or verruciform epithelial hyperplasia and hyperkeratosis with mild degree of atypical architecture.
Moderate dysplasia	Surface stratified squamous epithelium reveals cellular atypia and disordered maturation (dysplasia) extending from the basal layer to the mid portion of the spinous layer or verruciform epithelial hyperplasia and hyperkeratosis with moderate degree of atypical architecture.
Severe dysplasia	Surface stratified squamous epithelium reveals cellular atypia and disordered maturation (dysplasia) extending from the basal layer to a level above the midpoint of the epithelium or verruciform epithelial hyperplasia and hyperkeratosis with severe degree of atypical architecture.
Carcinoma in situ	Surface stratified squamous epithelium reveals cellular atypia and disordered maturation (dysplasia) involving the entire thickness of the epithelium.
Malignant	Islands and cords of malignant squamous epithelial cells arise from dysplastic surface epithelium and invade into the lamina propria.

Appendix Table 2. Odds ratios (95% confidence intervals) from univariate and multivariate logistic regression for early disease late disease models. The following predictors were log_{10} transformed: NA+, NA-, SR, and ML cells, lesion area, pack years. Sex corresponds to male (i.e., male=1, female=0). Age is in 10-year increments. Lichen planus is the clinical impression of lichen planus.

	Early Disease (2 | 3,4,5,6)				Late Disease (2,3,4 | 5,6)			
	Univariate OR	p	Multi-variate OR	p	Univariate OR	p	Multi-variate OR	p
NA+ cells	4.79 (2.45–9.74)	< 0.001	2.97 (1.22–7.41)	0.018	4.09 (1.86–9.44)	< 0.001	2.85 (0.76–11.16)	0.124
NA- cells	0.01 (0.002–0.04)	< 0.001	0.22 (0.03–1.16)	0.087	0.002 (0.0004–0.01)	< 0.001	0.28 (0.03–2.09)	0.231
SR cells	8.84 (4.94–16.54)	< 0.001	2.30 (0.97–5.58)	0.060	28.48 (12.48–71.69)	< 0.001	4.68 (1.34–17.50)	0.018
ML cells	4.45 (2.86–7.11)	< 0.001	1.65 (0.75–3.67)	0.215	13.77 (7.50–26.90)	< 0.001	4.03 (1.33–12.87)	0.015
Sex	1.76 (1.14–2.74)	0.011	1.58 (0.90–2.81)	0.112	2.65 (1.56–4.61)	< 0.001	4.23 (1.92–9.81)	< 0.001
Age	1.18 (1.00–1.39)	0.048	1.24 (1.02–1.51)	0.037	1.20 (0.99–1.45)	0.065	1.36 (1.04–1.81)	0.026
Lesion area	1.48 (1.23–1.84)	< 0.001	1.11 (0.90–1.39)	0.340	2.31 (1.61–3.52)	< 0.001	1.21 (0.89–1.75)	0.258
Lesion color	-	-	-	-	-	-	-	-
White	ref.	-	ref.	-	ref.	-	ref.	-
Red	1.14 (0.56–2.27)	0.703	0.63 (0.26–1.47)	0.294	5.14 (1.95–14.59)	0.001	2.81 (0.77–10.63)	0.119
Red and white	2.10 (1.30–3.44)	0.003	1.59 (0.84–3.03)	0.153	9.02 (4.20–22.43)	< 0.001	6.95 (2.58–21.21)	< 0.001
Lichen planus	0.13 (0.06–0.25)	< 0.001	0.16 (0.07–0.36)	< 0.001	0.08 (0.02–0.23)	< 0.001	0.12 (0.03–0.41)	0.002
Pack years	1.64 (1.20–2.23)	0.002	1.40 (0.94–2.09)	0.095	1.60 (1.12–2.29)	0.010	1.35 (0.78–2.33)	0.285

Appendix Table 3. Likelihood ratios. Positive and negative likelihood ratios (95% CI) for clinical and cytological predictors in distinguishing early disease and late disease. LR+ is the positive likelihood ratio. LR- is the negative likelihood ratio. Lesion area positive was defined as patients with lesion area > 200 mm^2. Tobacco positive was defined as patients with smoking pack years > 2.5 pack years. Alcohol use positive was defined as patients having 12 or more drinks in the past year. Alcohol and tobacco positive was denoted patients with both tobacco and alcohol use.

	Early Disease (2 | 3,4,5,6)		**Late Disease (2,3,4 | 5,6)**	
Predictor	**LR +**	**LR -**	**LR +**	**LR -**
Multivariate Model	2.70 (2.33–3.13)	0.38 (0.33–0.45)	5.59 (4.77–6.56)	0.16 (0.14–0.19)
NA- Cells	0.52 (0.38–0.72)	2.62 (1.90–3.59)	0.27 (0.16–0.45)	3.80 (2.29–6.30)
NA+ Cells	1.63 (1.38–1.94)	0.50 (0.42–0.60)	1.53 (1.20–1.93)	0.55 (0.43–0.70)
SR Cells	2.03 (1.74–2.38)	0.41 (0.35–0.48)	3.30 (2.71–4.00)	0.30 (0.25–0.36)
ML Cells	1.85 (1.56–2.20)	0.52 (0.44–0.61)	3.16 (2.59–3.84)	0.30 (0.25–0.37)
Lesion Color				
Red and White	1.43 (1.18–1.74)	0.71 (0.58–0.86)	1.88 (1.51–2.35)	0.42 (0.34–0.53)
White	0.68 (0.50–0.92)	1.24 (0.92–1.67)	0.21 (0.10–0.43)	1.66 (0.80–3.43)
Red	0.78 (0.51–1.19)	1.04 (0.69–1.59)	1.07 (0.65–1.78)	0.99 (0.60–1.64)
Lesion Area	1.44 (1.21–1.72)	0.53 (0.45–0.63)	1.55 (1.24–1.93)	0.38 (0.30–0.47)
Tobacco Use	1.44 (1.18–1.76)	0.74 (0.61–0.91)	1.41 (1.08–1.85)	0.73 (0.56–0.96)
Alcohol Use	0.87 (0.68–1.10)	1.25 (0.99–1.58)	0.82 (0.60–1.10)	1.34 (0.99–1.81)
Alcohol and Tobacco Use	1.35 (1.06–1.71)	0.87 (0.69–1.11)	1.22 (0.86–1.71)	0.91 (0.65–1.28)

Appendix Table 4. Predictor definitions.

Abbreviation	Description
NA-	percentage of differentiated squamous epithelial cells without nuclear F-actin (i.e., number of NA- cells divided by total cells, where ‘total cells’ is the number of cells of type NA-, NA+, SR, and ML)
NA+	percentage of differentiated squamous cells with nuclear F-actin (i.e., number of NA+ cells divided by total cells, where ‘total cells’ is the number of cells of type NA-, NA+, SR, and ML)
SR	percentage of small round cells (i.e., number of small round cells divided by total cells, where ‘total cells’ is the number of cells of type NA-, NA+, SR, and ML)
ML	percentage of leukocytes (i.e., number of leukocytes divided by total cells, where ‘total cells’ is the number of cells of type NA-, NA+, SR, and ML)
Age	age in years
Sex	male = 1, female = 0
Pack years	average cigarettes smoked per day times years smoked divided by 20
Lesion area	lesion area in mm^2 calculated using ellipse formula
Lichen planus	clinical impression of lichen planus--a binary measure completed by clinician at time of brush cytology sample collection indicating the presence (“1”) or absence (“0”) of the clinical features of lichen planus
Lesion color	variable indicating lesion color; white = 0, red = 1, red and white = 2

Appendix Table 5. Confusion matrices for OED spectrum diagnostic models for early disease (2 | 3,4,5,6), mild | moderate dysplasia (2,3 | 4,5,6), WHO binary classification (2,3,4L | 4H,5,6), late disease (2,3,4 | 5,6), benign vs malignant (2 vs 6), and healthy control vs malignant (1 vs 6) models. See **Table 2** in the main text for the diagnostic performance characteristics.

Early Disease - 2 | 3,4,5,6	Actual		
	Case		Non-case
Predicted	Case	89	62
	Non-case	35	179

2,3 | 4,5,6	Actual		
	Case		Non-case
Predicted	Case	68	44
	Non-case	18	235

WHO binary classification - 2,3,4L | 4H,5,6	Actual		
	Case		Non-case
Predicted	Case	68	51
	Non-case	17	229

Late Disease - 2,3,4 | 5,6	Actual		
	Case		Non-case
Predicted	Case	61	32
	Non-case	19	259

2 *vs* 6	Actual		
	Case		Non-case
Predicted	Case	58	23
	Non-case	7	217

1 *vs* 6	Actual		
	Case		Non-case
Predicted	Case	60	13
	Non-case	5	108

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