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'Cytology-on-a-chip' based sensors for monitoring of potentially malignant oral lesions

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ABSTRACT

Despite significant advances in surgical procedures and treatment, long-term prognosis for patients with oral cancer remains poor, with survival rates among the lowest of major cancers. Better methods are desperately needed to identify potential malignancies early when treatments are more effective.

Objective: To develop robust classification models from cytology-on-a-chip measurements that mirror diagnostic performance of gold standard approach involving tissue biopsy.

Materials and methods: Measurements were recorded from 714 prospectively recruited patients with suspicious lesions across 6 diagnostic categories (each confirmed by tissue biopsy -histopathology) using a powerful new 'cytology-on-a-chip' approach capable of executing high content analysis at a single cell level. Over 200 cellular features related to biomarker expression, nuclear parameters and cellular morphology were recorded per cell. By cataloging an average of 2000 cells per patient, these efforts resulted in nearly 13 million indexed objects.

Results: Binary "low-risk"/"high-risk" models yielded AUC values of 0.88 and 0.84 for training and validation models, respectively, with an accompanying difference in sensitivity + specificity of 6.2%. In terms of accuracy, this model accurately predicted the correct diagnosis approximately 70% of the time,

Abbreviations: PMOL, potentially malignant oral lesion(s); OED, oral epithelial dysplasia; OSCC, oral squamous cell carcinoma; HCA, high content analysis; AUC, area under the (receiver-operator characteristic) curve; NC ratio, nuclear-cytoplasmic area ratio.

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compared to the 69% initial agreement rate of the pool of expert pathologists. Key parameters identified in these models included cell circularity, Ki67 and EGFR expression, nuclear-cytoplasmic ratio, nuclear area, and cell area.

Conclusions: This chip-based approach yields objective data that can be leveraged for diagnosis and management of patients with PMOL as well as uncovering new molecular-level insights behind cytological differences across the OED spectrum.

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Introduction

It is estimated that 1–2% of adults in the United States present with a worrisome white or red patch or other potentially malignant oral lesion (PMOL) during a routine oral examination [1]. However, the vast majority of these lesions are benign, and only 1–2% will undergo progression into oral squamous cell carcinoma (OSCC) [2,3]. Oral healthcare providers are often the first line of defense in the early detection of oral cancer and are faced with the challenge of recognizing PMOL and deciding which patients to refer for tissue biopsy. This often difficult decision is becoming increasingly burdensome, confounded by the desire to reduce unwarranted biopsies and patient discomfort with the changing landscape of litigation directed at dentists for failing to refer patients [4]. To make matters worse, in PMOL, the histopathological diagnosis of oral epithelial dysplasia (OED) is not necessarily predictive of future malignant transformation, creating a demand for more sophisticated early risk assessment tools [5].

Though decades of studies aimed at developing non-invasive, adjunctive aids for monitoring oral lesions have not garnered widespread adoption [6], a new era of rapid, quantitative, and automated tools are beginning to pave the way towards data-driven clinical decision making. Recent advances in a diverse consortium of fields from automated sample processing to statistical machine learning, microfluidic-based single-cell analysis [7–9], and high content analysis/screening [10–14] have fueled a renewed interest in quantitative oral cytology. While offering strong potential for enhanced clinical insight relative to early disease detection, the "-omics" data derived from these new capabilities has a tendency to yield putative clinical models that do not perform as well in later validation studies. A recent review of 28 studies involving molecular classifiers by Castaldi et al. [15] found that the majority selected cross-validation practices that overestimated model performance [by ~17% (median) in terms of specificity].

To address these challenges, our team of bioengineers, oral medicine clinicians, oral and maxillofacial pathologists, and cancer biologists, designed and executed a prospective, international clinical study with the ultimate goal of equipping dental practitioners with simple, automated, quantitative risk assessment tools to assist in making difficult biopsy referral decisions. Here we describe this single-cell cytology-on-a-chip approach in the context of developing a multi-parameter image-based clinical decision tool.

The general method for collection and processing of cells within a microfluidic structure was demonstrated previously in the context of a small pilot study involving 52 patients using the single biomarker, EGFR, in order to differentiate between normal mucosa and OSCC [16]. The pilot yielded preliminary logistic regression models with sensitivity and specificity of 97% and 93% respectively, alongside area under the receiver operating characteristic (ROC) curve (AUC) equal to 0.94. These promising results paved the way for this more comprehensive follow-up with a Phase 2/3 clinical characterization-association study.

Many previous quantitative cytology studies have confirmed that measureable differences exist between the extreme phenotypes of normal mucosa/benign lesions and malignant lesions [17–19] such as the increased proportion of small, highly circular cells that resemble more primitive stem cells. Though these differences can be surmised by visual examination by experts and non-experts alike, they miss the more subtle spectrum of changes seen in PMOL representing the different grades of OED described by histopathologists [20]. An attempt to leverage the subtle measureable differences among OED cytology samples in order to develop objective classification models for PMOL has not existed until now.

Materials and methods

Study population

This study was approved by the Institutional Review Boards of all participating institutions. Informed consent was obtained for all participants in the trial after the possible consequences of the procedures were explained. The study design and clinical protocol for this study have been reported previously in detail [21]. Briefly, lesion samples from a total of 714 patients were measured, of which 85 were previously diagnosed malignant cases. The slight enrichment of the malignant cases allowed for a more substantial model development process with more equivalent class sizes. All other patients were prospectively recruited based on their exhibiting PMOL for which scalpel biopsy was a necessary part of the standard clinical practice. Histopathological assessment of biopsy specimens was used to place lesions in one of six categories of oral epithelial dysplasia (OED). These comprised 348 benign, 49 mild dysplasia, 18 moderate dysplasia, 12 severe dysplasia, 2 carcinoma in situ (CIS), and 135 malignant lesions in addition to 150 healthy controls. To obtain greater confidence in the gold-standard pathological diagnoses, which have been notoriously unreliable [22,23], a 3-stage adjudication and consensus review process was performed which achieved 100% consensus agreement from an initial 69.9% agreement rate between any two pathologists across all patient biopsy specimens [21].

A summary of the major variables analyzed is provided in the Supplementary Methods and Supplementary Tables 1–3. The molecular biomarkers EGFR, αvβ6, CD147, β-catenin, MCM2, and Ki67 were selected based on their capacity, through prior immunohistochemistry studies, to distinguish stages of disease progression towards OSCC for patients with PMOL. Due to the flexible design of this chip-based approach, future studies may easily adapt these protocols as new prognostic molecular markers are identified.

Cytology-on-a-chip sample processing

Specific details for cytology-on-a-chip sample processing can be found in the Supplementary Methods and are adapted from the indirect-immunoassay protocol described in Weigum et al. [16]. In summary, sample processing comprised the following steps: (1) the microfluidic device was primed with PBS at a flow rate of

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