A paradigm shift in the prevention and diagnosis of oral squamous cell carcinoma

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is a widespread disease with only 50%–60% 5-year survival. Individuals with potentially malignant precursor lesions are at high risk.

Methods: Survival could be increased by effective, affordable, and simple screening methods, along with a shift from incisional tissue biopsies to non-invasive brush biopsies for cytology diagnosis, which are easy to perform in primary care. Along with the explainable, fast, and objective artificial intelligence characterisation of cells through deep learning, an easy-to-use, rapid, and cost-effective methodology for finding high-risk lesions is achievable. The collection of cytology samples offers the further opportunity of explorative genomic analysis.

Results: Our prospective multicentre study of patients with leukoplakia yields a vast number of oral keratinocytes. In addition to cytopathological analysis, whole-slide imaging and the training of deep neural networks, samples are analysed according to a single-cell RNA sequencing protocol, enabling mapping of the entire keratinocyte transcriptome. Mapping the changes in the genetic profile, based on mRNA expression, facilitates the identification of biomarkers that predict cancer transformation.

Conclusion: This position paper highlights non-invasive methods for identifying patients with oral mucosal lesions at risk of malignant transformation. Reliable non-invasive methods for screening at-risk individuals bring the early diagnosis of OSCC within reach. The use of biomarkers to decide on a targeted therapy is most likely to improve the outcome. With the large-scale collection of samples following patients over time, combined with genomic analysis and modern machine-learning-based approaches for finding patterns in data, this path holds great promise.

KEYWORDS
brush biopsies, explainable AI, oral cancer, oral keratinocytes, precision medicine

1 | INTRODUCTION

Precision medicine is increasingly important for early detection, diagnostics, treatment, and prevention strategies for disease. Continuous efforts are being made in dentistry to find methods to ensure patients’ good oral health. However, odontology must expand its engagement in clinical research on preventable disease as a part of life science to ensure a healthy life for patients.
Specialists in oral and maxillofacial surgery and oral medicine diagnose and treat a number of complex conditions; among these, oral squamous cell carcinoma (OSCC) is one of the most difficult to manage, as it is potentially fatal—especially if it is diagnosed late. It is well known that OSCC mainly develops from oral potentially malignant disorders (OPMD), such as oral leukoplakia (OL), erythroplakia, and proliferative verrucous leukoplakia, as well as atrophic and erosive oral lichen planus and oral submucous fibrosis. A recent meta-analysis reported the malignant transformation rate from OL to OSCC, which is the dominant oral malignancy, to be 9.8%. Prevalence is increasing, according to a 2022 report requested by the Oral Health Foundation in the United Kingdom. OSCC is an aggressive tumour that spreads early and has a 5-year survival rate of only 20%–40% with a late diagnosis, in contrast to a 76%–94% 5-year survival rate when diagnosed early. During 2008–2020, the age-standardised incidence of head and neck cancer in Sweden increased by 20%. In 2019, 764 people in Sweden, of which 57% (432 cases) were men, were diagnosed with malignant tumours in the lip or oral cavity. In 2020, 336 people in Sweden (3.25 people per 100 000 inhabitants) died from cancer of the lip or oral cavity, mainly due to late OSCC diagnosis. Accordingly, there is an urgent need for efficient diagnostic methods to identify OL at risk of malignant transformation to OSCC.

1.1 | The future of oral care lies in predicting and avoiding diseases

By increasing opportunities for early diagnosis and treatment, including the use of refined methods to identify individuals at risk of developing OSCC, morbidity can be reduced. As an additional result, opportunities to preserve function, appearance, and quality of life for affected patients will lead to reduced care needs and costs. Current methods used to assess at-risk patients are either invasive and too costly for larger initiatives or insufficiently sensitive and specific. To address this problem, intensive research is being conducted to find non-invasive methods that can identify the risk of developing OSCC without requiring expensive equipment or expertise, allowing them to be handled at the point of care.

Although studies have indicated the great benefits of screening for oral cavity cancer, there are no guidelines for the systematic follow-up of at-risk patients, most of which are patients with leukoplakia, tobacco habits, excessive alcohol consumption, and/or chronic high-risk HPV infection. As the symptoms are often initially mild, patients tend to be diagnosed late, resulting in significantly more extensive and complicated treatment and a poorer quality of life during and after treatment.

1.2 | Improved diagnostics and treatment

In Sweden, at least 3.5% of OL develops into OSCC each year; thus, patients with OL require lifelong monitoring. Specialists in this field have a significant workload, as there are currently no methods to identify at-risk patients with high specificity and sensitivity based on only clinical and histopathological assessment. The World Health Organisation (WHO) Collaborating Centre for Oral Cancer suggests that the focus should be on identifying reliable predictive and prognostic molecular biomarkers. Since cancer is a genetic disease that affects cell cycle regulation, researchers should focus on identifying the specific genomic changes in DNA, RNA, or protein patterns that initiate the development and progression of a tumour. With such knowledge, treatment could be directed towards the specific cause, allowing each patient to receive a person-centred and cohesive course of care instead of following traditional standardised treatment such as surgery and radiation therapy.

1.3 | Precision treatment

For solid tumours, such as breast, colon, and skin tumours, it has been shown that identifying changes in the genome makes it possible to precisely target drug therapy. With new achievements in the field, immunotherapy has become increasingly important in cancer therapy and is emerging as a viable alternative to drug therapy. The difficulty in determining the best therapy lies in the fact that a tumour consists of many different mutations whereby new types of clones of tumour cells arise. Within the same tumour, many different clones can exist, each of which drives the development of cancer, making it extremely difficult to determine the correct treatment in individual cases. In some cases, it is possible to analyse the tumour cells prior to therapy using biomarkers in order to decide on the targeted therapy and immunotherapy that are most likely to help and improve the outcome; however, there is still a need for more advanced solutions for patient selection. The combination of large-scale sample collection from following patients over time with modern machine-learning-based approaches for finding patterns in data holds great promise as one such advanced solution.

1.4 | Non-invasive diagnostics

In a first step towards finding better, simpler, safe, and cost-effective methods for controlling OPMD, we have reviewed non-invasive methods that can detect high-grade cell changes and early tumours. The possible non-invasive techniques evaluated included fluid-based methods (e.g., saliva and blood analysis, and tissue samples with brush biopsy for cytology) and more direct screening with autofluorescence methods, optical coherence tomography (OCT), and Raman spectroscopy. Brush sampling for cytology and autofluorescence methods is currently attractive alternatives, while Raman spectroscopy and OCT did not meet the requirements of simplicity and low cost; nevertheless, with further technological development, they could be future alternatives. Brush biopsies for cytology are primarily liquid-based biopsies that have been shown to work well in precision medicine for cancer detection and treatment planning. Cytological diagnostics with brush biopsies are now reported to have high reliability, making this a viable alternative to tissue biopsy for the control of OL, and one that should be evaluated in systematic screening.
1.5 | Cytological sampling with brush biopsy and objective analysis with artificial intelligence

In a liquid-based brush biopsy for cytology, the cell material is suspended in a methanol-based medium and then machine prepared in the laboratory as a thin cell layer on glass—ideally without cell overlapping—for diagnostics. It is then possible to analyse the same sample both traditionally under a microscope and with digital cytology through whole-slide scanning, computerised image analysis, and artificial intelligence (AI)-assisted assessment (Figure 1). A great deal of research is underway to develop AI as a diagnostic aid and a complement in the control of potentially malignant lesions with cytology. Fast and objective characterisation with deep learning can become an easy-to-use, rapid and cost-effective methodology for identifying leukoplakia at high risk of developing malignancy or for diagnosing OSCC. Explainable AI (XAI) tools, which highlight the information used and make decision-making interpretable, may serve as a way to increase both reliability (i.e., by identifying cases where the AI system makes a questionable decision) and trust (i.e., by providing reasons for the provided output); they will also enable an improved understanding of the disease as such, by ‘explaining’ how different patterns and pieces of information in the sample may indicate different levels of risk and the future progression of the disease.

1.6 | Liquid-based brush sampling for the assessment of oral keratinocytes

Through an ongoing multicentre study in Sweden titled ‘Targeted screening to reduce the incidence of oral cavity cancer’ using liquid-based brush sampling and cytological diagnosis for the control of OPMD, a huge number of oral keratinocytes from OL are being collected. These cells will be investigated for reliable predictive and prognostic molecular biomarkers, in addition to analysis with XAI. A systematic review of the literature has been completed to explore possible biomarkers in oral keratinocytes that identify OL at risk of malignant cell transformation and OSCC.

1.7 | Biomarkers in oral keratinocytes indicating risk of OSCC

In a systematic search of the PubMed and Cochrane medical databases using MeSH terms and a combination of free word searches, 817 studies of potential interest were identified. After excluding those that did not meet the inclusion criteria (see the participants, intervention, comparison, outcomes, and studies in Table 1 and Figure 2), the full text of the 84 studies that remained were read, and further studies that did not align with the set criteria were eliminated. This left 10 articles dealing with biomarkers in oral...
keratinocytes from OL indicating a risk for cancer (Table 2). In these 10 studies, a total of 39 proteins or DNA biomarkers related to the risk of malignant transformation were reported. Since the search was restricted to proteins and DNA, additional studies will be needed to search for predictive genomic changes. The obtained data highlight the problem that a tumour usually develops during an extensive period and contains cells with different mutations, which makes it difficult to identify specific genomic changes that indicate a high risk of a tumour. There is a clear lack of conclusive data; although some markers have a weak correlation with the biological endpoint of OSCC, this is insufficient as a basis for clinical decision-making. In the systematic review, Hasan concluded that there is substantial heterogeneity and a lack of standardised reporting of data. No studies were found on the brush sampling of OL or the cytology analysis of human oral keratinocytes that could predict malignant transformation with high sensitivity and specificity. In the review, two publications were perceived to be more important than the rest, since they were the only longitudinal studies, although they were retrospective and had a short follow-up period. These two studies were especially interesting, since they illustrated the complexity of the analysis needed to find possible biomarkers and applied several methods (see Table 2, Nos. 1 and 8). More specifically, Graveland et al. (No. 1 in Table 2) followed a two-step process for the genetic screening of oral keratinocytes from brush biopsies from OL. In the first step, two methods were used: (a) microsatellites to investigate genes or mutations that could be responsible for malignant transformation and (b) polymerase chain reaction (PCR). Microsatellite DNA are short sequences of one to six nitrogenous bases that are repeated several times in a row and are scattered throughout the genome; the length of microsatellites and the number of repetitions can vary greatly, but the variations define different properties. PCR is a molecular biological and biochemical method used to amplify a few copies of a particular DNA sequence to generate thousands. The researchers found chromosomal

**TABLE 1** Inclusion criteria for the systematic literature review ‘Prognostic biomarkers in oral keratinocytes identifying OL at risk of malignant cell transformation’.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Patients diagnosed clinically and histologically with OL or OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intervention</td>
<td>Brush biopsy of the oral mucosa</td>
</tr>
<tr>
<td>Comparison</td>
<td>With status of molecular biomarker in healthy tissue</td>
</tr>
<tr>
<td>Outcome</td>
<td>Biomarkers regarding diagnosis, progression and surveillance of OL, OL at risk for OSCC</td>
</tr>
</tbody>
</table>

Abbreviations: OL, oral leukoplakia; OSCC, oral squamous cell carcinoma.

**FIGURE 2** Prisma flow diagram for a systematic review. Identification of studies via database and registers.
abnormalities, with heterozygosity on specific chromosome pairs (3p, 9p, 11q, and 17p), along with different variants of the genes; however, there was no correlation with increased risk of malignancy. In the second step, immunohistochemical staining was done on tissue biopsies from OL, to analyse mutations of the tumour suppressor gene p53, which normally prevents a cell from turning into a cancer cell. The researchers found that mutated—that is, inactivated—p53 in combination with histopathological grading with heterozygosity on

<table>
<thead>
<tr>
<th>No.</th>
<th>Author, Year, Country</th>
<th>Type of study</th>
<th>Sample size</th>
<th>Sex and age</th>
<th>Follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Graveland et al., 2013, The Netherlands</td>
<td>Retrospective</td>
<td>43 oral leukoplakia, 43 controls</td>
<td>17 male, 26 female Average age 61 (31–90)</td>
<td>11–31 months Mean 20.3 months Median 18 months</td>
</tr>
<tr>
<td>2</td>
<td>Yang et al., 2014, USA</td>
<td>Laboratory/observational</td>
<td>11 OPMD, 11 OSCC, 10 controls</td>
<td>OMPD: 6 male, 5 female 48–79 years OSCC: 6 male, 5 female 42–88 years Controls: 5 male, 5 female 40–84 years</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>Hartman et al., 2015, Germany</td>
<td>Retrospective</td>
<td>24 controls, 18 oral leukoplakia, 15 OSCC, 15 oral lichen</td>
<td>24 healthy: 16 male, 8 female 41.6 mean age Lichen: 11 male, 4 female 60.3 mean age Oral leukoplakia: 10 male, 8 female 58.4 mean age OSCC: 4 male, 11 female 65.7 mean age</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>Noda et al., 2016, Japan</td>
<td>Laboratory/observational</td>
<td>155 tissue biopsies, 61 smears, 37 smears combined with tissue biopsies</td>
<td>97 male 82 female 66 mean age (12–90)</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>Brands et al., 2016, Germany</td>
<td>Laboratory/observational</td>
<td>24 controls, 15 HNSCC, 18 leukoplakia, 15 lichen (20.8%)</td>
<td>41 male, 31 female 23–87 years 54.7 ± 15.1 mean age</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>Kujan et al., 2019, Australia</td>
<td>Cross-sectional study</td>
<td>42 leukoplakia, 3 erythroplakia, 10 OSCC</td>
<td>30 male (54.5%), 25 female (45.5%) 31–92 years 65.13 ± 13.2 mean age</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>Kujan et al., 2021, Australia</td>
<td>Semi-quantitative comparative study</td>
<td>Total: 110 samples, 38 excluded 72 included. Undetermined significance SIL: 28 Low-grade SIL: 14 High-grade SIL: 19 OSCC: 10</td>
<td>33 male (45.8%), 39 female (54.2%) 36–90 years 64.75 ± 11.7 mean age</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>Datta et al., 2021, Canada</td>
<td>Retrospective</td>
<td>Total: 149 patients, 19 excluded. Included 130 37 lost to follow-up Mild dysplasia: 45 Moderate dysplasia: 45 Hyperplasia or lichenoid lesions: 40</td>
<td>63 male, 67 female 61.7 ± 10.9 mean age</td>
<td>Progressors 11–217 months Non-progressors 7–248 months</td>
</tr>
<tr>
<td>9</td>
<td>Pal et al., 2022, India</td>
<td>Laboratory/observational</td>
<td>50 Oral leukoplakia, 44 Controls</td>
<td>94 male, 0 female Oral leukoplakia: 20–67 years 42.24 ± 12.08 mean age Control group: 20–69 years 36.36 ± 13.47 mean age</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>Poell et al., 2022, The Netherlands</td>
<td>Retrospective</td>
<td>Total: 68 oral leukoplakia, 27 excluded Included 41 with both brush biopsy and tissue biopsy Controls brush biopsy: 6, total 29 biopsies</td>
<td>No data shown</td>
<td>None</td>
</tr>
</tbody>
</table>

Abbreviations: OPMD, oral potential malignant disorders; OSSC, oral squamous cell carcinoma; SIL, squamous intraepithelial lesion.
chromosome (9p) indicated an increased risk of progression to malignancy. In the other study\textsuperscript{26} (No. 8 in Table 2), calculated the DNA content per cell nucleus (i.e., the DNA ploidy) in all the cells from brush biopsies from oral lesions, to determine a high or low risk of malignancy. The sum was then compared with normal DNA content to obtain an objective value of genomic instability, which was combined in turn with a chromatin organisation (CO) value. Chromatin is a complex combination of DNA and proteins that makes up chromosomes. The researchers analysed the samples using their own DNA image cytometry system. They concluded that the DNA ploidy and CO score of brush samples from OPMD can identify lesions at high risk of progression several years in advance and are thus stronger predictors than the conventional way of evaluating risk based on anamnesis, clinical picture, and tissue biopsy. Due to the study’s limitations, as it included only 130 analysed samples and 13 cases of progression to malignancy, this result must be interpreted with caution and requires verification through a longitudinal prospective randomised trial. Overall, the systematic review revealed significant heterogeneity and a lack of systematic approach in study design, with an absence of randomised controlled trials.

At present, there is a lack of biomarkers that identify OL at risk of developing into OSCC with high sensitivity and specificity and that thus have clinical implications. Since 2023, a prospective open randomised 5-year multicentre study titled ‘Targeted screening to reduce the incidence of oral cavity cancer’ has been ongoing, including 1036 individuals. The study’s objective is to assess whether non-invasive brush biopsy for cytological diagnostics is a viable alternative to invasive tissue biopsy. In addition, it elucidates whether explainable deep-learning-based AI can be trusted to identify OL with a high risk of developing malignancy. Due to its longitudinal follow-ups with continuous brush biopsies, this study is generating a large number of oral keratinocytes from patients that develop OSCC and patients that remain tumour free, which can be single-cell analysed for biomarkers that can forecast malignant cell transformation.

1.8 Single-cell analysis of RNA transcripts in oral keratinocytes to identify cell clones with proliferative activity

The genome sequencing of OL is difficult when tissue biopsies are analysed, because the cell populations contain inflammatory cells from the sub-epithelial tissue; thus, genomic and transcriptomic analyses do not only show keratinocyte genes. When exfoliative cytology (brush biopsy) is performed instead of tissue biopsies, the sub-epithelial tissue is not included, and the sample will (in principle) only contain keratinocytes whose cell nuclei can be analysed according to a single-cell RNA sequencing protocol. In this way, the entire transcriptome of keratinocytes from leukoplasia can be mapped. Single-cell analyses also make it possible to detect changes in gene signalling pathways predicting the initiation of OSCC.\textsuperscript{27} This approach may be a tool for detecting early signs of cancer transformation, which can support clinicians in treatment decisions. Using a brush biopsy methodology, cell samples from our prospective randomised multicentre study with patients with leukoplasia can be saved and analysed over time, allowing changes in the genetic profile, based on mRNA expression, to be followed and hopefully leading to the identification of biomarkers that predict cancer transformation.\textsuperscript{27}

2 DISCUSSION AND CONCLUSION

OSCC, including of the lips and throat, is one of the most common tumours, with an age-standardised global incidence rate of 4.1 per 100,000 for all genders and all ages.\textsuperscript{25} Early detection is crucial for survival and can be achieved through careful clinical examination and an invasive biopsy for histopathological diagnosis, which requires a referral to a specialist. Since most tumours are diagnosed late, due both to a lack of healthcare providers and specialists and to the fact that initial symptoms are mild and transient, the prognosis is poor.\textsuperscript{28} Even if the OL precursor lesions are excised, the recurrence rate is high, and patients with a history of OL must be subjected to continuous lifelong monitoring.\textsuperscript{6} There is a lack of guidelines for how at-risk patients should be monitored, as well as a lack of reliable screening methods. The gold standard is to obtain additional tissue biopsies for histopathological diagnosis during monitoring, at the discretion of the consultant. This is manageable in privileged societies with access to specialist care and with costs partly covered by insurance systems, but not for the majority worldwide. Without a viable alternative, it is clear that there is a need for practical, low-cost adjunctive methods that are easy to manage in primary care. Such methods should be non-invasive and have the potential to identify cellular changes or early cancer, thereby eliminating invasive tissue biopsies that can drive malignant transformation per se.\textsuperscript{29} This position paper highlights efficient non-invasive methods that can single out patients with oral mucosal lesions at the risk of malignant transformation with high sensitivity and specificity and thus can contribute to a reduced prevalence of OSCC. Brush biopsies are non-invasive and cost-effective, with high diagnostic accuracy; they can be managed in primary dental care and are a validated alternative to invasive scalpel biopsies.\textsuperscript{6} In liquid-based brush sampling, oral keratinocytes are collected for cytology. With digital cytology, the same sample can be assessed quickly and objectively through cost-effective and easy-to-use AI characterisation with deep learning, which has demonstrated great potential for finding early cancer or lesions at risk of malignant cell transformation.\textsuperscript{27} We also find it beneficial to analyse the oral keratinocytes with a single-cell RNA sequencing protocol, whereby the entire transcriptome of keratinocytes from OL can be mapped to provide prerequisites for identifying genomic anomalies and predictive and prognostic biomarkers that can affect clinical decision-making.\textsuperscript{30} The non-invasive methods of brush biopsies for cytology and AI-supported diagnostics in combination with predictive biomarkers open up an avenue for new screening initiatives for high-risk patients.

AUTHOR CONTRIBUTIONS

The study was conceptualised by Jan-Michaël Hirsch, Joakim Lindblad, and Bengt Hasséus, based on Refs. 6, 19, 24 Jan-Michaël Hirsch, Bengt Hasséus, Ronak Sandy contributed to the clinical aspects of the
study, and Joakim Lindblad contributed to the technical aspects of the study. All the authors contributed to drafting the article, and all authors approved the final version.

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CONFLICT OF INTEREST STATEMENT
The authors declare that they have no conflict of interest.

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