Oral Oncology 96 (2019) 51–59

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Review

The role of DNA image cytometry in screening oral potentially malignant lesions using brushings: A systematic review

Madhurima Datta[a], Denise Laronde[a,b], Branko Palcic[c], Martial Guillaud[c,d,*]

[a] Department of Oral Biological and Medical Sciences, Faculty of Dentistry, The University of British Columbia, 2199 Wesbrook Mall, Vancouver, BC V6T 1Z3, Canada
[b] Cancer Control Research, BC Cancer, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada
[c] Imaging Unit, Integrative Oncology, BC Cancer, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada
[d] Department of Statistics, The University of British Columbia, 2329 West Mall, Vancouver, BC V6T 1Z4, Canada

ARTICLE INFO

Keywords:
Oral dysplasia
Oral cancer
DNA ploidy
Aneuploidy
DNA image cytometry
Systematic review
Oral cancer screening

ABSTRACT

It is believed that the majority of oral cancers develop from oral potentially malignant lesions (OPML). Though they can be easily detected during screening, risk stratification is difficult. During screening clinicians often find it difficult to distinguish OPMLs from benign lesions, and predicting OPML at risk of malignant transformation is particularly challenging. DNA aneuploidy has been known to be a marker of malignancy in a number of sites including the oral cavity.

We performed a systematic review to evaluate the effectiveness of DNA-ICM using brushings in differentiating OPMLs from benign/inflammatory lesions during screening and in predicting malignant transformation. MEDLINE, Pubmed, EMBASE electronic databases were systematically searched using a combination of keywords and subject headings. A total of 11 articles satisfied our inclusion criteria. These studies reported a wide range of sensitivity (16–96.4%) and specificity (90–100%) due to the differences in study design, definitions of high risk or low risk lesions and DNA-ICM protocol used. No long-term longitudinal studies were identified to assess the role of DNA-ICM using brushings in predicting malignant transformation. No studies evaluated the role of DNA-ICM in community screening settings. A number of studies combined DNA-ICM with other techniques like cytology or argyrophilic nucleolar organizer region counts leading to improved test results. In spite of DNA aneuploidy being accepted as a marker of malignancy, there is limited evidence of DNA-ICM using brushings being successful as an adjunct oral cancer screening tool. Longitudinal studies and large community screening studies need to be undertaken to draw stronger conclusion.

Introduction

Oral cancer is the sixth most common cancer in the world, with more than 350,000 cases diagnosed annually [1]. This disease has a high mortality rate (~50% 5-year survival) due to the advanced stage at which it is diagnosed [2]. In high risk countries such as India, 60–80% of patients present with advanced disease as compared to 40% in developed countries [3].

The majority of oral cancers are presumed to develop from oral potentially malignant lesions (OPML) [4]. Lesions can be easily detected by visual inspection during screening. However, differentiating OPML from benign lesions can be challenging. Diagnosis of these OPMLs is based on histopathology and is defined by the presence or absence of dysplasia. Epithelial dysplasia consists of specific alterations in cell and tissue. Cell architectural changes includes the nuclear architecture, where the nucleus becomes enlarged (nuclear hyperplasia), dark-stained (hyperchromatism), and has an increased nuclear-to-cytoplasmic ratio due to an enlarged eosinophilic nucleolus [5]. According to 2017 WHO classification, dysplasia is graded as mild, moderate or severe, dependent on the architectural and cytologic changes being limited to the lower third, lower two-thirds, and more than two-thirds of the epithelium, respectively [6]. In OPMLs with dysplasia, only a small percentage (13%) progress to cancer [7]. The higher the grade of dysplasia, the greater the risk of progression [8].

Even though histopathology is considered the gold standard for predicting malignant transformation, a number of shortcomings are associated with it [9]. Histopathological grading has shown poor reproducibility and high inter and intra-observer variance rates [10].

Abbreviations: OPML, oral potentially malignant lesion; DNA-ICM, DNA image cytometry; CVOE, conventional visual oral examination; DNA-FCM, DNA flow cytometry; FV, fluorescence visualisation; TB, toluidine blue

* Corresponding author at: Imaging Unit, Integrative Oncology, Rm 6-108, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada.
E-mail address: mguillau@bccrc.ca (M. Guillaud).

https://doi.org/10.1016/j.oraloncology.2019.07.006
Received 10 May 2019; Received in revised form 17 June 2019; Accepted 5 July 2019
Available online 10 July 2019
1368-8375/

Moreover, oral cancer can develop without the presence of dysplasia, while severe dysplasias may not progress [11]. Risk stratification among mild and moderate dysplastic lesions has always been challenging due to their low transformation rates. Clinical risk indicators like size, appearance and site of the lesion may correlate with increased risk of progression, however it is difficult to predict the outcome for an individual patient [7]. The lack of reliable clinical indicators of malignant transformation warrants the use of additional biomarkers of progression.

Primary oral cancer screening is conducted in a dental office. One limitation of the conventional visual oral examination (CVOE) is that the majority of the clinically suspicious lesions are benign or inflammatory in nature and mimic OPMLs in presentation.

CVOE alone cannot differentiate high risk OPMLs from low risk or benign lesions during screening [12]. Adjunct screening devices like fluorescence visualisation (FV) and toluidine blue (TB) staining have been introduced to give clinicians additional information to aid in their decision-making regarding; (i) the probability that this lesion is dysplastic; and (ii) the risk of transformation if dysplastic [13,14]. FV is used to visualize alterations in tissue due to morphological and biochemical changes [13]. In high-risk settings, some studies have shown FV is capable of detecting occult oral lesions not detectable by CVOE alone [15,16]. However, shortcomings include high false positive for benign inflammatory lesions due to their increased vascularity [17]. A systematic review concluded that FV's usefulness is limited in the hands of an inexperienced clinician in primary care screening settings [18]. FV has not been found, to date, to aid in the identification of progressing OPMLs.

TB is an acidophilic stain which binds to the increased number of nucleic acids or penetrates into the tissue due to altered cell adhesion in high-risk dysplastic tissues [19]. A recent systematic review reported the sensitivity of TB staining for the detection of oral cancers ranges from 78 to 100% and the specificity from 31 to 100% [20]. Several authors have expressed conflicting views on the usefulness of TB as an adjunct screening tool as it shows low sensitivity in detecting lesions with dysplasia and frequently shows false positives for benign conditions [12]. In contrast, dysplastic OPML that stain TB positive have shown an increased risk of malignant transformation [21]. Thus, there is still a need for an objective, non-invasive, high throughput screening tool that can serve as an adjunct to CVOE.

DNA content imbalance has been recognised as an accepted biomarker of malignancy in a variety of sites including the cervix, colon and oral cavity [22,23,24]. In addition, it has shown increasing usefulness in predicting prognosis in prostate cancers [25]. Aneuploidy detected in biopsy *tissue* has been useful in predicting malignant transformation in oral lesions [26] and in Barrett's oesophagus [27]. The hazard ratio for malignant transformation was 3.3 when abnormal DNA content was found in *tissue* from oral lesions [26].

DNA ploidy analysis has been done using a variety of assays including DNA flow cytometry (FCM) and Image Cytometry (ICM) [24,29]. DNA-FCM measures the DNA content in nuclei or cells in a suspension while DNA-ICM obtains measurements from cells or nuclei affixed to a glass slide. DNA-ICM has been reported to show higher sensitivity than FCM in detecting a small percentage of near-diploid aneuploid cells [28,29].

Samples for DNA content analysis can be prepared from tissue or cell samples. DNA content analysis studies using tissue from fresh frozen or formalin-fixed paraffin-embedded (FFPE) tissue, uses primarily two methods: (i) A nuclei suspension created from thin FFPE sections after proteolytic digestion; [30] or (ii) sections from the FFPE tissue are directly fixed to the glass slide [31]. In comparison, cells can also be collected by brushing a lesion. Cells are obtained from the superficial and intermediate layers of the oral mucosa. Traditionally, brushings have been used for exfoliative cytology to detect abnormal tumour cells in the cervix or oral cavity [32,33]. Brushings for DNA-ICM pose an advantage over biopsy tissue as cells can be obtained for screening non-invasively. However, cell preparation from brushings can be inadequate and is limited to the superficial or intermediate layers of the oral mucosa making it difficult to detect mild dysplastic changes.

The aims of this review are to assess the role of DNA-ICM using oral lesion brushings as an adjunct screening tool to differentiate high risk OPMLs from benign conditions during screening, and to identify histologically proven dysplasias at an increased risk of progression to malignancy.

Materials and methods

Search strategy

A literature search of Medline, PubMed and Embase databases was conducted using a combination of text words and medical subject headings (MeSH). The search terms used included oral or mouth neoplasms, oral precancers, premalignant or potentially malignant, oral dysplasia, DNA ploidy, DNA cytometry-flow or image. Boolean operators 'AND' or 'OR' were used to combine the keywords. Two review authors individually searched the literature and extracted data from the chosen articles. Discrepancies were resolved through discussion with the third author. A manual sorting was done by reading the titles and abstracts. The systematic review was registered in PROSPERO (CRD42019128725).

The following questions were addressed:

- Does DNA-ICM serve as an adjunct screening tool in differentiating high risk lesions from benign or inflammatory oral lesions?

AND

- Does DNA-ICM identify oral potentially malignant lesions (OPMLs) at a risk of malignant transformation?

The questions were addressed using the PICO method:

P (Population): Oral potentially malignant lesions
I (Intervention): DNA content analysis by DNA-ICM using brushings collected from the lesions
C (Comparison): Histopathology
O (Outcome):
1. DNA-ICM can serve as an adjunct screening tool in differentiating high risk lesions from benign or inflammatory oral lesions
2. DNA-ICM can predict risk of malignant transformation in OPMLs

Inclusion and exclusion criteria

Inclusion criteria

- Primary oral potentially malignant lesions (OPML).
- DNA-ICM was used for DNA content analysis.
- Samples were collected using brushings.

Exclusion criteria

- Assessing the use of DNA-ICM in previously histologically confirmed oral cancers or inflammatory conditions like oral lichen planus.
- Other methods of DNA content assessment such as DNA-FCM were used.
- FFPE or fresh frozen tissue sections were used for DNA content analysis.
- Articles not written in English.

Data collection

A standardized data collection method was used for each study and

the following data was extracted: Author, study year, study objective, study design, number of cases and controls, grade of dysplasia/histopathology results, study endpoint (malignant transformation), type of brush, sample preparation method, staining type, scanner used, and the sensitivity or specificity of the methods. For studies which did not report sensitivity and specificity, values were calculated using a 2 × 2 table. Sensitivity and specificity of each study measured DNA-ICM's ability to distinguish between high-risk lesion/high-grade dysplasia and low-risk lesion/low-grade dysplasia in comparison to histopathology results. To assess the performance of DNA-ICM as an adjunct screening tool we used Littenberg's model of medical technology evaluation [34]. It considers biologic plausibility, technical feasibility, diagnostic performance and patient and societal outcomes. For biologic plausibility we looked at how DNA-ICM detects aneuploidy. Technical feasibility highlights the requirements needed to ensure accurate DNA-ICM results. Diagnostic performance relates to the sensitivity and specificity of the systems, recognizing the variety of study designs and biases that may affect the results. Finally, patient and societal outcomes were assessed in relation to ease of sample collection and projected ease and cost of using this technology as a screening tool.

Results

A total of 362 articles were identified through a literature search of PubMed, Medline and Embase. Three articles were included from references of the other studies. 153 articles were rejected after reading the titles and abstracts. A total of 44 articles were assessed for eligibility. Twelve studies which looked at oral lichen planus (supplementary data) and 133 studies primarily involving oral cancer were excluded. Six studies not written in English, 10 conference abstracts, 6 duplicates and 2 studies not looking at ploidy were excluded. Among the 44 shortlisted articles, 1 review, 1 case report and 3 retracted studies were excluded. The remaining articles were divided on the basis of DNA content analysis method used: DNA-ICM, DNA-FCM or other method of ploidy detection. Twenty- eight studies used DNA-ICM while 11 studies used DNA-FCM alone or in combination with other methods like fluorescence in situ hybridization (FISH) and array comparative genomic hybridization (aCGH). Out of the 28 DNA-ICM studies, 11 studies fulfilled the inclusion criteria of using brushings while the remaining used FFPE tissues. (Figure 1)

Table 1 summarizes the objective, sample size, patient selection criteria, type of brush, DNA-ICM system used, and the sensitivity and specificity reported by each study. The studies were conducted in specialist hospitals or clinics based on community referrals for suspicious lesions in the mouth. None of the studies looked at the effectiveness of DNA-ICM as an adjunct screening tool in a community screening setting.

None of the studies fulfilled our research question of whether DNA aneuploid OPMLs were more likely to show malignant transformation over time. No studies were longitudinal in design nor study the lesions over time until progression. The majority of the studies looked at the effectiveness of DNA-ICM in screening high risk OPMLs or differentiating malignant lesions from low risk or benign lesions.

The majority of studies [35–43] utilised automated DNA-ICM systems while two studies manually quantified DNA amount [44,45]. Histopathology was used as the gold standard in all the studies. The sensitivity of DNA-ICM in detecting high-risk lesions ranged from l6% [45] to 96.4% [42] while specificity ranged from 90% [39] to 100% [38,40,42–45]. However, the definition of high-grade lesions varied. Some studies [37–41] considered severe dysplasia, carcinoma-in situ and squamous cell carcinoma (SCC) as high risk while others included only SCC [42,44,45,43]. Further, in some studies poor differentiation between clinical diagnosis of leukoplakia and histopathological grade of dysplasia made it difficult to compare results [42,43]. Two studies [35,36] looked at effectiveness of DNA-ICM in differentiating between high and low grade dysplasia. Combining DNA-ICM with other diagnostic methods like cytology and argyrophilic nucleolar organizer region counts improved sensitivity and specificity rates [37,38,42,44,43].

Discussion

Littenberg's model of medical technology evaluation was used to estimate the effectiveness of DNA-ICM system. This model assesses new technology on four levels; does the technology make biological sense, can it be used in the proposed population, does it have an impact on the disease, and does it have an effect on the patient and society [34].

Biologic plausibility

In a normal cell, there are two copies of all 23 chromosomes, referred to as diploid (2c). Cells with one copy of the 23 chromosomes are known as haploid (1c). Cells that have complete multiples of the haploid set (1c) are called euploid, while aneuploid cells do not contain an integer multiple of the basic set of chromosomes [46]. DNA aneuploidy is the cytometric equivalent of chromosomal aneuploidy and is believed to drive carcinogenesis. There is debate whether chromosomal instability is the cause or effect of genetic mutation [47]. Cellular changes, including visible changes to the DNA organization in the cell, have been observed in breast and oral malignancies [48,40].

DNA-ICM is used to detect the presence of DNA aneuploidy. Cells are collected using a brush and deposited on a slide. The slides are stained using a DNA stoichiometric stain which stains the nucleus proportional to the DNA content of the cell [46]. Measurement of the DNA content of the nuclei is done by measuring the integrated optical density (IOD). The IOD, the cytometric equivalent of the cell's DNA content, is scaled to 'c' units using internal epithelial or lymphocyte cells as reference. To assess the presence of aneuploidy, a minimum number of epithelial cells need to be quantified. DNA-ICM system guidelines should follow the European Society for Analytical Cellular Pathology guidelines [50].

Technical feasibility

Assessment using DNA-ICM primarily depends on the clinician's ability detect a lesion and assess its risk prior to the decision to obtain a brushing. Other factors that affect the success of DNA-ICM includes the type of brush, sample preparation and staining, cellularity, and the use of an automated scanner to precisely measure cell DNA content with minimum human intervention.

Cell collection and preparation technique varied across the studies. All studies used a cytobrush to collect cells except Kaur et al who used a toothbrush [43]. Sample preparation used one of two techniques. In the majority of studies, the brush was directly rolled on a glass slide and fixed using a spray [37,38,39,42,43,44,45]. The remaining studies used liquid based cytology (LBC). In LBC, the brush is placed into a vial with a cell preservative and transferred to the lab [35,36,40,41]. Once in the lab, a thin monolayer of cells is deposited on the slide. This technique reduces blood, mucus and debris in the preparation [51].

For reliable results, a minimum number of cells need to be collected to detect the presence of aneuploidy. In most automated DNA-ICM systems, a minimum of 300–400 cells are required to provide a satisfactory diagnosis. Brushings from hard to access areas such as the posterior pharyngeal wall, or floor of mouth often result in low cell count. Remmerbach et al argued that the failure to detect a carcinoma on the posterior pharyngeal wall in their study was the result of inadequate cell collection due to limited access [42].

Diagnostic performance

The majority of the studies reported sensitivity and specificity as outcome when differentiating between high-risk and low-risk lesions as defined by histopathology. The wide variation in values can be attributed to the lack of an established DNA-ICM protocol, absence of a standardized definition of high-risk and low-risk lesions, and poor study design. Sensitivity was poor in studies that did not use an automated system. The study by Pektas et al reported the poorest sensitivity rates; DNA aneuploidy was detected in only 2 of 12 malignant lesions. The

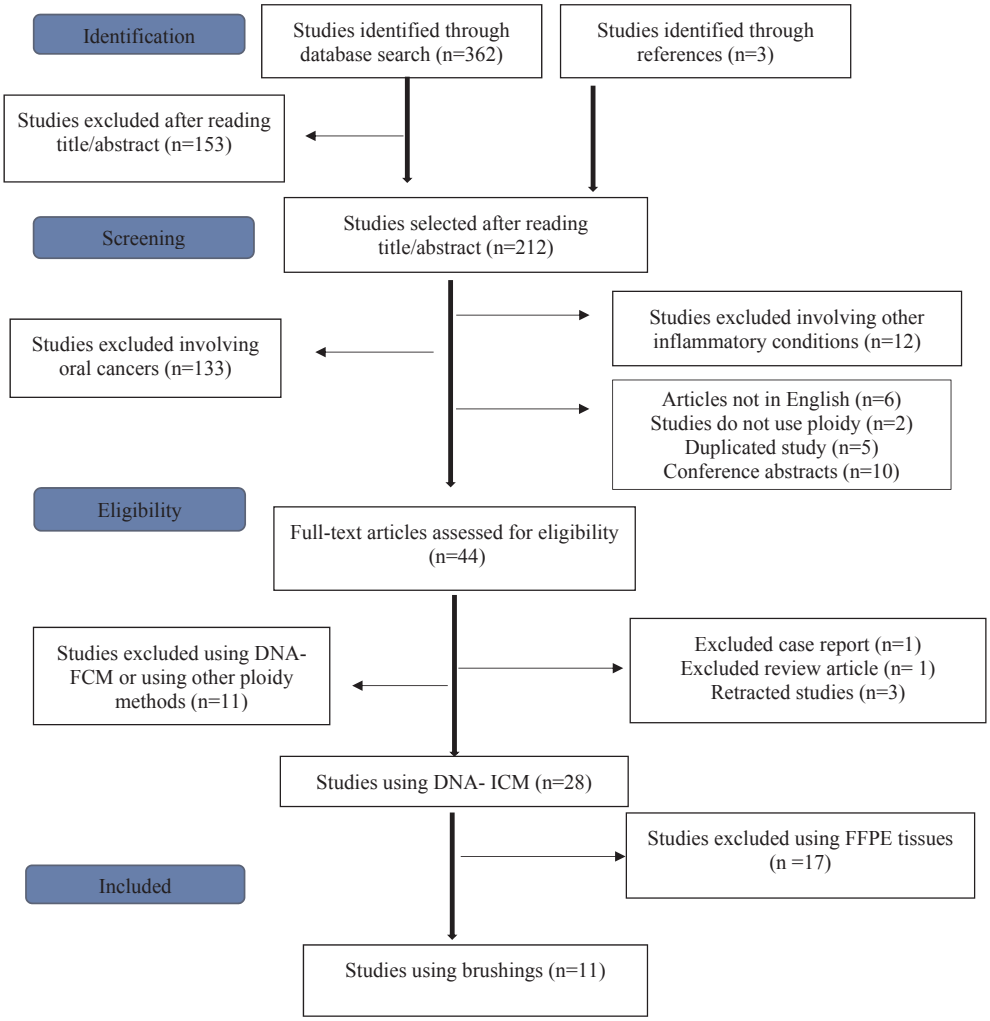

Fig. 1. Study selection flowchart.

authors compared their results with FCM studies and concluded that the low aneuploidy rates may be due to differences in cytometric assessment or tissue preparation methods [45]. However, it can be concluded that the poor performance is due to the lack of use of an automated DNA-ICM system. Similar conclusions can be drawn from the study by Kaur et al, where they manually measured the IOD of 100 traced nuclei obtained from photographs of Feulgen stained slides. In addition, assessment of a limited number of nuclei may be responsible for the low sensitivity rate of 68.7% [44]. Manual measurement of IOD are subjective and prone to error which explains the poor sensitivity rates of these studies. Similar findings were also reported by Böcking et al. where computerized assessment of nuclear morphometric features and DNA quantification showed higher sensitivity and specificity than manual DNA quantification [49].

Remmerbach et al reported the highest sensitivity rates of 96.4% [42]. However, these findings should be interpreted with caution as only OSCC was considered high risk and no pathological diagnosis was mentioned for the 83 leukoplakias classified as low risk. There was no break-down in the cases on the basis of severity of dysplasia.

MacAulay et al.'s study is the only study that used a training and test set to validate the performance of the DNA-ICM system [40]. The authors developed a series of machine learning algorithms of increasing complexity based on a combination of DNA amount and nuclear organization/texture features. The final results were derived from the test set which makes their findings more reliable and robust. In the same study, they also looked at the addition of FV in detecting OPMLs. This secondary aim was to test the potential of DNA-ICM to reduce the number of false positives, i.e. benign lesions showing FV loss. Sensitivity and specificity among the benign confounding set was lower compared to the malignant set. They concluded that inflammation or trauma increases the variability in cell features and their inclusion in the training set seemed to improve results.

Both Yang et al, and Xiao et al, looked at the association of grade of dysplasia in leukoplakia with DNA content [35,36] They reported a sensitivity of 68% and 58% respectively and a specificity of 67% in both studies, in detecting aneuploidy among high-grade dysplasia in comparison to low-grade dysplasia. Using the supplementary data from the Xiao et al study, we found the sensitivity and specificity values of detecting low-grade (mild dysplasia) and high-grade dysplasias (moderate and severe) varied significantly. The sensitivity of detecting mild dysplasia in comparison to benign lesions with no dysplasia was 34.4% while the specificity was 100%. Moderate and severe dysplasias grouped together in comparison to benign lesions with no dysplasia showed a sensitivity of 58.6% and specificity of 100%. The low sensitivity rates should be interpreted carefully as they were trying to differentiate between grades of dysplasia in contrast to other studies which compared benign lesions to malignant/dysplastic lesions. Differentiating between grades of dysplasia is difficult to achieve and highly sensitive algorithms are required. However, the authors were not clear if the data between the two studies were unique as: (i) both these studies were undertaken at the Ninth People's Hospital, China; (ii) recruited patients visited the department during the same time period and

Table 1
Table summarising the findings of each study on the role of DNA-ICM in differentiating high risk lesions from benign or low risk lesions.

Author/Study year/Study objective	Study design	Sample size [a] (Pathological diagnosis)	Clinical diagnosis[b]	Population Selection criteria	Type of brush	DNA– ICM scanner used	Sensitivity	Specificity	Study Findings
Yang et al. [36] Association of grade of dysplasia with DNA content and nuclear morphometric status	Cross- sectional	70 total 39 **low-grades** (LG) (No D + D1) 31 **high-grades** (HG) D2 + D3	No clinical diagnosis	Patients with suspicious lesions visiting the specialty hospital (2-year time span)	Liquid-based brush kit (MotiSavant)	MotiSavant DNA image cytometer	67.7%	66.6%	34 patients showed DNA aneuploidy out of which 21 were high-grade lesions. DNA content, DNA index, nuclear area, and nuclear radius of HG dysplasia was significantly larger than those of LG dysplasia ($p < 0.001$)
Xiao et al. [35] Association of grade of dysplasia with DNA content and nuclear morphometric status	Cross- sectional	65 total 36 **LG** (No D + D1) 29 **HG (D**2 + D3)	No clinical diagnosis	Patients with suspicious lesions visiting the specialty hospital (1-year time span)	Liquid-based brush kit (MotiSavant)	MotiSavant DNA image cytometer	58.6%	66.6%	27 patients showed DNA aneuploidy of which 17 showed moderate or severe dysplasia. The frequency of aneuploidy was reported higher in tongue (high-risk site) compared to other sites
Ma et al. [39] Estimate diagnostic ability of DNA-ICM as compared to histopathology in identifying high-risk lesions	Cross sectional	52 total 30 **low-risk** **(**Benign + D1 + D2) 22 **high-risk (**D3 + OSCC)	6LP, 21 OLP, 25 others[c]	Patients with suspicious lesions visiting the specialty hospital (8 months' time span)	Cytobrush	Automated DNA image cytometer	86.3%	90%	19 patients showed DNA aneuploidy among the 22 high-risk lesions.
Ng et al. [41] Evaluate whether QC can disclose abnormal DNA content and abnormal nuclear morphology of high-risk OPMLs	Retrospective	171 total 143 **low-risk** (99 benign + 25 D1 + 12 D2 + 7 VH) 12 **high-risk** (7 D3 + 5 CIS + 16 OSCC) 28 **controls** (fibroma + papilloma)	**Benign** 33 reactive epithelial cases 66 lichenoid mucositis (No further breakdown)	Patients who had both a biopsy and a concurrent QC assessment in a community referral-based oral medicine clinic (2-year time span)	Brushing Oral Advance kit (Perceptronix Medical Inc.)	ClearCyte Image cytometer (PMI)	89%	97%	30 patients were QC positive (11 severe dysplasia and 14 OSCC). QC positivity was significant with clinical features predictive of progression (high-risk site, lesions greater than 2 cm and non-homogenous appearance).
Pektas et al. [45] Efficacy of Nuclear morphometry and DNA ploidy measurement for detection of oral malignancies	Cross-sectional	22 total 12 **high-risk (**OSCC) 10 **low-risk** (Dysplasia grade not known) 22 **controls** (contralateral cheek brushing)	**High-risk** OSCC **Low-risk** 4 LP, 3 others [d]	Patients with suspicious lesions visiting the specialty hospital	Cytobrush	Images obtained from a colored video camera, image analysis using Zeiss Vision KS 400 (version 3.0) software	16%	100%	IOD and nuclear features manually calculated. Aneuploidy detected in only 2 OSCC samples out of 12.
MacAulay et al. [40] Using DNA-ICM to recognise high-risk lesions as detected by white light and FV	Retrospective	225 total 77 **low-risk (**Confounders) 148 **high-risk** (OSCC + CIS + D3) 144 **controls**	No clinical diagnosis	Patients enrolled in the Oral Cancer Longitudinal Prediction trial and (4 years' time span) Pathological proven sites enrolled	Innovatek cytology brush	Cyto-Savant automated quantitative system	89%	100%.	Used a training and test set to validate performance of series of algorithms which combined DNA ploidy with nuclear texture features. Also used algorithms to

(*continued on next page*)

Table 1 (*continued*)

Author/Study year/Study objective	Study design	Sample size [a] (Pathological diagnosis)	Clinical diagnosis[b]	Population Selection criteria	Type of brush	DNA– ICM scanner used	Sensitivity	Specificity	Study Findings
									correctly identify 77 benign confounders as detected by FV.
Kammerer et al. 2013 [38] Combination of brush cytology and DNA -ICM with histopathology to detect high-risk lesions	Cross sectional	87 total 57 **low-risk** (6 D1 + 51 benign) 31 **high-risk** (2 D2 + 4 CIS + 25 OSCC)	36 LP, 18 OLP, 34 others[e]	Patients with suspicious lesions visiting the specialty hospital. Patients with obvious signs of malignancy were excluded	Cytobrush	AutoCyte QUIC-DNA	DNA- ICM sensitivity = 70% Combined Sensitivity = 76%	DNA-ICM specificity = 100% Combined Specificity = 100%	DNA-ICM detected aneuploidy in 21 among 30 high-risk lesions (1 excluded due to low cell count). Sensitivity and specificity improved on combining cytology with DNA- ICM.
Maraki et al. [37] Combination of brush cytology and DNA -ICM with histopathology to detect high-risk lesions	Prospective	98 total 81 **low-risk** (79 No D, D1 + D2) 18 **high-risk** (15 OSCC + 3 D3) 1 (interobserver variability) D1 - D3	**Low –risk** 37 OLP, 21 LP, 25 others [f] **High risk** 15OSCC	Patients with suspicious lesions without obvious signs of neoplasm/ dysplasia that visited the speciality hospital.	Cytobrush	AutoCyte QUIC DNA	Combined Sensitivity = 100% DNA-ICM sensitivity and specificity data could not be extracted from the table	Combined Specificity = 97.4%	DNA -ICM predicted progression in one D1 lesion that was positive for tumour cell. Lesion showing discrepancy on histopathology did not reach outcome. Sensitivity and specificity improved on combining cytology with DNA- ICM.
Remmerbach et al [42] Combination of brush cytology and DNA -ICM with histopathology to detect high risk lesions	Cross-sectional	251 total 56 **high risk** (OSCC) 195 **low-risk** No grade of dysplasia	**High-risk** 56 OSCC **Low-risk** 83LP, 32 OLP, 80 others [g]	Patients with suspicious lesions visiting the specialty hospital. (3 years timespan)	Cytobrush	AutoCyte QUIC DNA	DNA-ICM sensitivity = 96.4% Combined Sensitivity = 98.5%.	DNA-ICM specificity = 100% Combined Specificity = 100%	DNA aneuploidy was detected in all neoplastic cases except one that was attributed to sample collection error. Sensitivity and specificity improved on combining cytology with DNA- ICM.
Kaur et al [44] Combination of brush cytology and DNA -ICM with histopathology to detect high risk lesions	Prospective However, does not follow up lesions	100 total 51 **low-risk (46** benign + 1 PH + 1 VH + 2 ulcer + 1 no epithelial abnormality) 49 **high-risk** (OSCC) No dysplasia grading	No clinical diagnosis	Patients with suspicious lesions visiting the specialty hospital.	Toothbrush	Images taken with camera at 40X magnification. 100 cells studied. Image Pro-Plus software used for image analysis.	DNA-ICM sensitivity = 68.7% Combined Sensitivity = 92%	DNA-ICM specificity = 100% Combined Specificity = 100%	IOD manually calculated. 33 cases showed aneuploidy among 49 malignant lesions. 42 cases showed positive on cytology for presence of malignancy.
Remmerbach et al [43] Sequential analysis of cellular characteristics in the same smear. Combines cytology, DNA-ICM & count of argyrophilic nucleolar organizing regions (AgNOR)	Prospective Lesions followed clinically over 3 to 6 months interval for 1 year	47 total 20 **high-risk** (17 OSCC + 1 large cell keratinizing carcinoma + 1 ASCC + 1 MEC 27 **low-risk** (7 LP + 20 OLP) No dysplasia grading	No clinical diagnosis	Patients with suspicious lesions visiting the specialty hospital	Brush-based cell collector	Multimodal Cell Analysis semiautomated system	DNA-ICM sensitivity = 90% Combined Sensitivity = 100%	DNA-ICM specificity = 100% Combined specificity = 100%	DNA-ICM improved false positive rates as detected by cytology. Combination of AgNOR analysis with cytology and DNA-ICM improved sensitivity and specificity

[a] No D – No dysplasia; D1 – Mild dysplasia; D2 – Moderate dysplasia; D3 – Severe dysplasia; VH – Verrucous hyperplasia; CIS – Carcinoma in-*situ*; OSCC – oral squamous cell carcinoma; HG – high-grade; LG – low-grade.

[b] LP – leukoplakia; OLP – Oral lichen planus; ASCC – Adenoid squamous cell carcinoma; MEC – Mucoepidermoid Carcinoma.
[c] 1erythroplakia, 1erythroleukoplakia, 1 hyperkeratosis.
[d] 4 palatal lesions in reverse smokers, 2 Discoid lupus erythematosus, 6 Gingivitis Desquamativa, 4 Tone ulcerous lesions, 4 neoplasm, 5 normal.
[e] 12 verruciform erythroplakia, 9 erythroleukoplakia, 7 erosion, 6 submucosal induration.
[f] 17 gingivitis desquamativa, 1 linear IgA disease, 1 apthous ulcers, 2 asthma spray stomatitis, 1 actinic keratosis, 3 erythroleukoplakia.
[g] 16 erosive lichen planus, 1 atrophic lichen planus, 12 pseudoepitheliomatous hyperplasia, 2 epulis granumotatosa, 1 desquamative gingivitis, 1 scar, 33 ulcus, 4 median rhomboid glossitis, 6 recurrent apthous ulcers, 1 denture stomatistis, 1 Lingua plicata, 1 lingual tonsil, 1 papillomatosis.

both studies report similar sample sizes; and (iii) the authors of the second study fail to cite the findings of the initial study.

Four studies looked at combining conventional oral cytology with DNA-ICM to improve sensitivity and specificity with histopathological diagnosis [37,38,42,44]. An improvement in sensitivity and specificity was noted when the methods were combined. Remmerbach et al used a novel semiautomated multimodal cell analysis (MCA) technology which can track individual cells when stained with Papanicolau, Feulgen and silver nitrate [43]. They used conventional cytology to spot abnormal and atypical cells which were then restained with Feulgen followed by silver nitrate to demonstrate argyrophilic nucleolar organizing region counts (AgNOR). AgNOR counts and size have shown to be correlated with increasing proliferation rates and with malignant potential [52]. The reported sensitivity and specificity rates of the three techniques combined were high however the author concluded that this system would not apply for routine screening as it is time-consuming and expensive. MCA technology would only be useful when a small number of abnormal or atypical cells are identified through cytology and application of the sequential stains would reduce the false negative and false positive rates.

Patient and societal outcomes

Most of the studies had better specificity than sensitivity rates. This is worrisome as it is likely that DNA-ICM may fail to detect true positives. However, the studies showing poor results demonstrate flaws in sample processing or used manual DNA quantification methods. DNA-ICM using FFPE tissues from oral lesions [27,53] have demonstrated superior results which indicates that the problem may lie with the sample collection and processing techniques used with brushings. A recent meta-analysis conducted by Alaizari et al. concluded that lesions found to be aneuploid using DNA-ICM had a 3.12-fold increased risk of turning into cancer. The meta-analysis was conducted on 5 longitudinal studies utilising FFPE tissue samples with malignant transformation as the end point. In contrast, the primary aim of our review was to investigate the effectiveness of DNA-ICM using brushings. Brushings are widely accepted by patients as they do not cause pain or bleeding making it an acceptable and cost-effective screening tool. DNA-ICM using brushings could help triage lesions in community settings and thus reduce morbidity associated with unnecessary biopsies. It should be noted that this technology is not a replacement for biopsy but a second step in the community screening pathway which can help clinicians identify lesions which need further investigation. This would allow the high-risk lesions to receive timely intervention without pressuring the health care system in low resource countries. In addition, DNA-ICM could supplement biopsies by providing further information on the risk of malignant transformation in low-grade dysplasias. As reported by MacAulay et al use of an automated DNA-ICM system requires minimal human intervention and can process a large number of samples in a short period of time [22]. Community screening studies involving a large number of patients using automated DNA-ICM systems are required to draw stronger conclusions.

Limitations

There are a number of limitations identified in the studies which need to be emphasized. The variation in sample collection, lesion risk definitions, analysis, and interpretation methods did not allow us to pool the sensitivity and specificity values. The definition of the disease state for determining sensitivity and specificity, and the poor definition of cases (clinical vs. histopathological diagnosis) makes it difficult to draw conclusions. Most studies are cross-sectional in nature, do not provide lesion outcome information and hence, are not useful to predict malignant transformation.

QUADAS-2 tool was used to assess the risk of bias for the selected studies [54] (Fig. 2). Patient selection applicability concern was commonly identified as studies recruited patients with oral suspicious lesions referred to speciality hospitals for treatment. It is assumed that

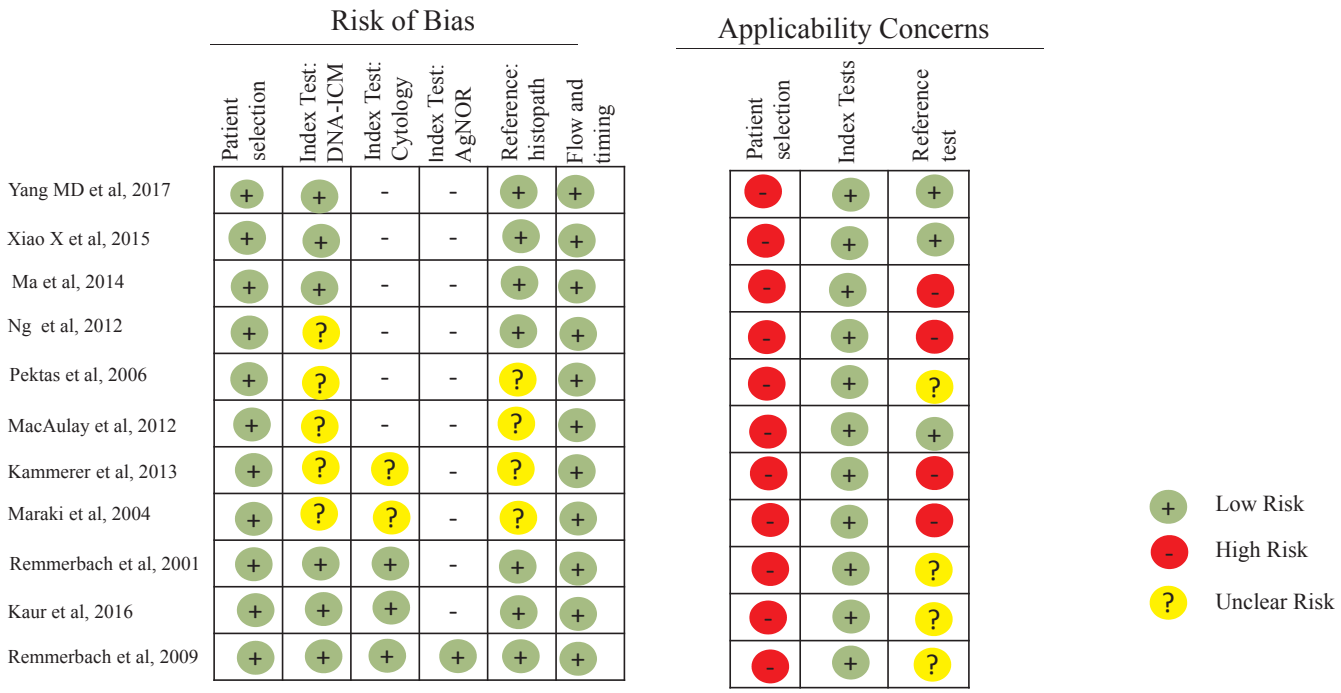

Fig. 2. Risk of bias and applicability summary. Quadas-2 tool was used to evaluate the risk of bias and applicability of the included studies on 4 key domains: patient selection, index test, reference standard, flow and timing of patients. Risk of bias in index tests (DNA-ICM and cytology) and reference (histopathology) tests was rated as unclear when re-evaluation was undertaken in case on contradictory test results. Patient selection applicability was a concern in all the studies as none of them were carried out in a community population and thus did not match our review question. Reference test applicability was also a concern due to varying high-risk lesion definition as determined by histopathology.

lesions referred to hospital settings are highly suspicious and more likely to be dysplasia or SCC. To adequately assess DNA-ICM's effectiveness as a screening tool, there is a need for community-based studies where a higher prevalence of benign lesions mimicking OPMLs may be encountered. Index test bias was also identified; three studies reassessed histology when the histology did not match cytology or DNA-ICM results. Reference test applicability was also commonly identified due to the inconsistent definition of low-risk lesions, none of which matched our research question.

Non-English articles were excluded from this review which may have led to omission of some important studies in this area.

Conclusion

In spite of DNA aneuploidy being accepted as a marker of malignancy, there is poor evidence to date of DNA-ICM using brushings being successful as an adjunct oral cancer screening tool. Studies with large sample sizes that follow established DNA quantification protocols for oral brushings are required (Fig. 3). In addition, there needs to be a clear distinction between histological and clinical diagnosis. There should be clear definitions of low and high-risk lesions. For screening studies, it is suggested that high-risk be defined as dysplasia or SCC.

Prospective studies are needed to establish the role of DNA-ICM in predicting malignant transformation of oral lesions using brushings. Repeated measures over time would also provide information on when changes in ploidy occur prior transformation. It is clear that further work is needed in this field before being adopted as an adjunct screening tool in clinical/community practice.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgements

The authors would like to acknowledge the literature search guidance provided by Dr. Kathryn Hornby, Medical Liaison Librarian, Woodward Library, The University of British Columbia, Vancouver.

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

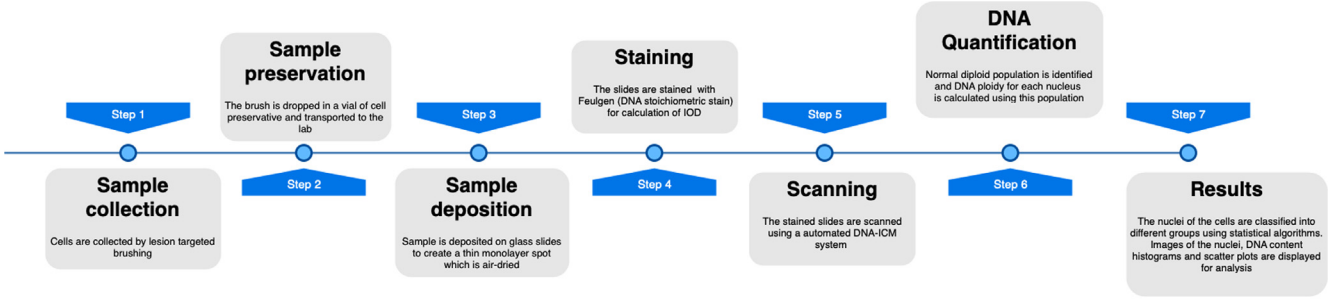

Figure 3: A simplified flow chart showing the ideal DNA-ICM protocol using brushings

Fig. 3. A simplified flow chart showing the ideal DNA-ICM protocol using brushings.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2019.07.006.

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68(6):394–424. https://doi.org/10.3322/caac.21492.

[2] Canadian Cancer Statistics publication – Canadian Cancer Society. www.cancer.ca. < http://www.cancer.ca/en/cancer-information/cancer-101/canadian-cancer-statistics-publication/?region=on > [Accessed August 24, 2018].

[3] Coelho KR. Challenges of the oral cancer burden in India. J Cancer Epidemiol 2012;2012. https://doi.org/10.1155/2012/701932.

[4] Warnakulasuriya S, Johnson NW, Waal IVD. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med 2007;36(10):575–80. https://doi.org/10.1111/j.1600-0714.2007.00582.x.

[5] Shirani S, Kargahi N, Razavi SM, Homayoni S. Epithelial dysplasia in oral cavity. Iran J Med Sci 2014;39(5):406–17.

[6] Cho K-J, Song JS. Recent changes of classification for squamous intraepithelial lesions of the head and neck. Arch Pathol Lab Med 2018;142(7):829–32. https://doi.org/10.5858/arpa.2017-0438-RA.

[7] Rock LD, Laronde DM, Lin I, et al. Dysplasia should not be ignored in lichenoid mucositis. J Dent Res 2018;97(7):767–72. https://doi.org/10.1177/0022034517748639.

[8] Schepman KP, van der Meij EH, Smeele LE, van der Waal I. Malignant transformation of oral leukoplakia: a follow-up study of a hospital-based population of 166 patients with oral leukoplakia from The Netherlands. Oral Oncol 1998;34(4):270–5. https://doi.org/10.1016/S1368-8375(98)80007-9.

[9] Speight PM, Khurram SA, Kujan O. Oral potentially malignant disorders: risk of progression to malignancy. Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125(6):612–27. https://doi.org/10.1016/j.oooo.2017.12.011.

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

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

[12] Lingen MW, Kalmar JR, Karrison T, Speight PM. Critical evaluation of diagnostic aids for the detection of oral cancer. Oral Oncol 2008;44(1):10–22. https://doi.org/10.1016/j.oraloncology.2007.06.011.

[13] Koch FP, Kaemmerer PW, Biesterfeld S, Kunkel M, Wagner W. Effectiveness of autofluorescence to identify suspicious oral lesions—a prospective, blinded clinical trial. Clin Oral Investig 2011;15(6):975–82. https://doi.org/10.1007/s00784-010-0455-1.

[14] Epstein JB, Zhang L, Poh C, Nakamura H, Berean K, Rosin M. Increased allelic loss in toluidine blue-positive oral premalignant lesions. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003;95(1):45–50. https://doi.org/10.1067/moe.2003.97.

[15] Marzouki HZ, Tuong Vi VuT, Ywakim R, Chauvin P, Hanley J, Kost KM. Use of fluorescent light in detecting malignant and premalignant lesions in the oral cavity: a prospective, single-blind study. J Otolaryngol – Head Neck Surg 2012;41(3):164–8.

[16] Farah CS, McIntosh L, Georgiou A, McCullough MJ. Efficacy of tissue autofluorescence imaging (VELScope) in the visualization of oral mucosal lesions. Head Neck 2012;34(6):856–62. https://doi.org/10.1002/hed.21834.

[17] Laronde DM, Williams PM, Hislop TG, et al. Influence of fluorescence on screening decisions for oral mucosal lesions in community dental practices. J Oral Pathol Med 2014;43(1):7–13. https://doi.org/10.1111/jop.12090.

[18] Rashid A, Warnakulasuriya S. The use of light-based (optical) detection systems as adjuncts in the detection of oral cancer and oral potentially malignant disorders: a systematic review. J Oral Pathol Med 2015;44(5):307–28. https://doi.org/10.1111/jop.12218.

[19] Pallagatti S, Sheikh S, Aggarwal A, et al. Toluidine blue staining as an adjunctive tool for early diagnosis of dysplastic changes in the oral mucosa. J Clin Exp Dent 2013;5(4):e187–91. https://doi.org/10.4317/jced.51121.

[20] Gray M, Gold L, Burls A, Elley K. The clinical effectiveness of toluidine blue dye as an adjunct to oral cancer screening in general dental practice. A West Midlands Development and Evaluation Service Report; 2000. 2000:57.

[21] Zhang L, Williams M, Poh CF, et al. Toluidine blue staining identifies high-risk primary oral premalignant lesions with poor outcome. Can Res 2005;65(17):8017–21. https://doi.org/10.1158/0008-5472.CAN-04-3153.

[22] Guillaud M, Benedet JL, Cantor SB, Staerkel G, Follen M, MacAulay C. DNA ploidy compared with human papilloma virus testing (Hybrid Capture II) and conventional cervical cytology as a primary screening test for cervical high-grade lesions and cancer in 1555 patients with biopsy confirmation. Cancer 2006;107(2):309–18. https://doi.org/10.1002/cncr.21993.

[23] Rabinovitch PS, Dziadon S, Brentnall TA, et al. Pancolonic chromosomal instability precedes dysplasia and cancer in ulcerative colitis. Can Res 1999;59(20):5148–53.

[24] Giaretti W, Monteghirfo S, Pentenero M, Gandolfo S, Malacarne D, Castagnola P. Chromosomal instability, DNA index, dysplasia, and subsite in oral premalignancy as intermediate endpoints of risk of cancer. Can Epidemiol Biomark Prev 2013;22(6):1133–41. https://doi.org/10.1158/1055-9965.EPI-13-0147.

[25] Merkel DE, McGuire WL. Ploidy, proliferative activity and prognosis. DNA flow cytometry of solid tumors. Cancer 1990;65(5):1194–205.

[26] Bradley G, Odell EW, Raphael S, et al. Abnormal DNA content in oral epithelial dysplasia is associated with increased risk of progression to carcinoma. Br J Cancer 2010;103(9):1432–42. https://doi.org/10.1038/sj.bjc.6605905.

[27] Reid BJ, Levine DS, Longton G, Blount PL, Rabinovitch PS. Predictors of progression to cancer in Barrett's esophagus: baseline histology and flow cytometry identify low- and high-risk patient subsets. Am J Gastroenterol 2000;95(7):1669–76. https://doi.org/10.1111/j.1572-0241.2000.02196.x.

[28] Giaretti W, Pentenero M, Gandolfo S, Castagnola P. Chromosomal instability, aneuploidy and routine high-resolution DNA content analysis in oral cancer risk evaluation. Future Oncol Lond Engl 2012;8(10):1257–71. https://doi.org/10.2217/fon.12.116.

[29] Faranda A, Costa A, Canova S, Abolafio G, Silvestrini R. Image and flow cytometric analyses of DNA content in human solid tumors. A comparative study. Anal Quant Cytol Histol 1997;19(4):338–44.

[30] Hedley DW. Flow cytometry using paraffin-embedded tissue: five years on. Cytometry 1989;10(3):229–41. https://doi.org/10.1002/cyto.990100302.

[31] Gouvêa AF, Santos Silva AR, Speight PM, et al. High incidence of DNA ploidy abnormalities and increased Mcm2 expression may predict malignant change in oral proliferative verrucous leukoplakia. Histopathology 2013;62(4):551–62. https://doi.org/10.1111/his.12036.

[32] Nieburgs HE, Pund ER. Detection of cancer of the cervix uteri: evaluation of comparative exfoliative cytologic diagnosis: a study of 10,000 cases. J Am Med Assoc 1950;142(4):221–6. https://doi.org/10.1001/jama.1950.02910220001001.

[33] Cowpe JG, Longmore RB, Green MW. Quantitative exfoliative cytology of abnormal oral mucosal smears. J R Soc Med 1988;81(9):509–13. https://doi.org/10.1177/014107688808100905.

[34] Littenberg B. Technology assessment in medicine. Acad Med J Assoc Am Med Coll 1992;67(7):424–8.

[35] Xiao X, Shi L, Li H, Song Y, Liu W, Zhou Z. DNA content status using brush biopsy with image cytometry correlated with staging of oral leukoplakia: a preliminary study. Oral Oncol 2015;51(1):59–63. https://doi.org/10.1016/j.oraloncology.2014.10.009.

[36] Yang X, Xiao X, Wu W, et al. Cytological study of DNA content and nuclear morphometric analysis for aid in the diagnosis of high-grade dysplasia within oral leukoplakia. Oral Surg Oral Med Oral Pathol Oral Radiol 2017;124(3):280–5. https://doi.org/10.1016/j.oooo.2017.05.509.

[37] Maraki D, Becker J, Boecking A. Cytologic and DNA-cytometric very early diagnosis of oral cancer. J Oral Pathol Med 2004;33(7):398–404. https://doi.org/10.1111/j.1600-0714.2004.0235.x.

[38] Kämmerer PW, Koch FP, Santoro M, et al. Prospective, blinded comparison of cytology and DNA-image cytometry of brush biopsies for early detection of oral malignancy. Oral Oncol 2013;49(5):420–6. https://doi.org/10.1016/j.oraloncology.2012.12.006.

[39] Ma J-M, Zhou T-J, Wang R, et al. Brush biopsy with DNA-image cytometry: a useful and noninvasive method for monitoring malignant transformation of potentially malignant oral disorders. Eur Arch Oto-Rhino-Laryngol 2014;271(12):3291–5. https://doi.org/10.1007/s00405-014-2935-4.

[40] MacAulay CE, Poh CF, Guillaud M, et al. High throughput image cytometry for detection of suspicious lesions in the oral cavity. J Biomed Opt 2012;17(8):086004. https://doi.org/10.1117/1.JBO.17.8.086004.

[41] Ng SP, Mann IS, Zed C, Doudkine A, Matisic J. The use of quantitative cytology in identifying high-risk oral lesions in community practice. Oral Surg Oral Med Oral Pathol Oral Radiol 2012;114(3):358–64. https://doi.org/10.1016/j.oooo.2012.06.008.

[42] Remmerbach TW, Weidenbach H, Pomjanski N, et al. Cytologic and DNA-cytometric early diagnosis of oral cancer. Anal Cell Pathol 2001;22(4):211–21.

[43] Remmerbach TW, Meyer-Ebrecht D, Aach T, et al. Toward a multimodal cell analysis of brush biopsies for the early detection of oral cancer. Can Cytopathol 2009;117(3):228–35. https://doi.org/10.1002/cncy.20028.

[44] Kaur M, Handa U, Mohan H, Dass A. Evaluation of brush cytology and DNA image cytomtry for the detection of cancer of the oral cavity. Diagn Cytopathol 2016;44(3):201–5. https://doi.org/10.1002/dc.23422.

[45] Pektas ZO, Keskin A, Günhan O, Karslioğlu Y. Evaluation of nuclear morphometry and DNA ploidy status for detection of malignant and premalignant oral lesions: quantitative cytologic assessment and review of methods for cytomorphometric measurements. J Oral Maxillofac Surg 2006;64(4):628–35. https://doi.org/10.1016/j.joms.2005.12.010.

[46] Garner D. Clinical application of DNA ploidy to cervical cancer screening: a review. World J Clin Oncol 2014;5(5):931–65. https://doi.org/10.5306/wjco.v5.i5.931.

[47] Pihan G, Doxsey SJ. Mutations and aneuploidy: co-conspirators in cancer? Cancer Cell 2003;4(2):89–94.

[48] Guillaud M, Ye Q, Leung S, et al. Large-scale DNA organization is a prognostic marker of breast cancer survival. Med Oncol Northwood Lond Engl 2017;35(1):9–9. doi: 10.1007/s12032-017-1068-1.

[49] Böcking AH, Friedrich D, Meyer-Ebrecht D, Zhu C, Feider A, Biesterfeld S. Automated detection of cancer cells in effusion specimens by DNA karyometry. Can Cytopathol 2019;127(1):18–25. https://doi.org/10.1002/cncy.22072.

[50] Haroske G, Baak JP, Danielsen H, et al. Fourth updated ESACP consensus report on diagnostic DNA image cytometry. Anal Cell Pathol 2001;23(2):8995.

[51] Navone R, Burlo P, Pich A, et al. The impact of liquid-based oral cytology on the diagnosis of oral squamous dysplasia and carcinoma. Cytopathology 2007;18(6):356–60. https://doi.org/10.1111/j.1365-2303.2006.00402.x.

[52] Zaini ZM, McParland H, Møller H, Husband K, Odell EW. Predicting malignant progression in clinically high-risk lesions by DNA ploidy analysis and dysplasia grading. Sci Rep 2018;8(1):15874. https://doi.org/10.1038/s41598-018-34165-5.

[53] Remmerbach TW, Weidenbach H, Müller C, Hemprich A, Pomjanski N, Buckstegge B, et al. Diagnostic value of nucleolar organizer regions (AgNORs) in brush biopsies of suspicious lesions of the oral cavity. Anal Cell Pathol 2003;25(3):139–46. https://doi.org/10.1155/2003/647685.

[54] Bristol U of. QUADAS | Bristol Medical School: Population Health Sciences | University of Bristol [Internet] [cited 2019 May 2]. Available from: < https://www.bristol.ac.uk/population-health-sciences/projects/quadas/ > .