Dna Image Cytometry as A Promising Noninvasive Diagnostic Approach for Oral Potentially Malignant Disorders and Oral Cancer – An Overview

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Abstract:

The majority of oral cancers are oral squamous cell carcinomas that arise from potentially malignant lesions in the oral mucosa. Early detection of neoplastic changes will significantly

increase the survival rate and improve patient quality of life. Chromosomal aberrations are one of the earliest neoplastic changes occurring in dysplastic oral mucosa. Current evidence indicates DNA aneuploidy to be an accepted biomarker of malignant transformation. DNA nuclear content measurement by image cytometry and its clinical relevance as a diagnostic tool has been explored in oral cancer detection. The combination of DNA ploidy and cytobrush techniques can offer non-invasive, economic, and early detection of carcinomas. However, substantiated evidence is required to establish the same, as large-scale population-based studies are lacking. This review describes DNA ploidy analysis by image cytometry, molecular aspects, principle, and methodology. A short note on studies evaluating the efficacy of DNA ploidy by brushings as a screening tool and the scope of DNA image cytometry as a predictive marker is mentioned along with limitations.

KEYWORDS:DNA ploidy, Oral Potentially Malignant Disorders

Introduction:

Any malignant neoplasia arising from lips or oral cavity is termed Oral cancer and it is the sixth most common type of all cancers occurring globally. World Health Organization (WHO) South East Asia Region reported the highest numbers with most cases from India and neighbouring countries.[1] The annual incidence rate in India is high with nearly 77,000 new cases and has a mortality rate of around 50,000 deaths yearly.[2] The general 5-year survival rate is also low in India compared to the West, as in most of the cases about 60%-80% are detected at advanced stages.[2,3]

Approximately 90 % of oral cancers are Oral Squamous Cell Carcinoma (OSCC) originating from the squamous epithelium of oral mucosa.[1,2] Carcinogenesis- the development of cancer is a complex multifactorial process in which the chronic exposure of epithelial cells to risk factors, and the subsequent effect on homeostasis causes genetic instability and alterations.[1] Smoking, tobacco consumption, betel quid chewing, Human papilloma Virus (HPV) of the oropharynx, poor oral hygiene including periodontitis, and nutrient-deficient diet are established risk factors for squamous cell carcinoma.[2,3] It is widely established that OSCC mainly develops from “premalignant lesions or conditions” like leukoplakia, erythroplakia, lichen planus, tobacco pouch keratosis, and oral submucous fibrosis.[4]

The term pre-malignant lesion or condition broadly describes certain oral mucosal areas with altered morphological and clinical appearances, that may have the potential to become cancer. This conveys the concept of a two-step or multi-step process of cancer development, but it is also acknowledged that there is no underlying uniformity regarding the progress of these tissues or in individual patients.[5] The Working group of WHO Collaborating Centre for Oral Cancer and Precancer in the UK proposed the term “potentially malignant disorders”, to the precancer areas as all of them do not progress to cancer.[5]

Early identification and monitoring of potentially malignant tissues allow clinicians to detect intraepithelial stages of oral carcinogenesis like mild, moderate, or severe dysplasia and carcinoma in situ, which generally are precursors of invasive OSCC.[6] The prognostic

implications of early diagnosis and treatment are advocated by the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) asserting that a third of a predicted 15 million cancer cases in the future can be reduced and another third can be more effectively manage by planning effective cancer control and screening strategies.[6]

Several diagnostic methods beginning with a clinical visual examination at a dental office, vital staining techniques using toluidine blue, Lugol's iodine, light-based fluorescence techniques, imaging techniques, spectroscopy, and cytological methods are used as screening and diagnostic tools, each having their advantages and limitations.[2,7] Biopsies with histological assessment have long been accepted and are even termed the "gold standard" in detecting suspicious lesions. But the invasiveness of the procedure, the dentist's efficiency to perform biopsies, and in some may not be feasible like patients taking anticoagulant medications are drawbacks of scalpel biopsies. Also, variations in inter-and intraindividual reproducibility of histological grading epithelial dysplasia and unequivocally identifying carcinoma in situ led to the development of further techniques.[8]

Recently, cytopathology is being explored as an adjunctive diagnostic method, and also potentially malignant areas demonstrated chromosomal, genomics, and molecular alterations similar to invasive cancers.[8,5] DNA content imbalance or aneuploidy was a proven biomarker in cervical, and colon malignancies and prognostic aid in prostrate cancer.[9] DNA content assessment is done by computer-aided analysis and DNA image cytometry (DNA-ICM) is one of the techniques used to quantify DNA content.[10] This review describes the DNA image cytometry technique and its application in oral cancer detection and potentially malignant disorders.

Biological aspects of cell:

Every organism has a unique amount of DNA in its nucleus within a cell and usually contains ‘diploid amount’ (2c) – euploidy of DNA. All the healthy cells in an organism contain the same amount except 3 types of cells 1) Gametes - cells that have gone through meiosis for sexual reproduction and contain a haploid amount of DNA(1c); 2) cells preparing for division or undergoing mitosis for a short period, contain double the amount of diploid DNA, i.e.,4c the DNA amount in these cells is between 2 c and 4c; and3) cells going through apoptosis in which DNA is fragmented and begins to lose.[11]

Rationale:

One of the early key events in malignancies is chromosomal aneuploidy characterized by numerical and/or structural aberrations caused by genetic instability. DNA aneuploidy is the cytometric equivalent of chromosomal aneuploidy and is considered a marker of neoplasia.[12]

The DNA ‘ploidy' cannot be the cytometric equivalent of chromosomal ploidy as the latter can be detected with cytogenic techniques in every single cell the former cannot be equated similarly. Hence DNA ploidy refers to the status of the typical large-scale genomic status of a cell population and can also be equivalent to the single-cell status.[12] The DNA content is expressed in a ‘*c*’ scale, for example- 1c denotes half of the mean nuclear content of cells from a normal diploid population undergoing the G0/G1 cell cycle phase.[12] DNA nuclear

quantity may be influenced by replication, polyploidization, gain, or deletion each having an impact on the size or the number of chromatids.[11]

Definitions of basic terms of DNA image cytometry:

It is important to know the terminology involved and definitions are by G.Haroske et al(Fourth Updated ESCAP consensus report 2004)-[12]

a) DNA stemline - a stemline means a proliferating cell population with a unique chromosomal outfit. A DNA-stemline is the G0/G1cell-phase fraction of a proliferating cell population (with a first peak and a second doubling one, or nuclei in the doubling region).[12]
b) DNA euploidy - the type of DNA distributions that cannot be differentiated from those of normal (resting, proliferating, or polyploidizing) cell populations.[12]
c) DNA aneuploidy - types of DNA distributions that are different at a statistically significant level from those of normal (resting, proliferating, or polyploidizing) cell populations. DNA aneuploidy can either be seen as DNA stemline aneuploidy or can be indicated by "rare events".[12]
d) DNA diploidy - type of euploid DNA histograms which is the cytometric equivalent of resting or proliferating cell population with a diploid chromosomal set.[12]
e) Polyploidization - means the (repeated) doubling of a chromosomal set.
    i) Euploid DNA polyploidization means the occurrence of peaks in the duplication (×2, ×4, ×8, ...) regions of euploid stemlines. In human tissues usually, the highest peak is at 2c.
    ii) Aneuploid polyploidization means the occurrence of peaks in the duplication region of aneuploid stemlines.[12]
f) DNA histogram - a frequency distribution of IOD values obtained by cytometric measurements of cells stained stoichiometrically for their DNA and rescaled by IOD values from reference cells in “c” units.[12]

    Rare events in DNA histograms are abnormal cells often called 5c or 9c exceeding events, having a nuclear DNA content higher than the duplicate or quadruplicate region of a normal G1/G0 phase population, i.e., not belonging to the G2M phase. They likely represent non-proliferating abnormal cells with different chromosomal aneuploidies and abnormally high numbers of chromosomes.[12]

The basic objective of DNA-ICM is to identify abnormal or aneuploid DNA stemlines at a defined statistical level of significance, in addition, should provide information on the number of abnormal stemlines, the euploid or aneuploid DNA stemlines with polyploidisation, the occurrence of rare cells with an abnormally high DNA and cell cycle fractions.[12]

Various methods have been prompted to assess nuclear DNA content by computer-aided analysis. Among the two most common techniques: one is DNA-ICM as stated earlier the other is flow cytometry (FCM).[10] In FCM, the DNA content is stained with a specific fluorophore -diamidino-2-phenylindole (DAPI). The DNA content is measured by the intensities of the fluorescence emitted when cells rapidly flow one by one across the focused

laser beam.[10] Whereas, in ICM the measurements are obtained from cells or nuclei stained with a dye that binds stoichiometrically to DNA content.[10]

Sample Collection:

The cells can be collected from superficial and intermediate layers of oral mucosa by excisional or incisional biopsies. Tissues from fresh frozen or formalin-fixed paraffin-embedded can be subjected to proteolytic digestion and nuclei suspension is created or affixed to a glass slide for DNA content analysis.[9]

Brush biopsies have an advantage over scalpel biopsies because of the non-invasive nature of the method.[9] The brush can be rolled over suspicious several times and immediately smeared on a glass slide and cells are fixed using an alcoholic spray.[13] Alternatively in liquid-based cytology, cells are collected and suspended in a vial of cell preservative and a monolayer of cells is prepared on a glass slide in the lab. This method has advantages like reduced blood and debris depositions in preparation. The disadvantages include higher costs for pathologists and a reduced retention rate of removed cell material.[7,13]

Technique:

In most automated DNA-ICM systems, a minimum of 300–400 cells are required to provide a satisfactory diagnosis with reliable results.[9] For DNA analysis the specimens are stained with stoichiometric nuclear stain- 'the Feulgen stain' and a cytoplasmic counterstain. The Feulgen stain is recommended rather than convention hematoxylin and eosin stains or pap stain. This stain binds stoichiometrically to DNA- each fixed molecule of Schiff's reagent correlates to an equal amount of DNA. So, the staining intensity is proportional to the amount of DNA present in the nucleus.[7]

The principle behind DNA-ICM is the measurement of light absorption by Feulgen-stained nuclei using a video camera with a charge-coupled device (CCD) sensor. The advantages of video-image cytometers like high-efficient, ease of usage, and less cost led to instrument development and research. A few first-generation systems were SAMBA (Unilog, France), AccuMed Cyto-Savant (AccuMed, Chicago, IL), and in them optical problems like glare and shading errors which influence densiometric measurements were not solved.[11]

Currently available systems are a new generation with high-resolution video cameras, automated scanning microscopes, and computers with high processing and storage capacities, with shading corrections provided by software programmes. Examples include CYDOK (Hilgers; Koönigs, Germany), and AUTOCYTE QUIC DNA (TriPath, Burlington, NC). Since a wide variety of DNA-ICM instruments are available, for quality assured work and reliable diagnosis, instruments that are in accordance with ESACP performance standards are recommended.[11]

In cytometry the DNA content cannot be measured directly, the nuclear IOD (Integrated optical density) which is the cytometric equivalent of a cell's DNA content is measured in arbitrary units (AU). The IOD values are rescaled to 'c' units and are compared against cells with known DNA content called reference cells like internal epithelial or lymphocyte cells. In

context to reference cells, the arbitrary unit scale (AU) is expressed in a reference unit scale for example 2c,4c, or, 8c.

The corrective factor is the mean ratio between the modal IOD values of the non-pathologic cells of the tissue under study and the reference cells. The accuracy of diagnostic DNA evaluation depends on the standard deviation (SD) of the corrective factor used during the rescaling procedure.[11,12]

The results of DNA measurements are expressed in DNA histograms. The European Society for Analytical Cellular Pathology guidelines is usually followed for diagnostic interpretation of DNA measurements.[12]

Algorithms in Diagnostic interpretation:[11]

DNA stemlines with a modal value between 1.8c and 2.2c possess diploid euploidy while between 3.6 and 4.4 c possess tetraploid euploidy. DNA stemlines with modal values < 1.80 c or >2.20 c and <3.60 c or >4.40 c has aneuploidy stemline. These are abnormal cells and often are called 5 c– or 9 c–exceeding events (5c-EE or 9c-EE), with nuclear DNA contents higher than the duplicate or quadruplicate of a normal G0/G1 phase population.

DNA ploidy as a predictive indicator for malignant transformation:

Diagnostic tests and screening tests help in assessing health by providing information about the presence or absence of disease. Diagnostic tests are definitive about a targeted condition or disease, while screening tests are generally used in a large-scale population, are less expensive, but are ambiguous. Sensitivity and specificity both analyze the predictive validity of a screening test.[14] Despite long-time acceptance, dysplasia grading by histological assessment also is not a definite risk indicator for malignant transformation of OPMDs. Few cross-sectional and case-control studies have shown DNA ploidy analysis by image cytometry as predictive of neoplastic changes in oral dysplastic lesions.[15]

Remmerbach W et al (2003) demonstrated early detection of carcinoma of DNA-ICM and exfoliative cytology against histological assessment in four patients. Cytology detected the occurrence of cancer in three out of four, DNA aneuploidy in all the cases but histologically proven in the second biopsy after a time duration of at least one to 15 months. The combined cytology and DNA ploidy techniques sensitivity was 98.2%, specificity-100%, positive predictive value-100%, and negative predictive valu-99.5%. They concluded DNA aneuploidy to be a promising marker for detecting cancer months before histological grading and suggested DNA-ICM as a mass screening tool in dental practice.[16]

Li C et al study, on a large-scale population, assessed DNA ploidy by ICM as a diagnostic marker for detection of dysplasia and OSCC. They optimized criteria of aneuploidy-noninvasive detection of oral dysplasia DI≥2.3 and carcinoma ≥3.5; concluded DNA aneuploidy using brushings to be a potential screening tool for noninvasive detection of oral dysplasia and OSCC in oral lesions.[17]

Shi L et al (2020) reviewed 9 studies of DNA aneuploidy as a marker of carcinoma in general lesions and reported pooled sensitivity and specificity as 84.8% and 99% respectively. The

authors opined that current evidence was not robust as the studies were carried out in small size populations and recommended more multi-center prospective studies.[18]

The systemic review by Datta M et al (2019) based on 11 studies have evaluated DNA-ICM as a screening tool using brushings. They reported a wide range of sensitivity (16–96.4%) and specificity (90–100%). The differences are attributed to study design, definitions of high-risk or low-risk lesions, and DNA-ICM protocol. The authors suggested more studies with a large sample size before consolidating DNA-ICM with brushings as an adjunct oral cancer screening tool. However, they concluded DNA-ICM with cytobrush would reduce unnecessary biopsies, and triage lesions in community settings. The detection of high-risk lesions and timely treatment would reduce the burden on the health care system in low resourceful countries.[9]

Alaizari et al performed a meta-analysis on 5 longitudinal studies- Sperandio et al. 2013, Bradley et al. 2010, Siebers et al. 2013, Bremmer et al. 2011, and Torres -Rendon et al. 2009 assessing aneuploidy as a risk marker for malignant transformations in OPMDs. The first three are retrospective, cohort studies of OPMD lesions. The remaining 2 studies were the retrospective case -controls, focusing on dysplastic lesions. There was found to be an association between aneuploidy, with a 3.12 -fold increased risk to progress into cancer.[19]

The study by Torres Rendon (2009) using DNA image cytometry, studied 86 lesions of oral epithelial dysplasia and their progression to carcinomas for a minimum follow-up of 5 years. They reported that 42 lesions progressed to carcinomas while 44 lesions did not show any progressions. The sensitivity was low but specificity was good with 74% of aneuploid lesions progressing to OSCC compared to only 42% of diploid lesions. Since only 14 out of 42 lesions with aneuploidy turned malignant, aneuploidy is not definitely associated with malignant progression. A contradiction to previous reports the article suggests that all cancers do not necessarily exhibit aneuploidy genomic aberrations. The authors do not rule out the probability of non-detecting the subtle chromosomal errors or nuclear changes by image cytometry but suggest the DNA content analysis as an adjuvant to histopathological examination.[20]

DNA ICM role in oral leukoplakia:

Chitturi et al observed the ploidy status of exfoliated mucosal cells in oral lichen planus patients by DNA-ICM. The cells in reticular and plaque types of OLP mostly exhibited diploidy whereas aneuploidy was exhibited by the cells in erosive and atrophic types of OLP. The association of majority cases with aneuploidy in erosive and atrophic subtypes was also reflected in *Hosni et al* study. Ploidy status by image cytometry in potentially malignant lesions like leukoplakia can help predict the prognosis. They concluded it is important to follow up on leukoplakia patients and cytology can be an adjuvant for ploidy status detection along with DNA-ICM. The cytological examinations are recommended for diagnostic purposes, follow-up, and mass screening of precursor lesions such as oral submucous fibrosis, leukoplakia, and erythroplakia.[21]

Sudbo J et al assessed 150 patients with leukoplakia classified as epithelial dysplasia and DNA ploidy status was evaluated by image cytometry. Carcinomas developed in 21(84%) out of 25 patients with aneuploid lesions, the study concluded DNA nuclear content analysis in leukoplakia cases can be a risk predictor of carcinoma.[22]

Limitations:

Another method to quantify DNA nuclear content is flow cytometry, it is relatively quick and gives more measurement precision in a large population of tumor nuclei, but does not offer visual control of measured objects, do not differentiate abnormal or normal cell and large sections of the tissue sample are required to prepare a suspension.[23] Russack v (1994) stated though ICM, is advantageous for small size populations, it is moderately labor tensive, and statistical evaluation is difficult. Feulgen staining procedure is not simple and at times cytopathologists require restaining for additional characterizations, which is not possible with Feulgen stain.[24]

DNA ploidy analysis in oral cancer has limitations owing to the heterogeneity of DNA content in malignant and potentially malignant lesions. Diwakar et al study among 42 samples of OSCC, found that 22(52%) are aneuploid, 1-tetraploid, 3-diploid, and 16-heterogeneity nature.

Hitherto, not all malignant cases are aneuploidy, and diploid cases can exhibit neoplastic changes as cited by Torres Rendon, Kaur, et al and a few other authors. Nevertheless, the combination of DNA ploidy with cytology or histological examination substantially improves the specificity and sensitivity of detecting malignant transformation.[25]

The majority of studies examined the samples from developed countries in which other risk factors in the development of cancer like nutritional deficiencies, and low socioeconomic status cannot be effectively correlated with the progression of cancer. Though DNA ploidy inspects nuclear aberrations irrespective of other initiating factors which influence the development of cancer, more studies are recommended from developing countries.[19]

And finding a very rare cell and segmenting it out in image cytometry can be difficult owing to overlapping and fragmentation.[10] In comparison with histological grading assessment ploidy analysis is an objective method and has well-established strict threshold criteria for ploidy classification.[19] The automated image analysis offers a quick and easy detection but instrument-based errors like unwanted glare in image, magnification, and sensitivity of digitizing tablet can occur.[25] Image cytometry offers only 2D analysis, and nuclear chromatin features are three-dimensional, hence for accurate results in early detection there can be limitations.[10]

Conclusion:

Aneuploidy is associated with a higher risk for neoplastic changes compared to a diploid sample however diploid status does not rule out malignancy transformation in the future. DNA content analysis can be a good predictive indicator in combination with cytology brushings or histological grading of dysplastic lesions. The histological assessment requires a

trained pathologist, is expensive, and is limited to health systems. In the case of brushings, they are more readily accepted by patients, less technique sensitive and the image cytometry can automatically detect the nuclear changes utilizing image analysis software. The coalition of DNA-ICM and cytobrush techniques can be supportive in screening potential malignant lesions and carcinomas, further research is warranted particularly in developing countries.

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