REVIEW ARTICLE

Diagnostic aids in detection of oral precancer and cancer: Past to present

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Abstract

Oral carcinogenesis is a stepwise accumulation of genetic damage over time. The older cancer diagnostic aids had less specificity, were time-consuming, and produced inter-observer bias. Technological and therapeutic advances in the recent years have helped to diagnose and treat this disease at an early stage. Advances in molecular biology over the past decade have helped us to enhance our understanding of the complex interplay between genetic, transcriptional, and translational alterations in human cancers. This review provides a summary of all the diagnostic modalities that were used earlier and the newer more advanced techniques with merits and demerits of each technique described briefly.

Introduction

"Oral cancer" encompasses all malignancies originating in the oral cavity. Oral cancer ranks sixth in the overall incidence for the 10 most common cancer sites worldwide and third in the developing countries.[1] Early diagnosis greatly increases the probability of cure, with minimum impairment and deformity.[2] Annually 75,000-80,000 new oral cancer cases develop in India. They are of utmost concern as the mortality rate of oral cancer for the past three and a half decades has remained high (over 50%) in spite of new treatment modalities. The 5-year survival rate has remained approximately 50% for the last 50 years, despite numerous advanced treatment approaches. Early diagnosis of cancer is essential to recognize high-risk people as this would allow for conservative treatment with a fast recovery and better quality of life.[3,4] Suspected patients should be more intensively examined and treated. The current trend in diagnosis focuses in the areas of molecular biology, and advanced diagnostic aids will transform our traditional approaches to oral disease management.

The field of cancer diagnosis has come a long way, from earlier traditional approaches to the newer advanced diagnostic aids. The older to newer techniques are discussed in detail below.

Vital staining

Toluidine blue, an acidophilic metachromatic dye has been used as a vital stain, for the identification of dysplasia cases and early oral squamous cell carcinoma. Dysplastic and anaplastic cells contain more nucleic acids quantitatively than normal tissues, hence used *in vivo*. In addition, intracellular canals of the malignant epithelium are wider than normal epithelium; this is a factor that would enhance penetration of the dye. The test is sensitive, simple, non-invasive, and highly cost-effective. It assists in identifying the preferred area of biopsy and marking the borders of the lesion. This may lead to early detection, diagnosis, and in directing surgical management.[4,5]

Oral brush biopsy

Oral brush biopsy provides cytological evaluation of cellular dysplastic changes, which uses the concept of exfoliative cytology. The procedure is rapidly conducted chair side that is cost-effective and perhaps the best approach for the initial evaluation and diagnosis of oral diseases. It permits better selection of cases for biopsy and to help localize the optimal site for brushing an abnormality, conventional oral brush biopsy combined with the application of toluidine blue is used to localize suspected mucosal

areas. Brush cytology is an advantageous diagnostic procedure because it is non-invasive, relatively painless with minimum bleeding, and requires a minimum of technical skills. Despite the advantages of brush cytology, it has certain disadvantages such as inadequate sampling and false-negative results.[6,7]

Liquid-based cytology (LBC)

LBC is a method of preparing and processing smears. LBC has recently become an alternative to conventional Papanicolaou (PAP) smear in the detection of intraepithelial lesions. The smear takes a sample of cells from the lesion and places them into a liquid solution (polymer solution containing agarose, polyethylene glycol, poly-l-lysine, and alcohol). The material collected in the liquid fixative preserves the cells. The centrifugation machine removes excess blood, mucous, and inflammatory cells and produces a thin layer of cells on a glass slide. The cells are stained and examined under the microscope in the same way as the conventional smear test.[8]

LBC provides uniform smear thickness and it has good sensitivity for detecting abnormalities as conventional smears, and the technique is simple. It also provides material for further investigations including immunocytochemistry, human papillomavirus testing, argyrophilic nucleolar organizer regions, DNA ploidy, or laser scanning cytometry with good inter-observer reproducibility. It is useful in screening programs and for patient follow-up. The limitations of LBC technique are that it involves expensive automated devices and materials, requires trained users for interpretations, increased processing costs, and loss of specificity.[9,10]

Centrifuged LBC (CLBC)

CLBC is a modification of LBC, where a smear is taken and flushed in a solution, centrifuged, the obtained cell pellet is resuspended in 95% alcohol, left for 2 h and then stained with PAP stain. The results obtained by this method are of superior quality in comparison with conventional techniques.[11]

CLBC does not require sophisticated equipment; hence, it can be used by many cytopathology laboratories with limited resources. It provides clear background, reduced number of unsatisfactory slides, and reduced false-negative results. It also provides material for further investigations similar to LBC.[12]

Histopathology

The gold standard for diagnosis and staging of many diseases is histopathology, evolved from an era of diagnosis based on hematoxylin and eosin stained slides. Grading systems have been developed to predict tumor aggressiveness, and the pathologist's report often guides clinical treatment decisions. Although it is a fairly reliable and inexpensive method for detection of precancer and cancer, there are several limitations. In this method, the quantitative measurement lacks objectivity and reproducibility, it is less sensitive and prone to a lot of errors, time-consuming, and there is an increase in inter-observer variability.[13]

Immunohistochemistry (IHC)

IHC is a technique for identifying cellular or tissue constituents (antigens) using antigen-antibody interactions, the site of antibody binding being identified either by direct labeling of the antibody, or by use of a secondary labeling method. It has an apparent advantage over traditionally used special and enzyme staining techniques that identify only a limited number of proteins, enzymes, and tissue structures.

The advantages of IHC are that it is compatible with standard fixation and embedding procedures, it can be performed retrospectively in archival material, it is sensitive and specific and is applicable to almost any immunogenic molecule.[14-16]

Photodiagnosis

It is a non-invasive procedure which provides tissue diagnosis in real time through optical spectroscopy. It can be used in performing guided biopsies, hemoglobin estimation and monitoring, tissue perforation in free flap surgeries and monitoring drug levels during chemotherapy, detection of dysplasia, assessment of surgical margins, and in sentinel node biopsy.[17]

Velscope system

The Velscope uses a blue light with peak intensity at approximately 436 nm; this wavelength especially stimulates a green fluorescence. The principle of tissue autofluorescence was used in screening and diagnosis of precancerous lesions in the lung, uterine cervix, and skin in the past. This concept of diagnosing dysplastic lesions in the oral cavity is based on the structural and metabolic changes of the epithelium as well as the connective tissue when interacting with the light.[18]

Identafi 3000

The Identafi 3000 ultra shines a violet light of approximately 405 nm, which especially stimulates a blue/violet fluorescence. This technology combines three concepts: Fluorescence, fiber optics, and confocal microscopy to screen the specific lesional area with its margins. This device is small and portable which makes it accessible to all areas in the oral cavity. It also examines tissue reflectance based on changes in angiogenesis with green-amber light.[19,20]

Flow cytometry (FCM)

FCM is a means of measuring certain physical and chemical characteristics of cells or particles as they pass in a fluid stream by a beam of light.

Flow cytometry is the instrument that determines the characterization of cells in a complex mixture. The cells or biological particles are led in a stream past an illumination and light detection system. As the cells traverse the illumination spot one by one, a micro objective collects the scattered and fluorescent light from the cells and directs it to a set of photomultipliers. The

temporal, spatial, and chromatic filters eliminate background light and separate the signals from different fluorophores.

The digital acquisition electronic equipment measures the intensity of the light pulses from each of the photomultipliers. A cell sorter adds a means for separating the cells of interest from a heterogenous mixture after they have been measured and classified based on the electrostatic charge of the particles.

FCM detects DNA-aneuploidy and gives information about the occurrence and number of abnormal stem lines, polyploidization of euploid or aneuploid stem lines, cell cycle fractions and occurrence of rare aneuploid cells with an abnormally high DNA content, detection of loss of heterozygosity and thus aids in detection of oral precancer and cancer.

FCM has certain demerits, as they are expensive, requires high current levels, and consumes water for the cooling system. Here, the cells need to be in a single cell suspension, the approximate size of particles should be known. The operator should be aware of the optimal excitation and emission wavelengths of the dyes used. Complex biosafety systems must be employed to reduce the potential of infection for the operator.[21,22]

Polymerase chain reaction (PCR)

PCR is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

It is used for DNA cloning for sequencing, DNA-based phylogeny or functional analysis of genes, the diagnosis of hereditary diseases, the identification of genetic fingerprints, and the detection of molecular markers.[23]

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) contain sequences complementary to the target region along with a DNA polymerase, which enables selective and repeated amplification.

Saliva is an excellent diagnostic tool that has various clinical diagnostic applications as it harbors a wide range of biomarkers that can be evaluated through PCR. Tissue fluid levels of hormonal, immunological, or toxicological molecules can be assessed by evaluating the molecular composition of saliva. It may be used to measure specific salivary macromolecules as well as examining proteomic or genomic targets such as enzymes, cytokines, growth factors, metalloproteinases, endothelin, telomerase, cytokeratins, mRNAs, and DNA transcripts.[24,25]

New technology has been developed, such as DNA microarray, which gives hundred to thousand times more genetic information in a shorter period than the original PCR techniques.

Microarray technology

Microarray technology helps in the quantitative study of mRNA. The expression levels of thousands of genes are assessed at the same time. This provides a unique profile of panel of genes, increased or decreased in a given disease. It is a multiplex lab-on-a-chip that is a two-dimensional array on a solid substrate that assays large amounts of biological material using high-throughput screening miniaturized, multiplexed and parallel processing and detection methods. It helps to detect precancer and cancer, perform guided biopsies, estimate the drug dosage during chemotherapy, assess the surgical margins, and has a role in sentinel node biopsy.[26-28]

Nanodiagnostics

Nanodiagnostics is the term used for the application of nanobiotechnology in molecular diagnosis which is based on pharmacogenetics, pharmacogenomics, and pharmacoproteomics information. It involves the application of nanoparticles, the use of manufactured nanorobots to make repairs at the cellular level. It is used in the discovery of biomarkers and the management of cancer through personalized medicine.[29]

Use of nanodiagnostics is cost-effective and has increased sensitivity. The tools include quantum dots (QDs), gold nanoparticles, and cantilevers. QDs are semiconductor nanocrystals characterized by high photostability, single wavelength excitation, and size-tunable emission. Barcoding of specific analytics can be done by QDs and magnetic nanoparticles. The QD technology can be used for detection of infectious agents and tumors, IHC, imaging of tissue and intracellular structures, advanced diagnostics, and fluoroimmunoassay.[30]

Laser-induced fluorescence (LIF)

LIF is a non-invasive, easy tool used for the detection of structural and chemical alterations of the cells. It is a spectroscopic method, in which an atom or molecule is excited to a higher energy level by the absorption of laser light and then followed by spontaneous emission of light. LIF finds its application in the study of the structure of molecules, detection of selective species, flow visualization, and measurements.[31] The wavelength is often selected to be the one at which the species has its largest cross section. The excited species de-excite and emit light at a wavelength longer than the excitation wavelength usually in the order of few nanoseconds to microseconds. This fluorescent light is typically recorded with filtered photodiodes or photomultiplier tube. Autofluorescence is due to the presence of fluorophores in tissue matrix and intracellular molecules such as elastin, collagen, and nicotinamide adenine dinucleotide hydrogen (NADH). As there are many fluorophores, e.g., pyridoxine, collagen, elastin, NADH, porphyrins, and flavins which have strong absorption bands in the 300-500 nm range, they give rise to fluorescence in the 350-700 nm range.[32]

In situ hybridization (ISH)

ISH is a powerful technique and unique in the way that allows the study of the macroscopic distribution and cellular localization of DNA and RNA sequences in a heterogeneous cell population.[33]

Hybridization of nucleic acid sequence to a probe in its cellular environment allows correlation with histologic alterations. DNA or RNA in a cell is identified *in situ* using a complementary probe of RNA or DNA. The labeled probe is detected using an antibody directed against the label. The complex is then visualized using a fluorochrome or by peroxidase reaction of a substrate, similar to IHC.

ISH helps in detection of various infective agents, the study of cell development, human gene mapping, and cytogenetics. However, the disadvantage is that it cannot provide information on translational and post-translational modifications.[34]

Ratio imaging

Ratio imaging is a popular method to analyze ion concentration change in living cells such as calcium ion and pH. Ratio imaging requires cameras with high stability and high quantitative precision for capturing multiple spectrum images.

It compares a photochemical or metabolic end-product of the intracellular compound, one which is increased in disease state, and another that is decreased in the same diseased state. The use of ratiometric fluorescent probes allows measurement of intracellular pH and calcium concentration at the single cell level, thus helping in the study of a multitude of cellular processes. They provide a non-invasive and semiquantitative assessment, thus used in biological applications and providing improved microscope optics.[35,36]

Elastic scattering spectroscopy

Elastic scattering spectroscopy generates a spectrum dependent on wavelength, which reflects changes in structure and morphology of tissues at scattering centers such as the chromatin, nucleus, sub-cellular organelles, structural proteins, lipids, and erythrocytes. The cellular and sub-cellular changes are identified using the refractive indices of the cellular components. The resultant scattered light is collected and analyzed by a spectrometer, and a spectrum is generated. The light emitted by cellular and sub-cellular organelles ranges from 330 to 850 nm, which is within the near ultraviolet and visible part of the spectrum.

Limitations include lack of tumor margin detection which would entail treating much larger area than that detected positively and the procedure is time-consuming.[37,38]

Optical coherence tomography (OCT)

OCT is an imaging technique that uses light to capture micrometer-resolution, three-dimensional (3D) images from within optical scattering media like tissue specimen. It is based on low-coherence interferometry, employing near-infrared light. It is a new optical technique that uses high-resolution, minimally invasive imaging of surface abnormalities in complex tissues. It provides cross-sectional, high-resolution subsurface tissue images.[39,40]

Raman spectroscopy

It is a spectroscopic technique used to observe vibrational, rotational, and other low-frequency modes in a system which provides a fingerprint by which molecules can be identified. It is a unique optical diagnostic technique that can detect changes in the biomolecules of the tissue. It is based on energy levels of tissue molecules by definite frequencies, above and below the incident photon that emerges out after interacting with the tissue. It is sensitive and real-time imaging at the molecular level and has high biomolecular specificity for *in vitro* and *in vivo* diagnosis of malignancies in various organs.[41,42]

Multiphoton excited fluorescence

Multiphoton excited fluorescence is an advanced optical method that uses high-resolution fluorescence imaging which allows imaging of living tissues up to a depth of about one millimeter. The excitation wavelength here is shorter than the emission wavelength in comparison with the traditional fluorescence microscopy. The wavelengths of the two exciting photons are longer than the wavelength of the resulting emitted light.

Changes in nuclear density with depth, keratin, and epithelial thickness can be noted along with providing insight into the changes in fluorescence intensity with precancer/cancer development even at the cellular level. 3D images of endogenous tissue fluorescence can effectively distinguish between normal, precancerous, and cancerous epithelial tissues with high-resolution capability.[43,44]

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy relies on the phenomenon of nuclear magnetic resonance and can

Table 1: Different diagnostics aids in detection of precancer and cancer

Chair side/routine tests	Molecular level techniques	Optical techniques
Vital staining	Polymerase chain reaction	Photodiagnosis
Oral brush biopsy	Nanodiagnostics	Flow cytometry
Oral fluid testing	*In-situ* hybridization	Nanodiagnostics
LBC	Microarray technology	Laser induced fluorescence
CLBC		Ratio imaging
Histopathology		Elastic scattering spectroscopy
IHC		Optical coherence tomography
		Raman spectroscopy
		Multiphoton excited fluorescence
		Nuclear magnetic resonance spectroscopy

LBC: Liquid-based cytology, CLBC: Centrifuged liquid-based cytology, IHC: Immunohistochemistry

Table 2: Summary of the merits and demerits of the various diagnostics aids in detection of precancer and cancer

Diagnostic aids used in detection of cancer	Merits	Demerits
Vital staining	• Determines the suspected site and margins of the lesions	↓Sed specificity
Oral brush biopsy	• Easy, non-invasive, and painless	• Inadequate sampling • ↓Sed specificity
LBC	• Uniform smear thickness • Simple and sensitive technique • Adequate material for further investigation	• Less specific and expensive • Mandates technicians for interpretations
CLBC	• Yields clear background • ↓Number of unsatisfactory slides • False-negative results • Retains adequate material for further investigations	Technique sensitive
Histopathology	Reliable and inexpensive	• Less sensitive, error prone and time-consuming • Inter-observer bias
IHC	• On par with routine fixation and embedding procedures • Archival material can be employed • Sensitive and specific	• Technique sensitive
Photodiagnosis	• Aids in guided biopsies • Detection of dysplasia • Assesses surgical margins	• ↓Specificity
Flow cytometry	• Detects DNA-aneuploidy, abnormal stem lines, and loss of heterozygosity	• Expensive • Prefers single cell suspension and uniform particle size • Optimal excitation, emission wavelengths of the dyes • Complex biosafety systems
PCR	• DNA cloning for sequencing • DNA-based phylogeny or functional analysis of genes • Diagnosis of hereditary diseases • Detects genetic fingerprints & molecular markers	• Expensive and sensitive • Fluctuates results by contamination
Microarray technology	• Quantitative study of mRNA • Gene detection in one go	• Time-consuming and labor intense • Mandates DNA preservation • Cross-hybridization
Nanodiagnostics	• Applicable for small samples • Sensitive, high speed, and flexible biological tests • Targeted therapy in cancer • Cost-effective	• Nanoparticles are hazardous to health • Legal/ethical concerns
LIF	• Non-invasive • Sensitive • Spatially resolved measurement • Accommodates laser imaging • Detects micro changes within cells	• Cumbersome technique • Potential photochemical effects
ISH	• Detects infective agents • Study of cell development, human gene mapping and cytogenetics	• Translational and post-translational modifications are hindered
Ratio imaging	• Non-invasive • Prefers the single focal plane • Improved optics and imaging	Cumbersome technique
Elastic scattering spectroscopy	• Sensitive and optically guided biopsy • Detects micro changes within tissues	• Fails to detect tumor margin • Time-consuming • Insensitive to subtle biochemical, structural changes
OCT	• Yields *In situ* imaging, without the need to excise a specimen • Provides 3D images in real time • Compact and portable device	• Blood hampers clear images • Low penetration ability

(*Contd...*)

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Table 2: Contd...

Diagnostic aids used in detection of cancer	Merits	Demerits
• Raman spectroscopy	• Highly specific • Facilitates remote analysis • Raman spectra can be collected from a very small volume (<1 μm in diameter) • Avoids sample preparation • Waterproof and non-damaging	• Not ideal for metals or alloys. • Sensitive and optimized instrumentation • Artifacts hinder the Raman spectrum
• Multiphoton excited fluorescence	• Suited for imaging in optically thick specimens • Higher axial resolution • Reduced photo-bleaching of marker dyes • Increased cell viability	• Photo and thermal damage
• Nuclear magnetic resonance spectroscopy	• Characterizes micro samples • Exhaustive information about a molecule's structure • Explores structural information of unknown compounds • Free of biologic hazard	• Less sensitive and expensive • Mandates consistent signal-to-noise level

LBC: Liquid-based cytology, OCT: Optical coherence tomography, ISN: *In situ* hybridization, LIF: Laser-induced fluorescence, PCR: Polymerase chain reaction, IHC: Immunohistochemistry, CLBC: Centrifuged liquid-based cytology

provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of a molecule. It allows the study of atoms in all its dimensions, helps in gene transcription and signal transduction. This method allows characterization of a very small amount of sample and can provide vast information about a molecule's structure, and it is free of biologic hazard.[45,46]

Different diagnostics aids in detection of precancer and cancer are discussed in Table 1, and a summary of the merits and demerits of each technique has been discussed in Table 2.

Conclusion

The detection, diagnosis, and management of oral diseases are complex. Refinements and continued research will undoubtedly improve our ability to detect any disease at the earliest possible stage. New technologies may emerge which will prove much more valuable in early diagnosis with probability of cure, with minimum impairment and deformity.

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