**To Do or Not To Do: Oral Cancer Diagnostic Modalities**

**Background of Oral Cancer**

Oral cancer is the sixth most common cancer globally with a 5-year survival rate of around 50%. Head and neck cancer in the oral cavity represents around 48% of the total reported cases and 90% of these cases are predominantly of oral squamous cell carcinoma (OSCC). Due to the aggressive nature of oral cancer, the epithelial cell morphology is greatly affected, thus the altered cells may undergo metastasis and even result in death. The twelve-month frequency of OSCC is ≥300,000. Oral cancers show high mortality rates with approximately 9,000 deaths every year. They tend to be more dangerous than breast cancer, cervical cancer, as well as prostate cancer and may kill one person, every hour, every day.

The tongue is reported to be the most common location, with a poor prognosis. The ratio of males to females being affected is 1.5:1 with males being affected more as compared to females.The danger of developing oral cancer increases with age and the majority of cases occur in people aged 50 years or over. About 6% of oral cancers occur in young people under the age of 45 years. As per the screening protocol for all head and neck cancers (including oral cancers) conducted by The American Society, asymptomatic individuals between the ages of 20 and 40 years should be screened every three years and asymptomatic patients after 40 years should be screened annually.

**Common Diagnostic Techniques For Screening Oral Cancer**

Severalrecent advances have been devised to improve the efficacy of oral cancer detection. We, therefore, suggest recent and innovative modalities for early identification and accurate diagnosis of malignant lesions (Table 1).

1. **Visual Examination**

To date, the intraoral and extraoral examination is still the standard method for oral cancer screening.The extraoral and intraoral examination includes visual and palpatory evaluation of the buccal and labial mucosa, lips, gingivae, labial mucosa, dorsal and ventral surface of the tongue, hard and soft palate, the floor of the mouth, uvula, face, ears, neck, and the regional lymph nodes.

1. **Biopsy and Histopathology Report**

The gold standard for diagnosing oral cancer cases is the histopathological assessment of a tissue biopsy. A tissue biopsy represents the alterations of the oral tissues such as variations in color, size, and shape.

1. **Vital Staining**

Vital staining is a conventionaltissue staining technique that includes various dyes like toluidine blue, methylene blue, and Lugol’s iodine for early cancer detection.

1. Toluidine Blue

Toluidine blue,an acidophilic metachromatic dye of the thiazine group displays a high affinity for acidic tissue components like deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Thus, an increased dye uptake is observed in tissues possessing high nucleic acid content, i.e., those undergoing dysplastic alterations. Furthermore, the widened intracellular canal can be seen in the malignant epithelium than in the normal epithelium, which may facilitate easy dye passage. Several ready-to-use kits like OraScan, OraScreen, and OraTestare available as three-component systems. Since one component comprises flavored 1% toluidine blue “o” 10 mL solution, the other two components consist of pre- and post-rinse solutions containing flavored 1% acetic acid.

Toluidine blue is widely used for stainingOSCCcases and inflamed traumatic regions. The pre-rinse is usually used to eliminate excessive saliva and provide a consistent oral environment. Moreover, using the post-rinse solutiondecreases the staining background area and helps in the precise determination of suspect lesions. Approximately 8–10% of the keratotic lesions as well as the regenerating ulcer and erosion edge cases can show false-positive staining (in cases of lesions staining blue, but no carcinoma is identified after a biopsy). Additionally, toluidine blue usage greatly improves the sensitivity and specificity of visual examinations in those cases having suspicious mucosal characteristics. Toluidine blue displayed improved detection of malignancy with sensitivity, specificity, and diagnostic accuracy of 92.6%, 67.9%, and 80%, respectively when compared with histopathologic examination results.Thus, toluidine blue staining of suspicious oral epithelial lesions can greatly help in detecting OSCC cases in high-risk populations, including patients with a prior history of previous oral cancer.

1. Methylene Blue

Methylene blue, a heterocyclic aromatic chemical compound with a molecular formula C16H18ClN3S, is utilized as a dye for various staining procedures, such as Wright’s and Jenner’s stains. Although it is a temporary staining modality, it is used for examining RNA or DNA.On one hand, toluidine blue is hazardous to health if ingested as it shows toxicity to fibroblasts. On the other hand, methylene blue has limited toxicity and is not interspersed in the nucleic acid chain.It is available in three-component solution systems. The first bottle consists of a pre-rinse solution containing raspberry flavor, 1% lactic acid, and purified water. The second bottle comprises a rinse solution containing 1% methylene blue while the third bottle is a post-rinse solution that includes 1% lactic acid, raspberry flavor, and purified water as its ingredients.

**Indications for methylene blue:**

* For screening high-risk populations for oral cancer andlesions with dysplastic characteristics.
* Biopsy’s site selection.
* Demarcating the outer margin of the cancerous lesion before the appropriate treatment isinitiated.

**Methylene blue possesses the following disadvantages:**

* Displays high false positive ratesin cases of inadequate follow-up.
* More effective for erythroplakia but not forleukoplakia, which is the area that demands the maximum attention.
* Enhanced efficacy than visual acuity but is inefficient in demarcating the true margins.
* Exhibits toxicity after ingestion.

**Methylene blue displays the following advantages:**

* Ease of execution.
* Inexpensive and rapid in action.
* Noninvasiveness.
* Helpful in the easy demarcation of the gross extent of the areas of interest.
1. Lugol’s Iodine

Richart used Lugol’s solution for the delineation of malignant changes. Thus, this solution displays a brown-black stain by an innate reaction of the iodine with glycogen, However, in this, normal tissue stains brown, but proliferative epithelium is sometimes poorly stained. It is observed that the glycogen content is inversely correlated to the degree of keratosis. Hence, the combination of toluidine blue and Lugol’s iodine can be used as a decisive adjunct to the visual examination of oral cancer patients and assess high-risk patients for adequate and reliable results.

1. **Oral Cytology**

Oral cytology is a traditional technique where oral mucosal cells are amassed for further examination by scraping, brushing, or rinsing the exfoliative cells using a tongue brush. Furthermore, the aggregated oral mucosal cells are fixed, stained, and their cellular morphology is subsequently examined under a microscope. In recent years, various other techniques have been developed for an early and precise assessment of suspicious lesions.

1. Exfoliative Cytology

Exfoliative cytology is a conventional diagnostic technique that is a painless, non-invasive, fast, and simple procedure. Thus, it is suitable for patients suffering from systemic diseases who are contraindicated for undergoing a biopsy. It reduces false negative biopsy and post-biopsy complications to a larger extent and can be repeated several times for diagnosis and follow-up purposes.

The working mechanism of exfoliative cytology is primarily based on epithelial physiology. In normal conditions, epithelial cells are tightly placed. However, the appearance of a benign disorder or malignant characteristics creates a loss of cohesion between these cells and thereby results in exfoliation. This loss of cellular cohesion enables the collection of the exfoliated cells for subsequent microscopic examination.

1. Brush Biopsy

Brush biopsy is a procedure that denotes an exfoliative biopsy of the oral mucosa. This modality is minimally invasive, easy to use, and efficacious in collecting mucosal representative cells when compared to the excision. Since this procedure is usually painless, it is the most sought-after technique for such patients.

1. Oral CDx Brush Test System

Over the years, standard exfoliative cytology for oral malignant lesions has been constantly judged for not giving adequate and reliable results. Therefore, in recent years, newer techniques, particularly the brush biopsy technique, have been developed for improved efficacy.

Computer-assisted transepithelial oral brush biopsy (Oral CDx) is a type of transepithelial oral biopsy procedure that can easily detect early cancerous lesions. In this modality, a small circular brush is utilized for penetrating the superficial, intermediate, and basal cell layers with minimal discomfort. Consequently, the resultant sample is placed onto a slide for computer analysis. Furthermore, these samples arefixed onto a glass slide and are further stainedand analyzed microscopically employing a computer-based imaging system that has the ability torank cells based on the degree of abnormal cellular morphology.

The oral CDx Brush Test System possesses the following benefits:

* It is an easy and rapidchair-side procedure that requires no topical anesthetic agent and results in minimal or no bleeding
* OralCDxsystem can be reliably used on oral lesions displaying epithelial abnormalities to confirm their benign nature, and to reveal clinically insignificant lesions that might have malignant potential.
1. **Optical Imaging**

Recently, a variety of optical imaging methods have been used for the detection of oral cancer. These techniques are based on the optical properties of the biological tissues. The most common optical imaging methods are chemiluminescence and autofluorescence.

1. Chemiluminescence

The chemiluminescence diagnostic technique is used for the examination of oral mucosa for the diagnosis of oral cancer. The chemiluminescence method involves the production of blue-white light by the chemical reaction of acetylsalicylic acid and hydrogen peroxide within the capsule rod. In this reaction, the light is reflected by the biological tissues with alterations such as an increased nuclear/ cytoplasmic ratio. This technique serves as an adjunct to conventional examination for the diagnosis of early-stage oral cancer. The two chemiluminescence-based diagnostic tests are ViziLite and VizLite Plus.

VizLite

 VizLite test kit contains an acetic rinse, retractor, and a light stick. During this procedure, the normal epithelium appears dark by absorbing ViziLite, whereas the abnormal epithelium appears acetowhite reflecting the ViziLite. This technology was applied to examine the characteristics of clinically diagnosed OSCC. The steps involved in the VizLite procedure are:

* A 1% VizLite acetic acid solution rinses are used by the patient.
* The VizLite light stick is continuously bent for activation till the inner capsule breaks.
* The investigator shakes and inserts the light stick into the hollow end of the retractor.
* Then the oral cavity is examined under dim light.
* Normal mucosa appears blue, whereas the white lesion appears acetowhite.

VizLite Plus

VizLitePlus serves as an adjunct to the VizLite test. The FDA approved the VizLite Blue Oral Lesion Identification and Marketing System in 2014. The three swab parts of the VizLite Plus system include two swabs of 1% acetic acid rinse and one swab of toluidine blue, a metachromatic vital tissue dye. The toluidine blue dye is applied to the white lesion so that it can be identified by the dentist under incandescent light.

1. Autofluorescence

Autofluorescence is defined as the natural fluorescence of the biological tissues, without applying any chemical substance. In this technique, fluorophores produce autofluorescence in living cells by excitation with a suitable wavelength. If the disease is present, alterations occur in the concentration of the fluorophores, light scattering, and absorption properties of the tissue. The autofluorescence device integrates a fiber-optic probe, two nitrogen–pumped dye lasers, and an optical multichannel analyzer. The probe is made up of a central fiber bordered by six fibers. This technique is utilized by dentists to investigate the most dysplastic location for biopsy. It is also reliable for differentiating oral premalignant and malignant tumors from healthy oral mucosa.

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|  **Wide Spectrum of Diagnostic Techniques** |
| Common diagnostic techniques  | * Visual Examination
* Biopsy and Histopathology Report
* Vital Staining
* Oral Cytology
* Optical Imaging
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| New diagnostic techniques under development | * DNA Methylation Biomarker
* mRNA Biomarker
* Protein Biomarker
 |
| Developing advanced diagnostic techniques | * Artificial Intelligence
* Oral Fluid Biosensor
* Lab–on–Chip
 |

**Table 1: Wide spectrum of diagnostic techniques for screening of oral cancer.**

**New Methods Under Development For Clinical Application**

The identification of biomarkers from biological fluids (blood, urine, saliva) has the potential for early diagnosis. Biomarkers are referredto as measurable variations in biological substances that link with normal or abnormal conditions. Biomarkers are molecular signs and indicators of normal biological, pathological, and pharmacological responses to therapy, and as such, they may provide useful information for illness detection, diagnosis, and prognosis In the field of oncology,the biomarkers can be divided into three types according to their clinical relevance- Diagnostic, Prognostic and Predictive biomarkers. In the era of OMICS, the biomarkers derived from Genomics, Transcriptomics, Proteomics, and Metabolomics can be of great help in screening, assessing, and predicting the course of oral cancer (Figure 1). Sensitive and specific biomarkers in the field of oncology used in many clinical trials can indicate the clinical outcomes of cancer in a relatively earlier stage. Biomarkers are considered as potential targets for drug design. Biomarkers also assist us in understanding the biochemical pathways and regulatory processesconnected with disease.

**Figure 1: Utilization of OMICS in oral cancer.**

The use of saliva and gingival crevicular fluid (GCF) for early cancer detection in the search for new clinical markers is a promising approach because of its non-invasive sampling and easy collection methods. Human whole-mouth saliva contains proteins, peptides, electrolytes, organic, and inorganic salts secreted by salivary glands, and complimentary contributions from GCF and mucosal transudates. This molecular diagnostic technique has resulted in the discovery and development of salivary biomarkers for the detection of oral malignancies like DNA, RNA, and mRNA (messenger RNA).Moreover, various protein biomarkers like cytokines (IL-8, IL-1b, TNF-), P53, defensin-1,Cyfra 21-1, dual specificity phosphatase,spermidine/spermineN1-acetyltransferase, tissue polypeptide-specific antigen, profilin, cofilin-1, transferrinhave already been discovered. Still, furtherresearchis required to assure the accuracy and reliability of salivary biomarkers for clinical settings.

But the biomarkers can also be utilized in cancer therapeutics for example clinical trials for head and neck squamous cell cancer (HNSCC) treated with immune checkpoint inhibitors are now underway. The US Food and Drug Administration has approved anti-PD-1 antibodies, such as nivolumab and pembrolizumab, for patients with recurrent or metastatic HNSCC, as well as cisplatin-resistant malignancies. Anti-epidermal growth factor receptor (EGFR) antibodies, such as cetuximab, have been approved for treatment in patients with HNSCC in conjunction with radiation therapy. Despite these advancements, their efficacy remains limited, and several side effects have been reported. As a result, more research into novel therapeutic techniques, such as customized medicines based on cancer biomarkers and novel molecular-targeted therapeutics with no or minimal adverse effects in patients with oral cancer, is required.DNA methylation, mRNA, and protein biomarkers in oral malignancies will be dealt with in brief in this section of the chapter. Before understanding each biomarker in detail the understanding of various laboratory techniques is a must. Table 2 summarizes the various laboratory methods/techniques to study biomarkers.

**Table 2. Various methods to study biomarkers.**

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| --- | --- |
| **Technique** | **Description** |
| Immunohistochemistry  | Immunohistochemistry (IHC) is a popular supplementary testing method in anatomic surgical pathology and cytopathology for cell classification and diagnosis of pathology. It employs antibodies directed against specific antigens in specific tissues and cells to aid in the identification of cell type and organ of origin. |
| Bisulfite PCR/Methylation-specific PCR | Bisulfite genomic sequencing is considered the gold standard for detecting DNA methylation since it provides a qualitative, quantitative, and efficient method for identifying 5-methylcytosine at single base-pair precision. This approach was developed by Frommer et al. and is based on the discovery that the amination processes of cytosine and 5-methylcytosine (5mC) had extremely different outcomes after treatment with sodium bisulfite. Cytosines in single-stranded DNA are transformed into uracil residues and recognized as thymine in subsequent PCR amplification and sequencing; however, 5mCs are resistant to this conversion and stay as cytosines, allowing 5mCs to be separated from unmethylated cytosines. Following the bisulfite treatment, a PCR technique employing particular methylation primers is required to detect the methylation status in the loci of interest or sub-group cloning sequence. Direct PCR product sequencing can be used to assess the actual methylation status. |
| Pyrosequencing | Quantifiable sequence data is required for characterizing complicated DNA alterations underlying gene expression patterns. Pyrosequencing is a sequence-based detection tool that allows for the quick and precise quantification of the sequence change. Pyrosequencing technology is a very versatile tool for exploratory and testing work in a wide range of disciplines due to its streamlined methods, analysis flexibility, and elegant output. |
| Microarrays | A microarray is a laboratory technique that detects the expression of thousands of genes simultaneously. DNA microarrays are microscope slides that have thousands of small spots in specific positions, each of which contains a recognized DNA sequence or gene. These slides are frequently referred to as gene chips or DNA chips. The DNA molecules linked to each slide serve as probes for detecting gene expression, which is also referred to as the transcriptome or the collection ofmRNA transcripts expressed by a group of genes. Comparative genomic hybridization has been accomplished using gene microarrays. In this method, genomic DNA is fluorescently labeled and utilized to detect gene loss or amplification. |
| Chromatin Immunoprecipitation | ChIP, or chromatin immunoprecipitation, is an antibody-based method that selectively enriches certain DNA-binding proteins as well as their DNA targets. ChIP is used to look into a specific protein-DNA interaction, a group of protein-DNA interactions, or interactions throughout the entire genome or a subset of genes. |
| Next Generation Sequencing | NGS platforms sequence millions of tiny DNA fragments in parallel. By mapping individual reads to the human reference genome, bioinformatics analyses are employed to piece together these fragments. Each of the human genome's three billion bases is sequenced numerous times, providing sufficient depth to offer reliable data and insight into unexpected DNA variation. The principle of NGS is SEQUENCING BY SYNTHESIS. |
| Mass Spectrometry | Mass spectrometry (MS) is a popular high-throughput method for protein research. MS-based protein identification begins with the digestion of proteins into peptides, which are subsequently separated, fragmented, ionized, and collected by mass spectrometers. |
| RNA sequencing | RNA-seq (RNA-sequencing) is a technique that uses next-generation sequencing (NGS) to investigate the quantity and sequences of RNA in a sample. It examines the transcriptome to determine which genes encoded in our DNA are activated or deactivated and to what extent. |
| Immunoassay | Immunoassays are bioanalytical methods that depend on the antigen(analyte) reactions and an antibody to measure an analyte. They make use of a competitive binding procedure between a predetermined amount of labeled analyte and varied quantities of unlabeled analyte for a few binding sites on a highly precise anti-analyte antibody. When these immunoanalytical reagents are merged and incubated, the analyte combineswith the antibody, producing an immunological complex. Physical or chemical separation techniques are used to separate this complex from the unbound reagent portion. The label activity including radiation, fluorescence, or enzyme in either the bound or free fraction is estimated. |
| Electrophoresis | Electrophoresis is a laboratory technique for separating DNA, RNA, and protein molecules based on size and electrical charge. The molecules are moved through a gel or other matrix using an electric current. Smaller molecules can flow faster than larger molecules because the pores in the gel or matrix act like a sieve. Standards of known sizes are separated on the same gel and compared to the sample to determine the size of the molecules in the sample. |

1. **DNA Methylation Biomarkers**

DNA methylation is an epigenetic modification that modulates gene expression without changing the DNA sequence. DNA methylation is seen in physiological and pathological processes. During embryonic development, the totipotent cells undergo DNA methylation and are directed to future specific lineage committed to performing specific functions.The differentiated cells will have stable and unique methylation patterns. In cancer biology, DNA methylation pattern is studied extensively as it is known to be associated with chromosomal instability and tumor suppressor gene silencing (Tables3 and 4). Thus, various methods to study DNA methylation are Immunohistochemistry, Bisulfite PCR/Methylation-specific PCR, Pyrosequencing, Microarrays, NGS, Mass spectrometry, and Chromatin immunoprecipitation (Table 2).

**Table 3: Examples of methylation patterns seen in oral neoplasia.**

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| --- | --- | --- | --- |
| **Methylation pattern** | **Target gene** | **Function**  | **Tumor** |
| Hypomethylation | Chloride Intracellular Channel Protein 3 (CLIC3) | Oncogene | Mucoepidermoid Carcinoma |
| Hypomethylation  | Survivin | Apoptosis  | Oral Squamous Cell Carcinoma |
| Hypomethylation | HCN2 | Oncogene  | Adenoid Cystic Carcinoma |
| Hypermethylation | MGMT | DNA repair | Oral Squamous Cell Carcinoma |
| Hypermethylation | *CDH1/E-cadherin* | Epithelial-Mesenchymal Transition, Adhesion | Oral Squamous Cell Carcinoma |
| Hypermethylation | P21 | Tumor Suppressor Gene  | Ameloblastoma, Adenomatoid Odontogenic Tumor |

**Table 4: Example of targeted interventions in DNA methylation pattern in neoplasia.**

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| --- | --- | --- | --- |
| **Author**  | **Sample** | **Intervention** | **Effect**  |
| Notarstefano V et al (2021)1 | On Oral Squamous Cell Carcinoma and Cancer Stem Cell lines | 5-azacytidine | Cytotoxic effect on Squamous Cell Carcinoma Cell lines by Demethylation of DNA which increases the transcriptional activity, conformationalchanges in DNA, and cell death by apoptosis mechanism. |
| Jeon YJ et al (2013)2 | Oral Squamous Cell Carcinoma Cell Lines  | Panobinostat (LBH589) | Induces apoptosis through the regulation of specificity protein 1 (Sp1) |
| Pettke A et al (2016)3 | Osteosarcoma Cell Lines | Vorinostat (Suberanilohydroxamic acid) | Induces apoptosis. Synergistic effect with Cisplatin. |
| Naganuma K et al (2014)4 | Oral Squamous Cell Carcinoma Cell Lines | 3-deazaneplanocin A | Reactivation Of Keratin 13 transcription. Anti-carcinogenic  |

1. **mRNA Biomarkers**

mRNA isa single-stranded RNA that is transcribed from a DNA strand, i.e., it carries genetic information and also helps in protein synthesis. mRNA is the only coding RNA in organisms. It acts as a direct template for passing genetic information and guiding protein synthesis. mRNA incorporates genetic information in DNA with protein translation and expression.It has a major role in living activities. Gene expression can be precisely identified by diagnosing mRNA levels. Therefore, recent research and development of new therapies for cancers, both the upstream DNA of mRNA, the downstream proteins of mRNA, and even the non-coding short RNAs that influence its processing and modification have been extensivelyinvestigated. mRNA is present in human cells and can also be seen in extracellular components like body fluids for example blood, saliva, urine, sperm, sputum, etc. Hence during malignancies the tissue sample and body fluids can be used to detect the mRNA transcriptomes related to carcinogenesis which serves as a biomarker (Table 5).

Exosomes can transport mRNAs across cells, which is known as "exosome shuttle RNA (esRNA)". The presence of mRNA in plasma exons has been demonstrated to be a promising liquid biopsy technique. EsRNAs have been found to have anticancer properties by suppressing genes involved in tumor formation.

**Table 5: Few studies on mRNA genes in neoplasia.**

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| **Author** | **mRNA Gene** | **Regulation**  | **Tumor** |  |
| Guo H et al 5 | HOXA1, HIST1H3J, and ZFP42 (High Risk mRNA) | Upregulated  | Oral Squamous Cell Carcinoma (Predicting Survival Outcome) |  |
| Guo H et al5 | CELSR3 and ASCL4 (Low-Risk mRNA) | Upregulated | Oral Squamous Cell Carcinoma (Predicting Survival Outcome) |  |
| Oh SY et al6 | NAB2, cytochrome P450, CYP27A1 ,NPIPB4, MAOB ,SIAE, COL3A1 | Downregulated  | Oral Squamous Cell Carcinoma (Early diagnosis ) |  |
| He W et al 7 | Phospholipase A1-alpha (PLA1A) and Dermokine (DMKN)  | Upregulated  | Melanoma (Vasculogenic mimicry and Epithelial-mesenchymal transition) |  |

Despiteadvanced research in oncology, malignant tumorsarestill the world's second-largest cause of death. Surgery, radiation, chemotherapy, targeted therapy, immunotherapy, and combination therapy are all common clinical therapies for malignancies. Furthermore, the successful treatment of certain cancers with immune checkpoint inhibitors (CPIs) has sparked new concepts regarding tumor immunotherapy. Tumor immunotherapy enhances the host's antitumor immunity, thus leading to a tumor-suppressive microenvironment, tumor reduction, and better patient survival. Cancer vaccines have great potential for antitumor immunotherapy. Since tumor antigens can be divided into tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs), vaccines against these antigens can specifically target malignant tumor cells having enhanced levels of antigen expression, thereby resulting in tumorreduction by means of immunological memory.

Cancer vaccinesare extremely precise and safe when compared with other immunotherapy modalities. mRNA vaccines can easily express TAA, TSA, and their related cytokines as well as boost both humoral and cellular immunities for greater adaptability towards various diseases. They also possess several advantages, like rapid production, adaptability, economical, and an innate potential for extracting protective immune responses. However, mRNA does not integrate into the host genome like other DNA vaccines. Thus, substantial amounts of customized mRNA cancer vaccines can be produced rapidly for positive patient outcomes.Several methods to study mRNA biomarkersare RT-qPCR, RNA-seq, and Microarrays (Table 2).

1. **Protein Biomarkers**

Oncoproteins are proteins that are encoded by oncogenes and are involved in the control or synthesis of proteins linked with malignant tumor cell proliferation. Antibodies that directly target oncoproteins in cancer cells and suppress the formation of invasive carcinoma are the focus of current oncoprotein research. There are numerous oncogenes that encode various oncoproteins. Growth factors, receptor tyrosine kinases, cytoplasmic regulatory subunits, transcription factors, and regulatory GTPases are a few examples of these oncoproteins. Normal genetic material mutations can result in gene expression as cancer proteins. Many tumor suppressor genes exist as well, which protect cells from cancer. These tumor suppressors typically interact with a stage in the progression of cancer. There are also many tumor suppressor genes that protect cells from cancer and usually interact with a step in cancer formation.

Scientists have uncovered the existence of new oncoproteins and the mechanism of oncoproteins on carcinogenesis as science and technology have progressed and medical levels have improved. In contrast to the proteins encoded by proto-oncogenes, tumor suppressor genes, which exist in cells under normal settings, can restrict cell growth. If it loses its function, it may encourage cell tumorigenesis. As a result, cancer could be caused by the activation of oncogenes and the inactivation of tumor suppressorgenes. Currently, the two most well-known tumor suppressor genes are the Rb and p53 genes. Their products are nuclear proteins that act as transcriptional regulators to control cell development. Various modalities to study protein biomarkersare Mass Spectrometry, Immunoassay, and Electrophoresis (Table 2).

**Developing Technologies For Oral Cancer**

Over 90% of malignant neoplasms (cancer) of the mouth are squamous cell carcinomas arising from the mucosal epithelium. It is the sixth most common cancer worldwide with a 5-year relative survival rate of 50%. In oral cancers, the innate aggressive nature affects the oral epithelial cells, where they may undergo metastasis and even result in death. The head and neck cancer in the oral cavity represents around 48% of cases and 90% of cases are of oral squamous cell carcinoma. The twelve-month frequency of oral squamous cell carcinoma is ≥300,000 and approximately 9,000 individuals die of this disease each year. They tend to be more dangerous than cancer being diagnosed in other parts of the body. The most commonly affected site is the tongue, with a poor prognosis. The ratio of males to females being affected is 1.5:1 with males being affected more as compared to females. The incidence of oral cancer rises steeply with age and, with an aging population, oral cancer will become more common. However, they may occur in young individuals (45 years) but the incidence is very low around 6%5. According to the screening protocol conducted by The Society of America for all head and neck cancers, healthy people between 20 and 40 years should be screened every three years, and people after 40 years should be screened annually.

 Various pain-free diagnostic, non-invasive tools havebeen used in the past such as toluidine blue staining (TB), autofluorescence (VELscope), and chemiluminescence (Vizilite) either solely or in combinations to detect potentially malignant lesions. For oral cancer detection, exfoliated cells, serum, and saliva are considered non-invasive tools due to their ease of availability, convenience, and cost-effectiveness. The visual tools are very subjective and reliant on the investigators' competence, which places certain restrictions on the non-invasive techniques. Microfluidics or Lab on Chip method and Oral fluid biosensors work on biological secretions such as blood, saliva, and gingival crevicular fluid. These methods have helped to reduce the anxiety and discomfort among people over routine biopsy procedures. They make use of biological reactions to detect the analyte of particular interest. Nowadays, radiographic imaging modalities including magnetic resonance imaging(MRI), cone beam computed tomography (CBCT), computed tomography (CT), and positronemission tomography (PET) are used to establish the stages of oral cancer in clinical settings and help to formulate an effective treatment plan. A few imaging techniques, including Raman spectroscopy, elastic scattering spectroscopy, diffuse reflectance spectroscopy, narrow-band imaging, and confocal reflectance microscopy were also developed to distinguish between cancerous cells and healthy, normal mucosa. The sensitivity for detecting small intraepithelial lesions is insufficient with these imaging techniques, which employ optical signals and offer real-time cell morphology. In recent times, nanotechnology has completely revolutionizedthe area of oncology. Imaging modalities make use of nanoparticles to deliver highly harmful drugs directly to cancerous cells. The discipline of oncology has seen a remarkable development of artificial intelligence (AI) in recent years. They assess the overall effectiveness of the categorization and diagnosis of oral potentially malignant disorders or OPMDs and oral cancer using deep convolutional neural network (CNN), a subset of machine learning.

1. **Artificial Intelligence-Based System And Oral Cancer**

AI, a branch of software engineeringis a technology-oriented process that mimics human behavior and thoughts such as learning, reasoning, adapting, and self-correction. AI and machine learning are two different terms that are used in the field of research interchangeably. Machine learning technique identifies distinguishable patterns from the existing data but relies on the knowledge of humans to differentiate the features. A subdivision of machine learning, deep learning makes use of CNN to emulate the brain of a human and directly extract features from raw images. AI has long been in the race for popular science invention. It originated from Alan Turing’s “Imitation game” or the “Turing test”. The first AI program- Logic Theorist was developed by Allen Newell and Herbert Simon in 1955.

Also, the term ‘artificial intelligence’ was coined by John McCarthy to describe machines that can perform intelligent actions without the involvement of humans. A collection of data is important for machine learning. This data can be in the form of clinical photographs, radiographs, patient symptom information, and audio files in the form of voices. The involvement of a variety of inputs in AI added revolutionary advantages in medical, dental, and healthcare delivery. The application of AI in the diagnosis of head and neck cancer has emerged rapidly with successes in the interpretation of medical images. They are developed as tools to guide the practitioner in providing solutions to various problems and diagnosis of disease through radiographic and clinical images.

The three fundamental steps to involve AI in clinical imaging of oral cancer are:

1. Pre-processing

2. Image segmentation

3. Post-processing

Pre-processing: The optical data is taken from the pictures and filters are utilized to decrease any conspiracy. Then contrast is changed to help in differentiation and outlining various structures; normal and dysplastic cells. Certain biomarkers are also used to avoid confusion at different levels. DL subdivision of AI succeeds at differentiating the complications among pictures, shifting the understanding of pictures from questionable results to a quantitative repeatable process that will only provide the important data required in the making of decisions.

Image segmentation: The area of interest is determined at this level. The diseased area is distinguished from a healthy area in imaging. Though there are four major classes of division, there are different paths to this interaction, and therefore multiple strategies are routinely used to increase its exactness.

Post-processing: In this stage, CNNs, recurrent neural networks, and multi-scale CNNs are used in the process of clinical imaging. Some extra relevant information was used to test the performance of the network and then the results were compared with the gold standard technique of histopathology.

AI provides a chief advantage by reducing the bulk visualization of slides manually. Also, they help pathologists in rapid decision-making with better accuracy. The computer-analyzed images of tissue slides provide more clear information that may be missed with routine methods. As, the more accurate and precise are histopathological findings the more early diagnosis, classification, prediction, and treatment planning for oral cancer can be made. Till now the specificity and sensitivity of CNN for differentiating oral cancer are reported to be 0.80 and 0.77, respectively.

1. **Oral Fluid Biosensor And Screening Of Oral Cancer**

In the present time, chair-side diagnostic techniques have gained importance over routine methods as they are easier and faster to perform. Biosensors are special forms of devices that utilize biological reactions for early diagnosis, and treatment of disease. These devices detect and measure the substrate (analyte) of interest. Over the period, blood has been the gold standard diagnostic fluid for the diagnosis of various diseases. However, oral fluids like saliva and GCF possess advantages over other types of body fluids like blood and serum, including noninvasive sample collection, easy storage and transit, and higher sensitivity. The first biosensor was introduced by Clark and Lyons, an enzyme-based glucose sensor.

Basically, a biosensor comprises six elements- bioreceptor, transduction element, electrochemically active interface, signal amplifier, signal processor, and display. The substrate binds to the biological product to form a product. The product-linked changes are converted by the transducer into electric signals which can be amplified, measured, and read out in the detector. After processing, the values are displayed on the monitor and controlling system.

Oral cancer is the most common cause of mortality and morbidity among developing countries. Hence for an early diagnosis, various biological markers have been developed. The potential biomarkers that can be used for diagnosis of oral cancer as IL-8, TNF- α, epidermal growth factor, and salivary transferrin and salivary genomes such as mi-RNA. These biomarkers are in direct contact with saliva and hence help in early diagnosis of disease. Pro-inflammatory chemokine (IL-8) plays an important role in tumor angiogenesis and metastasis. For the detection of IL-8, a surface-immobilized optical protein sensor is used. In this sensor, the substrate on the site reacts with a biotinylated monoclonal antibody (Ab) with the help of a capture probe. The light emitted from the fluorophore is then used as the detection signal, and the optical noise is reduced using confocal optics.

The saliva-based biosensor detects the exfoliated cells in the oral cavity and thereby allows screening and identification of potential biomarkers for the detection of oral cancer. Also, it helps reduce the patient's discomfort compared to routine biopsy procedures. Mi-RNA is non-coding short RNAs that are encoded throughout the genome sequence. Some regions in the genome are vulnerable to alterations; due to which deregulation in the mi-RNA can be seen during the detection of oral cancer. Therefore early detection can aid in better treatment. For the detection of oral cancer-related mi-RNA, an electrochemical biosensor method level was developed. This method detects mi-RNA using a magnetic-controllable gold electrode at attomolar levels. The advantage of this biosensor is magnetic beads-based enzymatic catalysis amplification which improves the sensitivity of the biosensor.

Oral fluid biosensors are easily available and noninvasivemethods for the collection of samples; thus, making them a novel method in the diagnosis of disease. However, its limitations such as low sensitivity and specificity are overcome by the introduction of new methods such as microfluidics and nanofluidics.

1. **Lab–On–Chip And Oral Cancer**

Microfluidics technology, micro-total analysis, or lab-on-a-chip (LOC) method is the integration and automation of analytical laboratory procedures into a single device or chip. It is often regarded as the chemistry that is equal to silicon-integrated chips and has revolutionized electronics, computers, and communications. In the present scenario, microfluidic systems are utilized in development for disease diagnostics, controlled drug delivery, detection of bioterrorism agents, and air and water quality monitoring. The diagnostic system accepts and processes a small biopsy or secretions such as a small –sample of blood, saliva, lung aspirations, and urine or intraductal breast fluid. Then it provides easy-to-interpret information regarding the presence and quantity of specific molecules, such as pathogen antigens, nucleic acids, antibodies, metabolites, toxins, drugs, and cancer markers.

In 1975, the first device was developed. In 1990, Manz et al. introduced a miniaturized open tubular chromatograph using silicon chip technology. The first (micro total analysis system) µTAS, capillary electrophoresis systemwas developed at the end of the 1990s. The Whiteside Group of Harvard University in 2007 invented and described the Concept of paper-based analytical devices. It comprises a microfluidic system, in which mixing and reaction take place. The detection and quantification take place with the help of a sensor system. In comparison to fluorescence and electrochemical detectors, colorimetric detection by optical absorption is the best alternative. There are eight inlets for the reactants and is fitted with a unique inlet for the sample to be tested. Then, the sample is mixed with the respective reactants. In biological and chemical applications, mixers help in enhancing the efficiency of mixing and homogenization. A small volume of samples can be used for the diagnosis of the disease with a minimally invasive technique which makes it a better method for early diagnosis of oral cancer.

The genetic changes in cancer cells produce alterations in the gene expression patterns, which can be recognized long before the cancer phenotype is expressed. In comparison to normal healthy mucosa, the variations that occur in cancerous cells can be used as biomarkers. The genes that are associated with OSCC tumor progression are p53, cyclin D1, and the epidermal growth factor (EGF) receptor gene. Microarray analysis of several tumor types helps in distinguishing tumor cells from normal cells. The introduction of high-density microarrays and advances in bioinformatics opened the door for the addition of these gene signatures into microfluidic LOC devices.

Studies have established a set of genes that are associated with down or up-regulation in OSCC. Approximately 1ml of saliva is provided by the patient, which is taken up by a sponge-tipped disposable collector. The collector is then implanted into the cassette to inject the collected oral fluid via a sample inlet port. The cancer diagnostic process involves an initial and first step in the removal of lymphocytes for the isolation of cancer cells from the sample. The cancer cells are collected from the sample using magnetic beads coated with anti-EpCAMantibody, which is abnormally expressed on the surface of cancerous epithelial cells. The separated cancer cells can be easily noticed and counted.

The separated cancer cells are then exposed to a thermal and/or chemical lysis step and the mRNA is isolated. Reverse transcription polymerase chain reaction, linear amplification, or bio-barcode technique can be used for amplification of multiplex m-RNA. Then, the transcription profile of the isolated sample cancer cells is compared with cancer signature profiles archived in a database using recognized statistical rules to identify the type of cancer.

**Conclusion**

Considering the size of the oral cancer research, most of the diagnostic methods are used in clinical settings or are commercially available. Thus, subsequent potential future technologies such as AI-based systems should be duly explored to optimize the efficacy of oral cancer diagnosis.

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