TO DO OR NOT TO DO: ORAL CANCER DIAGNOSTIC MODALITIES

Abstract

Oral cancer is one of the most common head and neck cancers, and the overall 5-year survival rate is less than 50%. Oral squamous cell carcinoma is the most common type of oral malignancy. Various diagnostic techniques have been developed for early diagnosis of oral cancer. The wide spectrum of diagnostic modalities used for early detection of oral cancer include common diagnostic techniques, new diagnostic techniques under development, and advanced diagnostic techniques. To date, intraoral and extraoral examination is still the standard method for the detection of oral cancer. The most common diagnostic techniques involved in oral cancer examination are visual examination, biopsy and histopathology report, vital staining, oral cytology, and optical imaging. The main new diagnostic methods under development include DNA Methylation Biomarkers, mRNA biomarkers, and protein biomarkers. The discovery of biomarkers in biological fluids such as blood, urine, and saliva provides significant hope for early diagnosis. The recently developing advanced diagnostic techniques include Artificial Intelligence, Oral Fluid Biosensor, and Lab – on – Chip. Considering the scale of oral cancer research, most of the diagnostic modalities are available commercially. Therefore, future tools such as AI-based technology should be explored to improve the effectiveness of oral cancer diagnosis.

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I. BACKGROUND OF ORAL CANCER

Oral cancer ranks as the sixth most prevalent cancer worldwide, boasting a 5-year survival rate of approximately 50%. Within the spectrum of head and neck cancers, oral cavity cancers account for about 48% of the total reported cases, with 90% of these cases predominantly classified as oral squamous cell carcinoma (OSCC). The aggressive nature of oral cancer significantly impacts the morphology of epithelial cells, potentially leading to metastasis and even death. OSCC occurs at a frequency of over 300,000 cases per year. Unfortunately, oral cancers exhibit high mortality rates, claiming the lives of roughly 9,000 individuals annually. They prove to be more lethal than breast cancer, cervical cancer, and prostate cancer, with the potential to cause one fatality every hour, every day.

The tongue is noted as the most frequent site for oral cancer, often associated with a grim prognosis. The incidence of this cancer tends to affect males more than females, with a ratio of 1.5 to 1. Additionally, the risk of developing oral cancer escalates with age, predominantly affecting individuals aged 50 and older. Remarkably, approximately 6% of oral cancer cases emerge in younger individuals under the age of 45. Following The American Society's screening protocol for all head and neck cancers, asymptomatic individuals between 20 and 40 years of age should undergo screening every three years, while asymptomatic patients aged 40 and above should receive annual screenings.

Common Diagnostic Techniques For Screening Oral Cancer: Several recent 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).

II. 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, dorsal and ventral surfaces of the tongue, hard and soft palate, the floor of the mouth, uvula, face, ears, neck, and the regional lymph nodes.

III. BIOPSY AND HISTOPATHOLOGY REPORT

The definitive method for diagnosing oral cancer cases involves the histopathological examination of a tissue biopsy. This biopsy allows for the evaluation of changes in oral tissue characteristics, including variations in color, size, and shape.

IV. VITAL STAINING

Vital staining is a traditional tissue staining method that involves the use of different dyes such as toluidine blue, methylene blue, and Lugol's iodine to detect early signs of cancer.

1. **Toluidine Blue:** Acidophilic metachromatic dye toluidine blue, belonging to the thiazine group, exhibits a strong preference for acidic tissue constituents such as 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 OraTest are 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 staining OSCC cases 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 solution decreases 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 oral cancer.

2. **Methylene Blue:** Methylene blue is a heterocyclic aromatic chemical compound with the molecular formula $C_{16}H_{18}ClN_3S$. It is commonly used as a dye in various staining procedures, such as Wright's and Jenner's stains. While it provides temporary staining, it is particularly useful for examining RNA or DNA. Unlike toluidine blue, which can be harmful if ingested and has toxicity towards fibroblasts, methylene blue has limited toxicity and does not become integrated into the nucleic acid chain. It is typically available in a three-component solution system. The first bottle contains a pre-rinse solution with raspberry flavor, 1% lactic acid, and purified water. The second bottle contains a rinse solution with 1% methylene blue, and the third bottle is a post-rinse solution containing 1% lactic acid, raspberry flavor, and purified water as its components.

- **Indications for Methylene Blue**

  ➢ For screening high-risk populations for oral cancer and lesions with dysplastic characteristics.
  ➢ Biopsy's site selection.
  ➢ Demarcating the outer margin of the cancerous lesion before the appropriate treatment is initiated.

- **Methylene Blue Possesses the Following Disadvantages**

  ➢ Displays high false positive rates in cases of inadequate follow-up.
  ➢ More effective for erythroplakia but not for leukoplakia, 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.

3. **Lugol's Iodine:** Richart employed Lugol's solution to highlight the malignant changes. Consequently, this solution induces a brown-black stain through an inherent reaction between iodine and glycogen. However, in this process, normal tissue takes on a brown stain, whereas proliferative epithelium may exhibit poor staining. It has been observed that the amount of glycogen present is inversely related to the degree of keratosis. Therefore, the utilization of both toluidine blue and Lugol's iodine can serve as a crucial supplement to visually examining oral cancer patients and evaluating high-risk individuals, yielding dependable and comprehensive results.

V. ORAL CYTOLOGY

Oral cytology is a conventional method that involves collecting oral mucosal cells through techniques such as scraping, brushing, or rinsing with a tongue brush for subsequent examination. These gathered oral mucosal cells are subsequently treated, stained, and their cellular structure is examined under a microscope. In recent times, several alternative methods have emerged to enable early and accurate evaluation of suspicious lesions.

1. **Exfoliative Cytology:** Exfoliative cytology is a traditional diagnostic method that offers a painless, non-invasive, quick, and straightforward procedure. This makes it an appropriate choice for patients with systemic illnesses who should not undergo a biopsy. It significantly reduces the chances of false-negative biopsy results and minimizes post-biopsy complications. Moreover, it can be performed multiple times for diagnostic 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.

2. **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.

3. **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 are fixed onto a glass slide and are further stained and analyzed microscopically by employing a computer-based imaging system that has the ability to rank cells based on the degree of abnormal cellular morphology.

The Oral CDx Brush Test System possesses the following benefits:

- It is an easy and rapid chair-side procedure that requires no topical anesthetic agent and results in minimal or no bleeding
- Oral CDx System 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.

VI. OPTICAL IMAGING

In recent times, several optical imaging techniques have been employed in oral cancer detection, leveraging the optical characteristics of biological tissues. The most prevalent optical imaging methods include chemiluminescence and autofluorescence.

1. **Chemiluminescence:** The chemiluminescence diagnostic method is employed to assess the oral mucosa for oral cancer diagnosis. This technique entails the generation of blue-white light through the chemical reaction of acetylsalicylic acid and hydrogen peroxide within the capsule rod. During this reaction, the light is reflected by biological tissues exhibiting changes like an elevated nuclear/cytoplasmic ratio. This approach complements the standard examination in identifying early-stage oral cancer. Two diagnostic tests based on chemiluminescence are known as 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.

2. **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.

Table 1: Wide Spectrum of Diagnostic Techniques for Screening of Oral Cancer

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

New Methods Under Development For Clinical Application: The discovery of biomarkers in biological fluids like blood, urine, and saliva holds significant promise for early diagnosis. Biomarkers represent measurable alterations in biological substances that correlate with normal or abnormal conditions. These molecular indicators serve as signals for normal biological processes, pathological conditions, and responses to therapy. Consequently, they offer valuable insights into disease detection, diagnosis, and prognosis. In the realm of oncology, biomarkers can be categorized into three types based on their clinical significance: Diagnostic, Prognostic, and Predictive biomarkers. In the era of OMICS, biomarkers derived from Genomics, Transcriptomics, Proteomics, and Metabolomics play a vital role in the screening, evaluation, and prediction of oral cancer's progression (Figure 1). These sensitive and specific oncology biomarkers, widely used in clinical trials, can provide early indications of clinical outcomes in cancer. Moreover, biomarkers are considered potential targets for drug development and offer insights into the biochemical pathways and regulatory mechanisms associated with diseases.
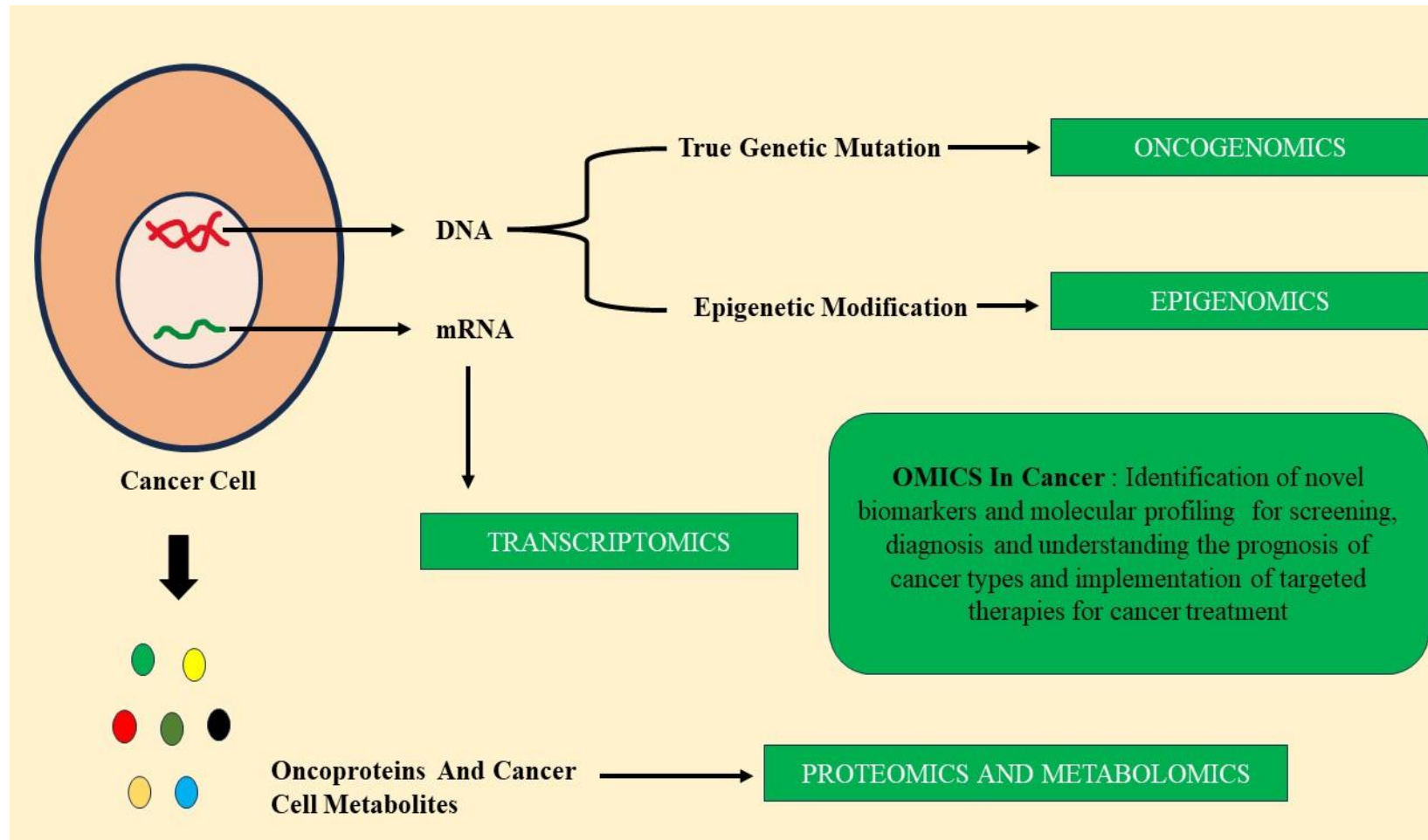
Figure 1: Utilization of OMICS in Oral Cancer

Utilizing saliva and gingival crevicular fluid (GCF) as a means for early cancer detection, while exploring novel clinical markers, holds significant promise due to its non-invasive sampling and straightforward collection procedures. Human whole-mouth saliva is rich in proteins, peptides, electrolytes, and organic as well as inorganic salts, which are secreted by the salivary glands. Additionally, it also incorporates contributions from GCF and mucosal transudates. This molecular diagnostic approach has led to the identification and development of salivary biomarkers that can detect oral malignancies, including DNA, RNA, and messenger RNA (mRNA). Furthermore, various protein biomarkers such as cytokines (e.g., IL-8, IL-1b, TNF-), p53, defensin-1, Cyfra 21-1, dual specificity phosphatase, spermidine/spermine N1-acetyltransferase, tissue polypeptide-specific antigen, profilin, cofilin-1, and transferrin have already been discovered. Nonetheless, further research is essential to ensure the accuracy and reliability of salivary biomarkers for clinical applications.

However, these biomarkers can also find application in cancer therapeutics. For instance, clinical trials are currently underway, exploring the use of immune checkpoint inhibitors in the treatment of head and neck squamous cell cancer (HNSCC). The US Food and Drug Administration has granted approval for anti-PD-1 antibodies, such as nivolumab and pembrolizumab, for patients with recurrent or metastatic HNSCC, including those with cisplatin-resistant malignancies. Additionally, anti-epidermal growth factor receptor (EGFR) antibodies like cetuximab have received approval for HNSCC treatment in combination with radiation therapy. Despite these advancements, their effectiveness remains somewhat limited, and adverse effects have been reported. Therefore, there is a pressing need for further research into innovative therapeutic approaches. This includes personalized medicines based on cancer biomarkers and novel molecular-targeted therapeutics with minimal or no adverse effects, specifically designed for oral cancer patients. In the following sections of this chapter, we will briefly explore DNA methylation, mRNA, and protein biomarkers in the context of oral malignancies. To better comprehend each biomarker, it is essential to first gain an understanding of various laboratory techniques. Table 2 provides an overview of the diverse laboratory methods and techniques employed for biomarker analysis.

Table 2: Various Methods to Study Biomarkers

Technique	Description
Immunohistochemistry	In cytopathology and anatomic surgical pathology, immunohistochemistry (IHC) is a widely used supplemental testing technique for pathology diagnosis and cell classification. In order to assist in identifying the type of cell and organ of origin, it uses antibodies that are directed against particular antigens in particular tissues and cells.
Bisulfite PCR/Methylation-specific PCR	Because it offers a qualitative, quantitative, and effective way to identify 5-methylcytosine with single base-pair accuracy, bisulfite genomic sequencing is regarded as the gold standard for detecting DNA methylation. The observation that the amination processes of cytosine and 5-methylcytosine (5mC) had remarkably different results following treatment with sodium bisulfite served as the basis for the development of this

	method by Frommer et al. 5mCs are resistant to this conversion and remain as cytosines, allowing 5mCs to be distinguished from unmethylated cytosines. Cytosines in single-stranded DNA are converted into uracil residues and identified as thymine in subsequent PCR amplification and sequencing. To find out the methylation status in the loci of interest or sub-group cloning sequence after the bisulfite treatment, a PCR method using specific methylation primers is needed. The real methylation status can be evaluated by direct PCR product sequencing.
Pyrosequencing	In order to characterize complex DNA changes that underlie patterns of gene expression, quantifiable sequence data is necessary. Pyrosequencing is a sequence-based detection technique that makes it possible to quantify sequence changes quickly and precisely. Pyrosequencing technology, with its streamlined techniques, flexible analysis, and elegant output, is a very adaptable tool for exploratory and testing work in a wide range of disciplines.
Microarrays	Through the use of a microarray, thousands of genes can have their expression simultaneously detected in a lab setting. A recognized DNA sequence or gene is contained in each of the thousands of tiny dots on a microscope slide known as a DNA microarray, which is arranged in a precise way. Many times, these slides are called DNA chips or gene chips. The DNA molecules that are connected to every slide function as indicators for identifying gene expression, which is also known as the transcriptome, or the assembly of mRNA transcripts that are produced by a set of genes. Using gene microarrays, comparative genomic hybridization has been achieved. This technique uses fluorescent labeling of genomic DNA to identify gene loss or amplification.
Chromatin Immunoprecipitation	ChIP, or chromatin immunoprecipitation, is an antibody-based technique that targets certain DNA targets and DNA-binding proteins to selectively enrich them. Using ChIP, one can investigate a single protein-DNA interaction, a collection of related interactions, or interactions spanning the whole genome or a subset of genes.
Next Generation Sequencing	In parallel, millions of minuscule DNA fragments are sequenced by NGS platforms. These fragments are assembled using bioinformatics methods, which map individual reads to the human reference genome. The three billion bases that make up the human genome have all been sequenced many times, giving us enough depth to provide accurate information and insight into unanticipated DNA diversity. NGS operates on the sequencing by synthesis principle.
Mass Spectrometry	Protein research frequently uses mass spectrometry (MS), a common high-throughput technique. Proteins are first broken down into peptides for MS-based protein identification. Mass

	spectrometers then use these peptides to separate, fragment, ionize, and gather them.
RNA sequencing	RNA-seq, or RNA-sequencing, is a method that looks at the amount and sequences of RNA in a sample using next-generation sequencing (NGS). To ascertain which of the genes encoded in our DNA are active or inactive and to what degree, it analyses the transcriptome.
Immunoassay	Immunoassays are bioanalytical techniques that rely on the measurement of an analyte using antibody and antigen (analyte) reactions. For a few binding sites on a very accurate anti-analyte antibody, they employ a competitive binding process between a fixed amount of labeled analyte and varying quantities of unlabeled analyte. An immunological complex is created when the analyte and antibody are combined with these immunoanalytical reagents and incubated. This complex is separated from the unbound reagent part using either physical or chemical separation procedures. The estimated label activity includes fluorescence, radiation, and/or enzyme in the free or bound fraction.
Electrophoresis	A laboratory procedure called electrophoresis is used to separate protein, RNA, and DNA molecules according to their sizes and electrical charges. An electric current is used to transport the molecules through a gel or other matrix. Because the holes in the gel or matrix function as a sieve, smaller molecules can pass through them more quickly than larger ones. To ascertain the size of the molecules in the sample, standards with known sizes are separated on the same gel and contrasted with the sample.

VII. 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 (Tables 3 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

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

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, conformational changes 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.

VIII. mRNA BIOMARKERS

mRNA is a single-stranded RNA that is produced by transcription from a DNA strand; it aids in the production of proteins and conveys genetic information. The only coding RNA found in organisms is mRNA. It serves as a direct template that directs the synthesis of proteins and transmits genetic information. Through the translation and production of proteins, mRNA combines genetic information found in DNA. It plays a significant part in

daily functions. Measuring mRNA levels allows for the accurate identification of gene expression. Because of this, recent studies and the creation of novel cancer treatments have focused a great deal of attention on the upstream DNA of mRNA, the downstream proteins of mRNA, and even the non-coding short RNAs that affect mRNA processing and modification. Moreover, 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 in malignancies, the tissue samples and body fluids can be used to detect the mRNA transcriptomes related to carcinogenesis which serves as a biomarker (Table 5).

Exosome shuttle RNA (esRNA) refers to the ability of exosomes to move mRNAs between cells. The presence of mRNA in plasma exons has been demonstrated to be a promising liquid biopsy technique. It has been discovered that EsRNAs have anticancer qualities by inhibiting genes linked to the development of tumors.

Table 5: Few Studies on mRNA Genes in Neoplasia

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 al[5]	CELSR3 and ASCL4 (Low-Risk mRNA)	Upregulated	Oral Squamous Cell Carcinoma (Predicting Survival Outcome)
Oh SY et al[6]	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)

Despite the significant advancements in oncological research, malignant tumors continue to be the second leading cause of death worldwide. Clinical therapies for malignancies encompass a range of approaches, including surgery, radiation, chemotherapy, targeted therapy, immunotherapy, and combination therapy. Notably, the success of immune checkpoint inhibitors (CPIs) in treating certain cancers has introduced innovative concepts in tumor immunotherapy. Tumor immunotherapy aims to bolster the host's antitumor immune response, fostering a tumor-suppressive microenvironment, reducing tumor size, and ultimately improving patient survival. Among the promising avenues in antitumor immunotherapy are cancer vaccines. These vaccines hold immense potential as they can target tumor antigens, which are categorized as either tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs). By focusing on these antigens, cancer vaccines can precisely

target malignant tumor cells characterized by elevated antigen expression levels, thereby achieving tumor reduction through immunological memory.

Cancer vaccines, in comparison to other methods of immunotherapy, might deliver specific, safe, and well-tolerated therapy. mRNA vaccines are a type of cancer vaccination that can encode and express TAA, TSA, and their related cytokines. mRNA cancer vaccines can promote both humoral and cellular immunity, thereby boosting their adaptability to varied illnesses and patients. mRNA cancer vaccines have various advantages, including rapid manufacture, flexibility, low cost, and the capacity to elicit a strong protective immune response. More crucially, unlike DNA vaccinations, mRNA does not integrate into the host genome. Large quantities of accurate and tailored mRNA cancer vaccines may be manufactured in a short amount of time, making them a promising treatment technique. Several methods to study mRNA biomarkers are RT-qPCR, RNA-seq, and Microarrays (Table 2).

IX. PROTEIN BIOMARKERS

Oncoproteins, encoded by oncogenes, play a crucial role in regulating the synthesis of proteins associated with the proliferation of malignant tumor cells. Current research in oncoproteins primarily focuses on antibodies that directly target these oncoproteins within cancer cells, effectively inhibiting the development of invasive carcinoma. Oncogenes encompass a wide range of genes, each encoding distinct oncoproteins. Examples of such oncoproteins include growth factors, receptor tyrosine kinases, cytoplasmic regulatory subunits, transcription factors, and regulatory GTPases. Mutations in normal genetic material can lead to the expression of these cancer-associated proteins. Additionally, there are tumor suppressor genes that function to safeguard cells against cancer development. These tumor suppressors typically intervene at various stages of the cancer progression process. In summary, both oncoproteins and tumor suppressor genes play critical roles in regulating cancer-related processes.

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 suppressor genes. 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 biomarkers are Mass Spectrometry, Immunoassay, and Electrophoresis (Table 2).

Developing Technologies For Oral Cancer: Over 90% of malignant neoplasms of the mouth are SCCs 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 of oral squamous cell carcinoma. The twelve-month frequency of OSCC 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%. According to the screening protocol conducted by The American Cancer Society 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 have been 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 the Lab-On-Chip (LOC) method and oral fluid biosensors work on biological secretions such as blood, saliva, and GCF. 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 positron emission 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 revolutionized the 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 (ML).

X. ARTIFICIAL INTELLIGENCE-BASED SYSTEM AND ORAL CANCER

AI, a branch within the field of software engineering, is a technology-driven process that aims to replicate human behaviors and cognitive functions, including learning, reasoning, adaptation, and self-correction. In the realm of research, AI and ML are frequently used interchangeably, although they represent distinct concepts. ML techniques are employed to identify discernible patterns within existing data but require human expertise to differentiate key features. Within the domain of ML, deep learning, a specialized subset, leverages CNNs to simulate the human brain's capabilities and directly extract features from unprocessed images. The quest for advancements in AI has been a prominent pursuit in the realm of scientific discovery. Its origins can be traced back to Alan Turing's "Imitation Game" or the renowned "Turing test." Notably, the inaugural AI program, known as Logic Theorist, was developed by Allen Newell and Herbert Simon in the year 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 ML. 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 involving AI in the clinical imaging of oral cancer are:

1. **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. Deep learning (DL), a 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.

2. **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.

3. **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 offers a significant advantage by automating the labor-intensive process of manually reviewing slides, reducing the workload for pathologists. Moreover, it assists pathologists in making rapid decisions with enhanced accuracy. Computer-analyzed images of tissue slides reveal valuable information that might go unnoticed using conventional methods. Increased accuracy and precision in histopathological findings enable earlier diagnosis, classification, prediction, and treatment planning for oral cancer. To date, the reported specificity and sensitivity of CNNs in distinguishing oral cancer are 0.80 and 0.77, respectively.

XI. ORAL FLUID BIOSENSOR AND SCREENING OF ORAL CANCER

In the current era, chair-side diagnostic techniques have gained prominence due to their ease and speed of use, surpassing the traditional methods. Biosensors, which are specialized devices utilizing biological reactions for early disease diagnosis and treatment, play a crucial role in this regard. These devices are designed to detect and quantify specific substrates or analytes of interest. While blood has traditionally served as the gold standard diagnostic fluid for various illnesses, oral fluids such as saliva and GCF offer distinct advantages over other bodily fluids like blood and serum. These advantages include

noninvasive sample collection, convenient storage and transport, and heightened sensitivity. The first biosensor, an enzyme-based glucose sensor, was introduced by Clark and Lyons.

In essence, a biosensor consists of six essential components: a bioreceptor, a transduction element, an electrochemically active interface, a signal amplifier, a signal processor, and a display. The bioreceptor facilitates the binding of the substrate to the biological product, resulting in the formation of a product complex. The transducer then converts the changes associated with this product complex into electrical signals, which can subsequently be amplified, quantified, and recorded by the detector. Following data processing, the values are presented on the monitor and control system.

Oral cancer poses a significant health concern, particularly in developing nations, where it is a leading cause of both mortality and morbidity. To enable early detection, various biological markers have been developed. Potential biomarkers for oral cancer diagnosis include IL-8, TNF-α, epidermal growth factor (EGF), salivary transferrin, and salivary genomes like mi-RNA. These biomarkers are inherently present in saliva, aiding in the early identification of the disease. The pro-inflammatory chemokine IL-8 plays a crucial role in tumor angiogenesis and metastasis. To detect IL-8, a surface-immobilized optical protein sensor is employed. In this sensor, the substrate at the site interacts with a biotinylated monoclonal antibody facilitated by a capture probe. The emitted light from the fluorophore serves as the detection signal, and confocal optics are utilized to reduce optical noise effectively.

The biosensor based on saliva is designed to identify exfoliated cells in the oral cavity, facilitating the screening and identification of potential biomarkers for oral cancer detection. Additionally, it offers a more comfortable experience for patients compared to traditional biopsy procedures. Mi-RNA, which consists of non-coding short RNAs encoded in the genome sequence, is susceptible to alterations in certain genome regions. Such deregulation in mi-RNA can be indicative of oral cancer. Therefore, early detection plays a crucial role in improving treatment outcomes. An electrochemical biosensor method has been developed to detect oral cancer-related mi-RNA, capable of detecting mi-RNA at attomolar levels using a magnetic-controllable gold electrode. This biosensor leverages magnetic beads-based enzymatic catalysis amplification to enhance sensitivity.

Oral fluid biosensors offer a convenient and noninvasive means of sample collection, making them an innovative approach to disease diagnosis. However, their limitations, including low sensitivity and specificity, have been addressed through the implementation of emerging techniques like microfluidics and nanofluidics.

XII. LAB–ON–CHIP AND ORAL CANCER

Microfluidics technology, also known as micro-total analysis or LOC methodology, involves the integration and automation of various analytical laboratory procedures within a single device or chip. It is often likened to the equivalent of silicon-integrated chips and has had a transformative impact on the fields of electronics, computing, and telecommunications. In today's context, microfluidic systems find applications in disease diagnostics, precise drug delivery, the detection of bioterrorism agents, as well as monitoring air and water quality. These diagnostic systems are capable of accepting and processing small biopsy samples or

bodily secretions, such as blood, saliva, lung aspirates, urine, or intraductal breast fluid. They subsequently provide easily interpretable information regarding the presence and quantity of specific molecules, including pathogen antigens, nucleic acids, antibodies, metabolites, toxins, drugs, and cancer markers.

The inception of this technology dates back to 1975 when the first device of its kind was developed. Notably, in 1990, Manz et al introduced a miniaturized open tubular chromatograph, showcasing the early strides in silicon chip technology adoption. The emergence of the first micro total analysis system (µTAS), specifically a capillary electrophoresis system, occurred towards the end of the 1990s. Subsequently, in 2007, the Whiteside Group at Harvard University innovated and introduced the concept of paper-based analytical devices. These devices incorporate microfluidic systems, facilitating mixing and chemical reactions, while the detection and quantification processes are carried out using sensor systems. In contrast to fluorescence and electrochemical detectors, optical absorption-based colorimetric detection is considered a superior alternative. These devices feature eight inlets for reactants and a dedicated inlet for the sample under evaluation. The sample is then mixed with the corresponding reactants. Mixers play a pivotal role in biological and chemical applications, enhancing the efficiency of mixing and homogenization. This technique allows for the use of a minimal sample volume in disease diagnosis, making it a less invasive and more effective method for the early detection of oral cancer.

The genetic transformations within cancer cells lead to modifications in gene expression patterns, which can be identified well in advance of the manifestation of the cancer phenotype. These alterations in cancerous cells, when compared to normal healthy mucosal cells, can serve as valuable biomarkers. Notably, genes such as p53, cyclin D1, and the EGF receptor gene have been associated with the progression of OSCC. Utilizing microarray analysis across various tumor types aids in the differentiation of tumor cells from their normal counterparts. The integration of high-density microarrays and advancements in bioinformatics have paved the way for incorporating these gene signatures into microfluidic LOC devices.

Research has identified a specific set of genes that exhibit either downregulation or upregulation in OSCC. To begin the cancer diagnostic procedure, the patient provides approximately 1ml of saliva, which is absorbed by a sponge-tipped disposable collector. Subsequently, the collector is inserted into a cassette to introduce the collected oral fluid through a sample inlet port. The initial step in the cancer diagnostic process involves the removal of lymphocytes, facilitating the isolation of cancer cells from the sample. Magnetic beads coated with anti-EpCAM antibody, are anomalously expressed on the surface of cancerous epithelial cells and are used to capture and extract the cancer cells from the sample. The separated cancer cells can be readily observed and quantified.

Following the separation of cancer cells, they undergo a thermal and/or chemical lysis process, after which the mRNA is extracted. For the amplification of multiplex mRNA, techniques like reverse transcription polymerase chain reaction, linear amplification, or the bio-barcode method can be employed. Subsequently, the transcription profile of the isolated cancer cells from the sample is compared to cancer signature profiles stored in a database using established statistical criteria to determine the specific cancer type.

XIII. 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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