Utilization of Saliva as a Diagnostic Fluid in Determination of Oral Cancer

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ABSTRACT

Today’s world technology has evolved and we have been able to evaluate certain phenomenon that occurs before manifestation of observable clinical symptoms. Every health care worker has a dream of a perfect diagnosis so that correct service whether medical or surgical, care can be facilitated to the patient. Cancer is one such disease where tracking the tell-tale signs is essential. The one factor behind oral cancer’s high mortality is the challenge in its early detection. Despite the scepticism in the scientific community and the conservatism of the patients, saliva seems to emerge as a valuable tool in cancer diagnostics and mass screening of the population. An attempt to integrate the simultaneous testing of different salivary molecular markers in order to raise the possibility of an accurate diagnosis by simply using micro- and nanoelectricmechanical systems biosensors is on the way raising much hope in its future applications.

Keywords: Saliva, Oral cancer, Diagnostic fluid.


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Conflict of interest: None

INTRODUCTION

The human body is riddled with complex phenomena going on in different somatic systems. These are well programmed and seem to be systematic. It is left to the researchers to pick up the clues to predict the disease and health of the population. But, in today’s world technology has evolved and we have been able to evaluate certain phenomenon that occurs before manifestation of observable clinical symptoms. Every health care worker has a dream of a perfect diagnosis so that correct service whether medical or surgical, care can be facilitated to the patient. Cancer is one such disease where tracking the tell-tale signs is essential. The one factor behind oral cancer’s high mortality is the challenge in its early detection.

CURRENT SCENARIO IN ORAL CANCER SCREENING

The most definitive procedure for oral cancer diagnosis is a scalpel biopsy followed by careful histopathological evaluation by a qualified pathologist. For this to be an effective procedure, it requires three consecutive visits to the dentist and physician’s office, biopsy by the licensed health care provider and a pathologist’s evaluation.

Detection of an oral cancer at stage I will carry a prognosis of 80% survival, while the same lesion at stage III carries a 20% survival. This is a dramatic difference that affects not only the quality of life for the patient but have significant savings on the health care costs.

Researchers have been looking for alternative approaches to biopsy, with the hope to find a test for oral cancer detection that is similar to the Papanicolaou smear, which has significantly improved the mortality in cases of cervical cancer. Since most oral cancers arise as asymptomatic small lesions, formal diagnosis procedures begin only when the clinician or patient notices abnormal tissues. Microscopic investigation of the progressive cancer is often conducted too late for any further intervention. It is also impractical to use imaging techniques for cancer screening since they are time-consuming and expensive. These techniques are typically used for confirmation due to their lack of sensitivity toward small lesions.

Studies have demonstrated that good positive predictive values can be achieved by oral cancer tissue staining with toluidine blue. However, extensive experience is required in applying this technique and in interpreting its results. Exfoliative cytology may be a less invasive method for oral cancer detection but exfoliated cancer cells tend to correlate with tumor burden and lower rates of detection are seen in those with minimal or early disease. Many DNA markers, like TP53, HPV EBV genomic sequence, have been studied. Cytokeratins have been used for RNA diagnostics, while squamous cell carcinoma, CD44, cytokeratin 19 fragments and telomerase have been used as protein markers. However, none of them is able to identify squamous cell carcinoma.

SALIVA AS A DIAGNOSTIC FLUID

The oral cavity is the ‘gateway to our body.’ More specifically speaking, saliva is a mirror of the body. The ability
4. Saliva is easier to handle for diagnostic procedures because
3. Can be procured easily, does not require any specialized
2. Relatively safe, with no use of invasive devices like the
1. Colorless liquid in contrast to blood (which can undergo
Advantages of saliva for diagnostics are as follows:
• Studies done on saliva for detection of breast cancer are
5. It has better sensitivity and specificity than reference
Disadvantages: A major drawback to the use of saliva
as a diagnostic fluid has been the notion that informative
analyses are generally present in lower amounts in saliva
than in serum.
It is riddled with bacteria and other detritus that would
yield adulterated samples incapable of generating reliable
and reproducible results.
Saliva has long been used for the monitoring of drug
abuse (drugs and addictive substances), such as cocaine,
heroin, amphetamine, barbiturates, etc. Moreover, salivary
testing has been largely performed for the diagnosis of HIV
infection. Analysis of salivary parameters, such as salivary
flow rate, pH, buffer capacity, *Lactobacillus*, and yeast
content, presence of IgG, IgM and anti-La autoantibodies
and raised protein levels, such as that of lactoferrin and
cystatin C as has been proposed for the diagnosis of Sjogren’s
syndrome. Concerning cancer diagnostics and follow-up
altered levels of certain mRNA molecules have been detected
in saliva in oral cancer patients and of certain proteins in
several cancers.

Studies done on saliva for detection of breast cancer are
as follows:
• Salivary picture in breast cancer: HER 2—It is found on
the surface of regularly growing cells. Overexpression
of this factor causes uncontrolled cell growth, more
common in breast cells.
• Epidermal growth factor (EGF): It is a small protein
known to stimulate tumor cells. A salivary study of
this factor showed higher levels in women with breast
cancer. Testing for proteins associated with EGF pathway
appears to be promising in detection of breast cancer.
• Erb and CA 15-3: Studies have shown that these
are in the proportion of 45 to 50% higher in women
with breast cancer than those without. Salivary erb tests
were able to detect 87% of the women with breast cancer.

<table>
<thead>
<tr>
<th>Year</th>
<th>Reference</th>
<th>Study findings</th>
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<tbody>
<tr>
<td>2000</td>
<td>Liao PJ, Chang YC, Huang MF, Tai KW, Chou MY.</td>
<td>Claimed that exon 4 codon 63 of the p53 gene is mutated in salivary DNA from five of eight (i.e. 62.5%) oral cancer patients. Five of 27 control subjects (i.e. 18.5%) had similar mutations in their p53 gene.</td>
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<td>2001</td>
<td>El-Naggar AK, Mao L, Staerkel G, et al.</td>
<td>Demonstrated genetic heterogeneity in saliva from patients with oral squamous carcinoma and suggested the use of epithelial cells in saliva from patients with head and neck squamous tumorogenesis for genetic analysis. Again a similar approach was conducted on submandibular and sublingual saliva by method of comparative proteome analysis.</td>
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An increasing number of systemic diseases and conditions have been shown to be reflected diagnostically in saliva. Along with these developments are technology advancements that have overcome barriers to the widespread implementation of salivary diagnostics. These barriers include technological problems related to achieving high sensitivity, high specificity, miniaturization, high throughput (that is, assay a large number of samples concurrently), automation, portability, low cost, high functionality and speed; overcoming them has enabled researchers to detect and measure multiple disease markers.

Salivary contents in HFN tumors: In order to utilize the full diagnostic potential of saliva, one needs to decipher and catalog the informative components comprehensively. Comparison of such a catalog with a disease population will reveal diagnostic signatures that can discriminate between normal individuals and those with disease. The salivary proteome presents one such resource.\(^{16}\)

Cancer-related genetic alterations identified in bodily fluids: In the development of neoplastic disease, progressive genotypic and phenotypic alterations, such as the activation of protoncogenes and oncogenes and inactivation of tumor suppressor genes associated with tumorigenesis are detected in the affected cells, establishing the model of multistep tumorigenesis.\(^{17}\) It has been shown that identical mutations can be identified in bodily fluids draining a tumor,\(^{18}\) but also lately in bodily fluids secreted away from the initial point where a solid tumor is developing.\(^{19,20}\) Nucleic acids and proteins related to cancer cells have been detected in plasma/serum,\(^{21,22}\) urine,\(^{23,24}\) saliva,\(^{25,26}\) broncho-alveolar lavage fluid, cerebrospinal fluid\(^{27}\) and other bodily fluids. These nucleic acids and proteins have been used as molecular markers for the early diagnosis of the disease,\(^{26-29}\) recurrence markers survival and metastasis predictors\(^{30,31}\) and decide the therapeutic approach.\(^{32,33}\)

Speculations about possible mechanisms that lead to the presence of genotypic and phenotypic markers in the saliva: Cell-free nucleic acids and proteins in saliva may be derived from serum or can be locally produced.\(^{34}\) Serum derived nucleic acids and proteins in the saliva may be part of the normal salivary secretion (by the acinar cells)\(^{35}\) or come there either via intracellular routes, such as active transport or passive diffusion\(^{36}\) from the serum to saliva across cell membranes or extracellular routes, such as ultrafiltration through tight junctions\(^{37}\) or as constituents of the outflowing gingival crevicular fluid.

Cell-free nucleic acids and proteins in saliva however can be locally produced by cell necrosis, lysis or apoptosis and trauma and may even be actively released by normal epithelial or cancerous cells. Cell necrosis is a possible mechanism leading to the release of cell free nucleic acids and proteins in the saliva and this idea is also supported by the large amount of DNA in the plasma of patients with cancers in an advanced stage. Moreover, evidence exists concerning the presence of cell-free nucleic acids and proteins in apoptotic bodies\(^{38}\) which also protect these molecules from degradation.\(^{39}\) The active release of these molecules in exosomes or microvesicles is another strong possibility.\(^{40}\) Exosomes or microvesicles are released by living cells. They are membrane vesicles, 40 to 100 nm in diameter,\(^{41}\) originating from the endoplasmic reticulum and are released when fused with the cell membrane. They contain mRNA,\(^{42}\) miRNA,\(^{43}\) and proteins\(^{44}\) and are thought to play a role in the cell-free intercellular communication.\(^{45-47}\) Refer the below table for ease of understanding.

<table>
<thead>
<tr>
<th>Year</th>
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<tr>
<td>2006</td>
<td>Philips C.</td>
<td>Rinse and spit. Saliva as a cancer biomarker source. In NCI Cancer Bulletin 2006;3-5.(^{14})</td>
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Mechanisms that lead to the Presence of Genotypic and Phenotypic Markers in Saliva

Cell-free nucleic acids and proteins in saliva (diagrammatic representation):

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<th>Serum derived</th>
<th>Locally produced</th>
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<td>Normal salivary secretion</td>
<td>Cell necrosis, lysis</td>
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<tr>
<td>Passive diffusion</td>
<td>Apoptosis</td>
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<tr>
<td>Active transport</td>
<td>Trauma</td>
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<tr>
<td>Ultrafiltration through</td>
<td>Active release</td>
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<tr>
<td>tight junctions</td>
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<td>Outflow of crevicular fluid</td>
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Salivary markers for oral cancer detection: Molecular markers for the diagnosis of OSCC can be questioned in three levels: (1) changes in the cellular DNA, (2) altered mRNA transcripts and (3) altered protein markers.

- Changes in the cellular DNA: Typical changes in the host DNA of dysplastic or cancer cells include point mutations, deletions, translocations, amplifications and methylation.
- Changes in protein markers: (1) cyclin D1, epidermal growth factor receptor (EGFR), microsatellite instability and HPV presence. Allelic loss on chromosomes 9p has been observed in OSCC. Mitochondrial DNA mutations have also been useful targets to detect exfoliated OSCC cells in saliva. The same mitochondrial DNA mutations were detected in 67% of saliva samples from OSCC patients by direct sequencing alone. p53 gene mutations are also present in approximately one-half of head and neck cancers. Using plaque hybridization, Boyle et al identified tumor specific p53 mutations in 71% saliva samples from patients with head and neck cancer.

Promoter hypermethylation of several genes has been reported in head and neck cancer. Rosas et al identified aberrant methylation of at least one of three genes (p16, MGMT or DAP-K) in OSCC. Abnormal promoter hypermethylation was also detected in the matched saliva sample in 65% of OSCC patients.

Cyclin D1 gene amplification has been found to be associated with poor prognosis in OSCC. In another study, Ki67 markers were increased, while 8-oxoguanine DNA glycosylase, phosphorylated-Src and mammary serine protease inhibitor (Maspin) were found decreased in the saliva of patients with OSCC.

Microsatellite alterations of DNA were also observed in the saliva of patients with small cell lung cancer. In the same study, it was further demonstrated that 93% of the patients with microsatellite instability in tumor DNA also had similar microsatellite alterations in the corresponding plasma DNA.

The presence of HPV (human papilloma virus) and Epstein-Barr virus genomic sequences have been identified as possible DNA molecular markers in detecting OSCC and tumor progression.

Altered mRNA Transcripts

For several years, RNA was believed to be quickly degraded in saliva due to the various RNAses that the saliva contains. Despite the opposite reports, cell-free RNA molecules, however, seem to exist in saliva both intact but also fragmented. An intriguing question that remains to be answered is the mechanism by which mRNA in saliva is protected by degradation. A speculation is that salivary mRNA is contained in apoptotic bodies or actively released in exosomes or microvesicles. Lately, microRNAs, small RNA molecules, 18 to 24 molecules in length, which seem to regulate transcription were also discovered existing in saliva. mRNA detection in saliva has been extensively reported enabling body fluid identification in forensic medicine. Moreover, mRNA markers in the saliva have been proposed for the diagnosis of primary Sjögren’s syndrome and for the identification of sleep drive both in flies and humans.

Various mRNA molecules were found upregulated in the saliva of patients suffering from OSCC by the team of Li et al. Seven mRNA molecules transcripts of the following:

1. Interleukin 8 (IL-8) playing a role in angiogenesis, replication, apoposis, calcium-mediated signaling pathway, cell adhesion, chemotaxis, cell cycle arrest, immune response.
2. Interleukin 1B (IL-1B) which takes part in signal transduction, proliferation, inflammation and apoptosis.
3. DUSP1 (dual specificity phosphatase 1) with a role in protein modification, signal transduction and oxidative stress.
4. H3F3A (H3 histone, family 3A) having a DNA binding activity.
5. OA1 (ornithine decarboxylase antizyme 1) taking part in polyamine biosynthesis.
6. S100P (S100 calcium binding protein P) with a role in protein binding and calcium ion binding.
7. SAT (spermidine/spermine N1-acetyltransferase) which takes part in enzyme and transferase activity were found significantly elevated in OSCC patients rather than in healthy controls.

Altered Protein Markers

Several salivary protein markers for OSCC have been investigated in various studies and have shown relatively moderate sensitivity and specificity values relative to prognosis prediction.
For example, defensins are peptides which possess antimicrobial and cytotoxic properties. They are found in the azurophil granules of polymorphonuclear leukocytes. Elevated levels of salivary defensin-1 were found to be indicative for the presence of OSCC, since higher concentrations of salivary defensin-1 were detected in patients with OSCC compared with healthy controls.

In another study, soluble CD44 was found to be elevated in the majority of patients with OSCC and distinguished cancer from benign disease with high specificity. Whereas the solCD44 test lacks sensitivity by itself, methylation status of the CD44 gene seems to complement the solCD44 test and provides very high sensitivity and specificity for the detection of OSCC.

St John et al investigated whether IL-6 and/or IL-8 could serve as informative biomarkers for OSCC in saliva. IL-8 was detected at higher concentrations in saliva, while IL-6 was detected at higher concentrations in serum of patients with OSCC. Thus, they concluded that IL-8 in saliva and IL-6 in serum hold promise as biomarkers for OSCC.

A group of leading researchers using new and sophisticated approaches, such as, Luminex Multianalyte Profiling (xMAP) technology, shotgun proteomics, capillary reversed-phase liquid chromatography with quadrupole time-of-flight mass spectrometry and matrix-assisted laser desorption/ionization-mass spectrometry (MALDI–MS), has contributed significantly in recent years to the research in saliva for cancer diagnosis. Their studies have shown that saliva contains proteins that may serve as biomarkers for OSCC, since 46 peptides/proteins were found at significantly different levels between the OSCC and control groups. For example, Arellano-Garcia et al using Luminex xMAP technology showed that both IL-8 and IL-1b were expressed at significantly higher levels in OSCC subjects.

Molecular markers for the diagnosis of oral squamous cell carcinoma

Changes in the cellular DNA
- Allelic loss on chromosomes 9p mitochondrial DNA mutations
- p53 gene mutations
- Promoter hypermethylation of genes (p16, MGMT or DAP-K)
- Cyclin D1 gene amplification
- Increase of K67 markers
- Microsatellite alterations of DNA
- Presence of HPV

Altered mRNA transcripts
- Presence of IL-1B
- DUSP1 (dual specificity phosphatase 1)
- H3F3A (H3 histone, family 3A)
- OAZ1 (ornithine decarboxylase antizyme 1)
- S100P (S100 calcium binding protein P)
- SAT (spermidine/spermine N1-acetyltransferase)

CONCLUSION

Saliva, a biofluid seems to hold a promise as a diagnostic fluid as compared to blood in parameters, such as sensitivity, specificity and applicability of the method, cost and duration of the procedure, with superb patient compliance. Due to the recent advances and emerging technologies in molecular biology new molecular markers (DNA, RNA and protein markers) have been discovered existing in the saliva in measurable quantities. OSCC can be diagnosed with high sensitivity and specificity by merely testing saliva samples from the subjects. This does not of course undermine the value of screening tests by visual examination neither the importance of the tissue biopsy.

We still need to explore and exploit technologies to get the best outcome of the laboratory tests. Despite the scepticism in the scientific community and the conservatism of the patients, saliva seems to emerge as a valuable tool in cancer diagnostics and mass screening of the population. An attempt to integrate the simultaneous testing of different salivary molecular markers in order to raise the possibility of an accurate diagnosis by simply using micro- and nanoelectromechanical systems biosensors is on the way raising much hope in its future applications.

Finally, since the present methods are not ready for immediate clinical use as diagnostic tools, much work is necessary and it can be envisaged that simple, fast, portable and cost-effective clinical diagnostic systems could be available in the near future.

REFERENCES