

Salivary Tumor Markers- A Review

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Abstract: The first tumor marker reported was Bence Jones protein in 1846 by precipitation of a protein in acidified boiled urine. The measurement of Bence Jones protein has been a diagnostic test for multiple myeloma. The first modern tumor marker used to detect cancer was human chorionic gonadotrophin (HCG), a marker used in pregnancy tests. High level of HCG in the blood may indicate the presence of a placental malignancy. After pregnancy if the uterus continues to enlarge; then a high level of HCG in the blood may indicate the presence of a cancer of the placenta called as Gestational trophoblastic disease. This marker is still used to help in their diagnosis & to monitor their response to therapy. The first success in developing a blood test for a common cancer was in 1965, when Carcinoembryonic antigen was found in the blood of some patients with colon cancer. By the end of 1970s several other blood tests had been developed for different cancers. Some proteins are secreted and/or cleaved into the extra cellular milieu and may represent valuable serum biomarkers for diagnostic purpose. This review is all about the various salivary tumor markers which help in diagnosis of oral squamous cell carcinoma.

Keywords: Tumor Markers, Saliva, Oral Squamous cell carcinoma, Proteins, Peptides, MicroRNA, Cyfra.

INTRODUCTION

Tumor markers are substances that are produced by the body, in response to cancer growth or by the cancer tissue itself. These molecules can be present in blood referred to as serum markers and in tissues referred to as tissue markers.

Thus, they are biochemical indicators of the presence of a tumor. They include Cell surface antigens, Cytoplasmic proteins, Enzymes, Hormones and most of them are proteins. They can be products of cancer cells themselves or of the body in response to cancer or other conditions. Increase in the levels of a tumor marker can indicate cancer; however this is considered after ruling out other causes leading to elevation of these markers and due to this, tumor markers cannot be construed as primary modalities for the diagnosis of cancer. At present, it is estimated that cancer deaths are over 6 million people per year around the globe, with over 10 million new cases being diagnosed every year. Mortality due to cancers is mainly attributable to spread of primary cancer focus to various distant organs; on which there is only little effective treatment is available [1].

In recent years, a number of studies have attempted to determine the prognostic relevance of certain molecular markers. The prognostic value of this biological parameter has been shown different in various malignancies. These are used to predict the local recurrence, potential for metastasis and thereby assessing the disease free survival and survival to death. Markers for proliferation have been used for more than a decade as molecular indicators of malignancy without having shown a clear relation to the clinical behavior of the disease [2]. With many advances in technology in the modern era, levels of certain genetic materials (DNA or RNA) can now be measured.

USES OF TUMOR MARKERS

Tumor markers are not diagnostic in themselves. A definitive diagnosis is made by

microscopic examination and assessment of the biopsy specimen. However, tumor markers provide information that can be used for:

Screening

Some of the organ specific markers can be used for screening with a strong family history of a particular cancer. PSA testing for prostate cancer is an example.

Help in diagnosis

Elevated tumor markers help in detecting the primary cancer such as CA-125 for ovarian cancer, and to help differentiate it from other conditions.

Staging

Elevations in levels of tumor markers help to determine how far the cancer has spread into other tissues and organs.

Determine prognosis

Some tumor markers can be used to determine its aggressive nature of certain histological variants of tumors.

Guide treatment

Some tumor markers, such as Her2/neu, give information about treatments their patients may respond to (for instance, breast cancer patients who are Her2/neu positive are more likely to respond to Herceptin treatment).

Monitor treatment

Tumor markers can be used to monitor the effectiveness of treatment, especially in advanced cancers. CEA, for instance, is used to monitor colorectal cancer, but not every colorectal cancer patient will have elevated levels of CEA.

Determine recurrence

Currently, one of the biggest uses for tumor markers is to monitor for cancer recurrence. Elevation or suppression of tumor markers is inversely proportional to the treatment given.

Oral squamous cell carcinoma

Squamous cell carcinoma is defined as “a malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and/or the presence of intercellular bridges”. The reorganization that different degree of differentiation occurs in epidermoid carcinoma prompted Borders to suggest a system of grading tumors. The one advantage of grading a tumor is that the grade reflects the anaplasticity of the Study of prevalence of p53 tumor suppressor gene, bcl-2 apoptotic indicator and Ki-67 proliferative indicator in oral SCC by Piattelli A found strong correlation between p53 over expression and cell proliferation (MIB-1) and an inverse relationship was found between bcl-2 expression and MIB-1. A good

positive correlation was present between apoptotic index and MIB-1 expression lesion, which in turn indicates the general rapidity of growth, the rapidity of metastatic spread, the general reaction to expected after X-ray radiation and the prognosis [3].

Study of prevalence of p53 tumor suppressor gene, bcl-2 apoptotic indicator and Ki-67 proliferative indicator in oral SCC by Piattelli A found strong correlation between p53 over expression and cell proliferation (MIB-1) and an inverse relationship was found between bcl-2 expression and MIB-1. A good positive correlation was present between apoptotic index and MIB-1 expression. PCNA/AgNOR and Ki-67/AgNOR double staining in oral squamous cell carcinoma by Costa et al concluded there is a significant correlation between cell proliferation and AgNORs score [3].

A comparative study between oral benign and malignant lesion including 50 cases of Squamous cell carcinoma, 14 cases of leukoplakias, and 11 patients of pleomorphic adenomas by Tsuji T *et al.* DNA histogram of 20 cases of Squamous cell carcinoma was measured with flow cytometry. P53 was seen in Squamous cell carcinoma, but rare in leukoplakia and pleomorphic adenoma. And also p53 markedly increased in smokers than nonsmokers.

liao *et al.* analyzed the proliferative activity of dysplastic leukoplakia, oral SCC, and salivary gland tumor specimens using Ki-67 immunohistochemistry. The Ki-67 labeling indices (LI) were significantly higher in dysplastic leukoplakia and oral SCC with high telomerase activity than in dysplastic leukoplakias and oral SCC with low and negative telomerase activity ($P < 0.01$ and $P < 0.05$). These results indicate that telomerase activity has some correlation with the progression of multistep oral carcinogenesis with the cellular proliferation, and also indicate that telomerase may be a specific marker used to distinguish malignant salivary gland tumors from their benign counterparts. Serum concentrations of AMDL DR-70 were estimated by enzyme linked immuno-sorbent assay in 52 patients with carcinoma of the tongue and compared with 40 controls and 42 patients with benign lesions in the tongue. Thirty-nine patients with carcinoma of the tongue had results above 6 mg/L (75%), compared with 3/40 (7%) in healthy controls and 4/42 (10%) in those with benign tumours. The concentration of AMDL DR-70 in serum correlated significantly with 3-year survival [4].

An interesting study by Saito T “Immunohistochemical analysis of cell cycle-associated proteins p16, pRb, p53, p27 and Ki-67 in oral cancer and precancer with special reference to verrucous carcinomas” Comparing squamous cell carcinomas with VCs, there was a great difference in expression levels of p27, Ki-67 and p53, which seemed to reflect the

different cell proliferative activities of these two tumors. Expression of p16 was low in both dysplasia and Squamous cell carcinoma, whereas p16 expression was high in VCs. Epithelial differentiation specific marker cytokeratin-4 expression when compared with p53 status and ki67 in 30 cases of oral papilloma and 30 cases of normal mucosa. Twenty-eight of 30 (93%) papilloma specimens were positive p53. The percentage of p53-positive cells in the basal layer was 60.4 +/- 14.8, and that of Ki-67-positive cells was 26.7 +/- 14.4. There was no correlation between expression of p53 and that of Ki-67. Expression of CK4 was inversely correlated with the expression of Ki-67 but not correlated with the expression of p53. Indicating that proliferation cells cannot be precluded due to imbalanced proliferation [5].

Recent study has shown that low haemoglobin level is of prognostic value for Oral squamous cell carcinoma patients. This may be due to poor general and nutritional conditions which reportedly have been of prognostic value. Furthermore, anaemia has been associated with increased risk of developing Oral squamous cell carcinoma and Tumors of the salivary glands constitute a heterogeneous group of lesions of great morphologic variation. Changes in iron concentration have been shown to alter squamous cell kinetics. It is thus possible that low haemoglobin level may influence the development and growth of Oral squamous cell carcinoma.

In another study it was found that Rhesus negative patients had significantly poorer prognosis than Rhesus positive. The Rhesus gene is located on the short arm of chromosome 1 (1p36.2- 1p34), and the chromosome instability on this area reportedly occur in some head & neck and other cancers. Three oncogenes L- myc, N- ras and jun which may be involved in cancer progression, are also located in this chromosome area.

Saranath *et al.* in 1990 reported a significant correlation between prognosis of Oral Squamous cell carcinomas and aberrations on the L- myc gene area which is located very close to the Rhesus gene. This chromosome area may also include a tumor suppressor gene. In addition an association between a hereditary malignant melanoma and Rhesus gene has been found. Thus, a link between prognosis, Rhesus gene, Chromosome instability and Oncogene activation is highly speculative. Further studies of genetic events on chromosome 1 are needed to elucidate possible interactions.

Salivary gland neoplasm

Benign tumors of salivary gland need no treatment other than surgical removal. Malignant tumors on other hand may require radiation or chemotherapy or both after surgery. It's very important to understand the biological behavior of both types of

tumors, since both needs different type of treatments. Possibility of carcinoma arising in benign tumor cannot be overlooked [3].

Alves FA et al. have studied the correlation between expression of two proliferative marker namely PCNA and ki67 in submandibular salivary gland tumors and also to compare the tumor suppressor gene p53 expression. In this study 15 cases of pleomorphic adenoma, mucoepidermoid carcinoma and adenoid cystic carcinoma are used. The results showed that all pleomorphic adenomas were negative for p53 and Ki-67 with 66.6% being positive for PCNA. However, p53 was positive in 53% of the mucoepidermoid carcinomas and in 20% of the adenoid cystic carcinomas. Ki-67 was expressed in 47.7% of the mucoepidermoid carcinomas and 40% of the adenoid cystic carcinomas. All malignant tumors were positive for PCNA [6].

Martinez-Barba E reviewed for clinically, histologically and immunohistochemically for prognostic significance (p53, Ki67, c-erbB-2 and DNA content) in 9 cases of salivary ductal carcinoma [7]. The average age of the patients was 62.8 years. Tumor size ranged from 1 to 6 cm. Recurrences was found in 3 patients, regional metastases in 4 patients and systemic metastases in 3 patients. Three patients died of their disease, one is alive with the disease and 5 are alive without evidence of disease. When compared for prognostic biomarkers expression p53 protein nuclear immunostaining was positive in 66.6% and c-erbB-2 overexpression was observed in 100% of the tumors. Tumors or malignancy growth cannot grow beyond 2-3cms without neovascularisation. Formation of blood vessel not only helps in growth but also helps in metastasis [8].

Lim JJ have studied the vascular endothelial growth factor and compared with expression of p53 and ki67 in 45 cases of salivary gland carcinoma. When the results of expression with clinicopathological features were compared, VEGF expression was low in 14 cases, moderate in 15 cases, and high in 16 cases. It was significantly correlated with a variety of clinicopathological factors of p53 but not with that of Ki-67. VEGF showed significant association with the expression [9].

Lida W Chan *et al.* determine the predictive value of urinary levels of two angiogenic factors, vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMPs), in a longitudinal study to determine their correlation with 1-year progression-free survival in patients with cancer. This small exploratory study suggests that the angiogenic urinary trends of VEGF and MMPs may be useful predictive markers for progression-free survival in cancer patients after the completion of radiotherapy. PCNA Immunohistochemical expression revealed stronger quantitative labeling index for the follicular

ameloblastoma, while for p53 protein the strongest quantitative labeling index was detected in the plexiform type.

Van Heerden WF have studied pleomorphic adenoma, Polymorphous low-grade adenocarcinoma, adenoid cystic carcinoma, mucoepidermoid carcinoma, carcinoma ex mixed tumor, undifferentiated carcinoma and epithelial-myoepithelial carcinoma, total of 43 cases were studied. The difference between the means of benign and malignant tumors and polymorphous low-grade adenocarcinoma and adenoid cystic carcinoma were highly significant. Since AgNOR is more strongly expressed in malignant tumors so the study concludes that AgNORs count can be diagnostic aid in differentiating between salivary gland neoplasms [10].

ODONTOGENIC TUMORS

Odontogenic tumors represents a spectrum of lesions ranging from malignant and benign neoplasm to dental hamartomas, all arising from odontogenic residues, i.e. odontogenic epithelia and/or ectomesenchyme with variable amounts of dental hard tissue formed generally in the same sequence as in normal tooth development. PCNA expression has been evaluated in both ameloblastoma and adenomatoid odontogenic tumors by Barboza CA [11]. Ameloblastomas are reported to be more than one histological subtype, in this study follicular ameloblastoma, plexiform ameloblastoma, acanthomatous with follicular ameloblastoma, basal cell ameloblastoma and adenomatoid odontogenic tumor were used. This study suggests that the different histologic patterns of ameloblastoma did not show a direct correlation with their clinical behavior and consequently with the prognosis of the cases. The study results concluded that the ameloblastoma has greater proliferative potential than the AOT, which can contribute to explain its more aggressive and invasive characteristics.

Coleman HG *et al.* subjected various odontogenic cysts and the unicystic ameloblastoma with AgNOR staining. They conclude that AgNOR counts are not of diagnostic significance and cannot be used to distinguish the various odontogenic cysts from one another nor from the unicystic ameloblastoma [12].

Kumamoto H *et al.* have examined in 8 tooth germs and 31 ameloblastomas, to determine whether cell cycle regulation or alteration plays a role in oncogenesis and cyto-differentiation of odontogenic epithelium [13].

Cell cycle-related factors, including cyclin D1, p16INK4a, p21 (WAF1/Cip1) and p27Kip1 proteins, DNA topoisomerase II-alpha and histone H3 mRNA, were examined. Cyclin D1 was expressed in epithelial cells near the basement membrane in tooth germs and

ameloblastomas, suggesting that this protein participates in cell proliferation in odontogenic epithelium. P16 protein was observed in most epithelial cells in tooth germs and ameloblastomas. Expression of p21 protein was detected in most epithelial cells in tooth germs and ameloblastomas, but not in keratinizing or granular cells in variants of ameloblastomas. Expression of p27 protein was chiefly found in central polyhedral cells and keratinizing cells in tooth germs and ameloblastomas. DNA topoisomerase II alpha and histone H3 mRNA were localized in scattered epithelial cells attached to the basement membrane in tooth germs and ameloblastomas.

Salivary tumor markers: a new diagnostic tool

The oral cavity is an ideal site for screening smokers and tobacco chewers who are considered as high risk group for developing head and neck squamous carcinoma (HNSC) because of the availability of cells shed in saliva and the convenience of visualizing and sampling lesions at these locations. Saliva contains locally expressed proteins such as alpha amylase, lactoferrins, lysozymes, proline-rich proteins, mucins, histatins, cystatins and transferrin [14]. Diagnosis of OSCC can be done at three different molecular levels. This was proved by Markopoulos *et al.* in their original study on salivary biomarkers for oral cancer detection [15].

Salivary molecular markers for diagnosing oral squamous cell cancer help in three ways 1) Changes in the cellular DNA, 2) alterations and aberrations in mRNA transcripts, 3) Alterations in intracellular and extracellular proteins. Thus salivary biomarkers can be a genetic marker, a protein or a metabolic marker. Identifying novel and reliable biogenetic markers for the biological assessment of squamous lesions may assist in early diagnosis and treatment of head and neck squamous tumorigenesis. Microsatellite DNA motifs consisting of highly polymorphic short tandem repeat (STR) sequences distributed throughout the genome have been widely and successfully used as markers for molecular analysis of tumorigenesis in head and neck and other neoplasm's [16]. Studies using microsatellite markers from different chromosomal arms in HNSC have shown that alterations at certain regions on chromosomes 3p, 9p, 17p and 18q to be associated with the development of these tumors. Different studies have shown high incidence of loss of heterozygosity (LOH) in non-invasive lesions indicating an early association with tumorigenesis [16]. Analysis of selected microsatellite markers at these regions on epithelial cells from patients' saliva is a convenient and noninvasive approach for molecular screening and early detection of the disease.

According to a study conducted on 37 patients; significant correlation between LOH in tumor at certain markers and smoking and alcohol has been found.

The results indicate that

- Epithelial cells in saliva from patients with head and neck squamous tumorigenesis provide suitable material for genetic analysis.
- Combined application of certain markers improves the detection of genetic alterations in these patients.
- Clonal heterogeneity between saliva and matching tumor supports genetic instability of the mucosal field in some of these patients; and
- LOH at certain chromosomal loci appears to be associated with smoking and alcohol consumption.

Molecular genetic studies have demonstrated frequent genetic alterations at certain chromosomal regions in premalignant lesions and invasive tumors. The studies also showed that certain regions on chromosomes 3p, 9p, 8p and 17p are frequently altered in dysplastic lesions and may constitute an early event in lesion development.

Analysis of these markers in oral secretions and other accessible specimens may allow for rapid, inexpensive and objective assessment of the genetic abnormalities at these sites for early detection, screening of individuals at high risk, and follow up of patients with cancer.

SALIVARY BIOMARKERS

There are various salivary biomarkers detected till date and they can be detected in saliva by various techniques (Table 1). Few studies have already documented many potential salivary biomarkers for detection of oral squamous cell cancers (Table 2) [15, 17]

PEPTIDES - Defensins possess antimicrobial and cytotoxic properties. The acidophilic granules of the neutrophils contain defensins. Studies by Mizukawa *et al.* have reported that OSCC can be detected even in their earlier stages by the elevated levels of salivary defensin-1 compared with healthy controls [18].

PROTEINS- Several salivary protein markers such as interleukins [8, 6, 1b], matrix metalloproteinase (MMP 2, 9), transforming growth factor (TGF-1), Ki67, cyclic D1, Cyfra 21.1, transferrin, a amylase, tumor necrosis factor (TNF-a) and catalase have been detected in oral squamous cell carcinoma by various studies[19]. Franzmann *et al.* reported raised levels of CD 44 in saliva (oral rinse) of oral squamous cell carcinoma patients (n = 102) compared to controls (n = 69) [20]. St John *et al.* detected higher concentrations of IL-8 in saliva and higher concentrations of IL-6 in serum of patients with OSCC and concluded that IL-8 in saliva and IL-6 in serum are the informative biomarkers for OSCC [21].

DNA- Boyle *et al.* in their comparative study identified and documented that p53 mutations in 71% of saliva samples from patients with OSCC by using

plaque hybridization technique. Rosas *et al.* identified aberrant meth-ylation of p16, MGMT and DAP-K in OSCC patients. Salivary mRNAs- According to Liu *et al.*, oral carcinogenesis can be detected by the elevation of salivary mRNAs which includes six mRNA molecules such as DUSP1, H3F3A, IL 1B, IL 8, SAT and S100 [22].

DUSP1 (dual specificity phosphatase 1)- DUSP mRNA participates in the MAPK (Mitogen Activated Protein Kinase) pathway. It is involved in protein modification, oxidative stress, and signal transduction. Molecular studies conducted by Khor *et al.* revealed that hypermethylation of DUSP1 gene is an important event in oral carcinogenesis [23].

H3F3A - H3 histone family 3A is a protein encoded by the H3F3A gene which is situated on chromosome 1. These proteins are nuclear proteins responsible for the structural integrity of chromosomal nucleosome and acts as a proliferative marker for oral cancer.

IL 1B- Interleukin 1 beta is a member of interleukin 1 family of cytokines. It is a chemical mediator of cell proliferation, differentiation, and apoptosis. Elevated serum levels of IL 1B are detected in patients with oral squamous cell carcinoma.

IL 8 - Interleukin 8 is a pro-inflammatory cytokine, also known as neutrophil chemotactic factor. It plays an important role in tumor angiogenesis, cell adhesion, and cell cycle arrest. St John *et al.* by their various studies on salivary biomarkers concluded that IL 8 in saliva is the best biomarker for squamous cell carcinoma.

SAT - Spermidine / spermine N1-acetyltransferase 1 a protein encoded by the gene SAT, belongs to the acetyl transferase family. It participates in the catabolism of polyamines. The levels are elevated in the saliva of oral cancer patients compared to the healthy controls.

S100 - P S100 calcium binding protein P, a member of the S100 family is located in the cytoplasm or in the nucleus. It is responsible for cell cycle regulation and differentiation. 6.5. Salivary microRNA MicroRNAs (miRNAs) are short RNA transcripts. Their dysregulation affects cell growth, apoptosis, differentiation, motility, and immunity. Compared to mRNA, microRNA's are significant potential biomarkers for diagnosis of oral cancer because they can accurately differentiate even poorly differentiated carcinomas. These include miR-125a, miR-200a and miR-31.

MiR-125a- It plays an important role in cell proliferation and can affect the genes involved in MAPK metabolism. The levels of miR-125 in saliva are reduced in patients of oral cancer compared to healthy individuals.

MiR-200a- It is involved in tumor suppression and in early metastasis. The levels of miR-200 are also reduced in the patients of oral cancer compared to healthy individuals.

MiR-31- This is a tumor suppressor microRNA. Genetic and molecular studies conducted by Liu *et al.* have shown elevated miR-31 levels in all stages of squamous cell carcinoma patients and has advocated the use of miR-31 as one of the earliest biomarker to detect oral cancer[22]. Lajer *et al.* also suggested miR-31 as one of the potential biomarker to detect oral cancer by their microarray analysis using 51 biopsies from the representative sites [24].

Advantages and disadvantages of salivary biomarkers

Salivary biomarkers have many advantages over serum because they are

- Inexpensive, non-invasive, cost effective and easily accessible media and play a vital role in diagnosis, prediction of prognosis and monitoring of patient's health.
- It is a convenient medium for multisampling and safe for health care professionals compared to blood and is also used for detection of other types of cancers.

Disadvantages of the salivary biomarkers include:

- Lack of standardization procedures like sample collection, processing and storage.
- Variability in the levels of salivary biomarkers.
- Validation in oral inflammatory conditions.

LITERATURE FROM THE PAST

Rafael Nagler *et al.* examined tumor markers in the saliva of oral squamous cell carcinoma (OSCC) patients. They measured the concentrations of six most studied epithelial serum circulatory tumor markers in the saliva of OSCC (tongue) patients. Significant increase in salivary concentration of Cyfra 21-1, tissue polypeptide antigen (TPA), and CA 125 was seen. Salivary concentration of CA 19-9, SCC, and carcinoembryonic antigen (CEA) increased without statistical significance [19].

TPS: assayed using monoclonal immunoradiometric assay

Cyfra 21-1: evaluated using Cyfra 21-1 immunoradiometric Assay kit

SCC, CEA, CA19-9, and CA125 were determined with a microparticle enzyme linked immunoassay distributed by Abbot.

Concurrent analysis showed that out of six; three tumor markers found to be most substantially and significantly increased in the cancer patients were Cyfra 21-1, TPS, and CA 125; which were all increased by

about 400%. The most important result found is that several salivary tumor markers are found to be significantly increased (by 400%) in the saliva of oral (tongue) cancer patients. It is important with respect to both clinical and pathogenesis related aspects of oral cancer.

Rafael *et al.* concluded that the increase shown in salivary tumor markers of the cancer patients can be used as a diagnostic tool. They suggest that this new diagnostic tool is of special importance for patient monitoring, as it is often difficult to distinguish clinically between a post-operatives and/or irradiated scarred oral mucosa and a recurring cancer lesion.

Salivary testing is noninvasive, making it attractive, effective alternative to serum testing, and the possibility of developing home testing kits would further facilitate it as a diagnostic aid, enabling patients to monitor their own health at home.

It is important for those who live far from their treatment centers and especially for those at the risk of developing Oral Squamous cell carcinoma.

CONCLUSION

Availability of advanced molecular diagnostic techniques made salivary biomarkers as a promising diagnostic and prognostic tool. Collection of saliva is easy, inexpensive and multisampling also possible without pain when compared to blood collection for investigations. OSCC can be diagnosed with high sensitivity and specificity by merely testing saliva samples from the subjects. More research with large sample size and on various types of carcinomas can be done to provide greater insight.

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