Review Article

A review on oral cancer biomarkers: Understanding the past and learning from the present

ABSTRACT

Biomarkers are broadly classified as genomic, proteomic, or metabolomic. Molecular biology and oncology research studies on oral cancer biomarkers focus on identifying key biological molecules or markers that could be linked to cancer development, risk assessment, screening, recurrence prediction, indicating prognosis, indicating invasion/metastasis and monitoring therapeutic responses of cancer. Cluster of differentiation factor 34 is a salivary biomarker that can identify recurrence potential of oral squamous cell carcinoma (OSCC). Integrin $\alpha 3$ and integrin $\beta 4$ are genomic biomarkers that are helpful in estimating the risk of regional and hematogenous dissemination of malignant oral squamous cells. Other examples are vascular endothelial growth factor, B-cell lymphoma-2, claudin 4, yes-associated protein 1 and MET proto-oncogene, and receptor tyrosine kinase, which are genomic biomarkers that are used to predict radio-resistance in OSCC tissue. The present article reviews the clinical application, methodologies and steps in developing candidate biomarkers, protocols in reporting, evaluating candidate biomarkers, and challenges in biomarker research with a focus OSCC.

KEY WORDS: Biomarkers, evaluation, oral cancer, oral squamous cell carcinoma, reporting

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INTRODUCTION

Carcinogenesis is a complex process that occurs at the phenotype and genotype levels. Cancer development is driven by the accumulation of genetic and epigenetic changes that disturb the homeostatic equilibrium between cell proliferation and cell death.[1] The molecular level changes that occur in carcinogenesis are: (i) cancer cell proliferation without external stimuli, (ii) insensitivity to inhibitory growth signals, (iii) evasion of apoptosis or cell death mechanisms and/or activation of anti-apoptotic genes, (iv) unlimited replicative potential, (v) sustained angiogenesis, (vi) invasion and metastasis ability, (vii) genomic instability, and (viii) protooncogenes mutation caused by defects in DNA repair.[1]

Research on cancer tissues has revealed that there may be a link between molecular level and tissue level changes that drive malignant changes in the tissue and play a pivotal role in disease progression.[2] The inference can be drawn is that a study of the biological molecules involved in the molecular mechanism of carcinogenesis could provide valuable diagnostic data, i.e., biomarkers, on the cancer disease process. The National Cancer Institute has defined "biomarker" as a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition of disease such as cancer.[3] Biomarkers play an important role in distinguishing between the presence or absence of disease. The underlying tissue changes in the disease process could be categorized as genomic, proteomic, or metabolomic expressions [Figure 1]. Biomarkers include nucleic acids, proteins, peptides, enzymatic changes, antibodies, metabolites, lipids, and carbohydrates.[4] Biomarkers can be derived from one, or a combination, of the following body fluids blood, serum, plasma, body secretions (sputum, saliva), or excretions (stool, urine). Body fluids sample for biomarker investigation can be obtained by noninvasive, minimally invasive or invasive methods.[5] Nucleic acids (DNA/RNA)

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extracted from blood, saliva, oral exfoliative cells, or buccal smear cells are instrumental in identifying mutations and will help to correlate and confirm the diagnosis, monitor the disease progression, or act as prognostic indicators in treatment.[6]

CLINICAL APPLICATIONS AND CONSIDERATIONS OF ORAL CANCER BIOMARKERS

Biomarkers can be used for patient assessment in multiple clinical settings. They can be used for estimating disease risk, screening for occult primary cancers, distinguishing benign from malignant findings/one type of malignancy from another, determining prognosis, acting as predictors/screening, and monitoring disease status. Biomarkers can be used to either detect recurrence or determine progression/response to therapy. The determination of a patient's risk of developing oral cancer is helpful if risk reduction strategies or screening have been effective. These strategies when applied to high-risk groups are much more efficient than wholesale application to the entire population.[7]

Salivary biomarkers such as L-phenylalanine serve as screening biomarkers and help in the early diagnosis and monitoring of oral squamous cell carcinoma (OSCC).[8] Cloning of an acidic laccase gene 2 is a proteomic biomarker that is used to differentiate between squamous cell carcinoma and adenocarcinoma.[9] The angiogenetic marker cluster of differentiation factor 34 (CD34) serves as an important predicting tool for recurrent cases of OSCC.[10] Genomic biomarkers such as integrin α3 and integrin β4 have been positively correlated with distant metastases and prognosis of tumors.[11] Sixty vascular endothelial growth factor, B-cell lymphoma-2, claudin 4, yes-associated protein 1 and MET proto-oncogene, and receptor tyrosine kinase were suggested as a novel group of biomarkers that function as therapeutic monitors and radioresistance predictors in OSCC patients.[12] Hu *et al*. reported immunoassay validation of salivary proteins such as Mac-2-binding protein (M2BP), profilin, CD59, MRP14, catalase, histone H1, S100A12, rat sarcoma viral oncogene homolog (Ras)-related protein Rab-7, moesin, involucrin, S100 calcium binding protein *P* (S100P), and hematopoietic lineage cell-specific protein are differentially abundant in OSCC and healthy control subjects.[13] Aberrant expression of miR-375, miR-200a, and miR-200c-144 methylation was initially identified in OSCC and suggested as a potential clinical application for OSCC diagnosis.[14] The clinical significance of the above mentioned oral cancer biomarkers is summarized in Table 1.

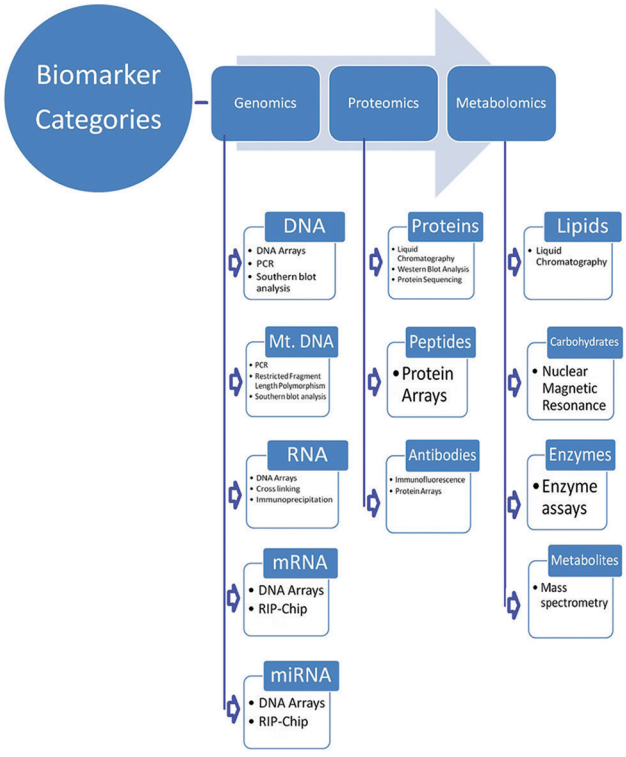

Figure 1: Classification of oral cancer biomarkers

Biomarkers can be used as screening tools in healthy individuals and in patients who are clinically or histologically negative for oral cancer. Screening and early diagnosis of oral cancer are a useful and important risk-reduction strategy. Other strategies are lifestyle changes, habit cessation, and prophylactic treatment in cancer prevention, which increase the survival rate of patients. In 2014, Wang *et al*. successfully isolated potential salivary biomarkers for the early diagnosis of OSCC. Their study investigated eight up-regulated and six down-regulated groups of salivary metabolomic biomarkers. Their study employed an integrated separation approach of reversed phase liquid chromatography and hydrophilic interaction chromatography combined with a time-of-flight mass spectrometer to study the salivary metabolomic analysis methods. The results revealed that five salivary metabolomic biomarkers, propionyl choline, N-acetyl-L-phenylalanine, sphinganine, phytosphingosine, and

Table 1: Clinical significance of oral cancer biomarkers

Clinical significance	Oral cancer biomarker category	Reference
Screening for oral squamous cell carcinoma	Salivary biomarkers such as L-phenylalanine, sphinganine, phytosphingosine, and S-carboxymethyl-L-cysteine	Wang *et al*. in 2014[8]
Differential diagnosis	Proteomic marker CLAC2	Shinmura *et al*. in 2014[9]
Recurrence potential marker	CD34 expression	Kademani *et al*. in 2009[10]
Predicting prognosis and distant metastasis	Genomic biomarkers such as ITGA3 and ITGB4 expression	Nagata *et al*. in 2013[11]
Predicts radio-resistance in oral squamous cell carcinoma	Genomic markers such as VEGF, BCL-2, Claudin-4, YAP-1, and c-MET	Akervall *et al*. in 2014[12]

CLAC2=Cloning of an acidic laccase gene 2, CD34=Cluster of differentiation factor 34, VEGF=Vascular endothelial growth, BCL-2=B-cell lymphoma-2, YAP-1=Yes-associated protein 1, c-MET=MET proto-oncogene, receptor tyrosine kinase, ITGA3=Integrin α3, ITGB4=Integrin β4

S-carboxymethyl-L-cysteine provided a significant statistical correlation and could be considered as candidate biomarkers in screening for OSCC.[8]

Biomarkers can be used to determine the prognosis and recurrent potential in posttreatment (surgical, radiotherapy, or chemotherapy) oral cancer patients. Huang *et al*. in 2010 investigated the GRP78 protein expression in OSCC tissue using the Western blotting assay and immunohistochemistry, and their study results revealed that decreased GRP78 protein expression was significantly correlated with the prognosis of the tumor tissue.[15] Coexpression of phosphoprotein 53 (p53)/p-glycoprotein, coexpression of combined cytoplasmic and membranous epidermal growth factor expression (EGFR) and p53, coexpression c-erbB-2,3 and 4, phosphoprotein 16 (cyclin-dependent kinase inhibitor 2A) (p16)/cyclic D1 amplification, and RAR alfa/phosphoprotein 21 (potent cyclin-dependent kinase inhibitor) expression might provide more prognostic information about OSCC affected tissues. The positivity of prognostic biomarker mechanistic target of rapamycin (serine/threonine kinase) (p-mTOR) may be an important instrument in identifying high-risk subgroups and/or poor prognostic tissues.

Biomarkers can be used to identify therapeutic targets, and as a tool to detect efficacy of therapy.[16-21] Monteiro *et al*. in 2011 suggested that higher expression of a p-mTOR protein may be used as a potential therapeutic target in patients with OSCC.[22] Yang *et al*.'s 2014 investigation of biopsy specimens of OSCC tissues revealed that the GF 15 expression can be used as a prognostic and predictive biomarker for patients under docetaxel, cisplatin, and 5-fluorouracil (TPF) induction chemotherapy.[23,24]

The biomarkers that identify germ-line mutations are significantly important in predicting individuals at risk for developing cancer, and who may have an adverse reaction to specific cancer therapy. Gene polymorphism in p53/p73, CCND1, MDM2, and Harvey Ras (H-Ras) are related to cell cycle, apoptosis, and cancer risk.[25] Tandle *et al*. in a 2001 report suggested that an association between p53 genotypes and oral cancer was not observed in Indian patients.[26] Misra *et al*. in 2009 studied the polymorphisms at p53, p73, and MDM2 and analyzed the risk of oral cancer development at the combined three loci. Their study results suggested that the presence of at least one risk allele at all three loci increases the risk of tobacco-associated leukoplakia and the development of oral cancer.[27] Sathyan *et al*. in 2006, studied the CCND1 gene and potential oral cancer risk, and their results revealed no association with cancer development. However, their study results suggest that the variant "C" allele of the H-Ras (C81T) is associated with a higher risk for oral carcinoma development, particularly in male populations, and thus, this polymorphism could be a low-penetrance gene predisposition factor of oral carcinoma.[28]

Biomarkers are useful in detecting recurrent malignant potential in patients who have received adjuvant therapy. Sulzyc-Bielicka *et al*. in 2013 investigated thymidylate synthase (TS) gene polymorphism in colorectal cancer patients receiving adjuvant 5-fluorouracil. Their study results revealed that patients with a higher expression of TS are at significantly greater risk of early recurrence of oral cancer in the posttreatment period.[29] The efficacy of the EGFR in head and neck squamous cell carcinoma (HNSCC) is currently in Phase III clinical trial investigation. These studies indicate that identification of biomarkers for determining efficacy and toxicity of adjuvant therapies is an important area of investigation in the treatment of OSCC.[30]

Biomarkers are also useful tools for identifying invasion, metastasis, and monitoring therapeutic responses in patients who receive therapy for cancer metastases. Huang *et al*. in 2014 investigated miRNA-459-5p and GIT1 in OSCC tissues as potential biomarkers for invasion and metastatic phenotypes. Their results revealed that expression levels of miRNA-491-51-5p and GIT1 correlated inversely in OSCC tissues. Their results also supported the view that miRNA-491-5p and GIT1 may serve as metastatic prognostic biomarkers, and as targets for intervention in cases of OSCC metastasis.[31]

The miRNA microarray analysis was first reported by Yang *et al*. and explored on low-grade dysplasia, i.e., oral premalignant lesion and analyzed the progression into high-grade dysplasia or OSCC.[32] Their report revealed that 13 miRNA were down-regulated, and 12 miRNAs were up-regulated in progressive low-grade dysplasias. Salazar *et al*. revealed that miR-9, miR-191, and miR-134 as a novel noninvasive biomarkers in HNSCC.[33]

METHODOLOGIES OF IDENTIFYING ORAL CANCER BIOMARKERS AND FURTHER DEVELOPMENT OF DISCOVERED ORAL CANCER BIOMARKERS

The candidate biomarker can be identified by multiple approaches. These approaches can lead to identifying biomarkers from the tumor cell, tumor microenvironment, tumor adjacent tissue, or by the metabolism of pharmaceutical or therapeutic agents. A clear understanding of cancer biology is thus very important in identifying the candidate biomarker.

Methodologies of identifying oral cancer biomarker

Biomarkers are identified by various molecular techniques such as DNA arrays, high-throughput sequencing, polymerase chain reaction, gene expression arrays, restricted fragment length polymorphism, ribonucleoprotein immunoprecipitation-gene chip, cross-linking immune-precipitation, liquid chromatography, nuclear magnetic resonance, mass spectroscopy, enzyme assays, and immunohistochemistry [Figure 1]. Studies focusing on identification of the candidate biomarker need to statistically

standardize their study design, sample group, data analysis, and provide accurate, valid and reliable results on the newly identified biomarkers.

Chai *et al*. in 2014 discovered potential serum biomarkers for lymph-node metastasis in oral cancer. Their study quantified serum proteins using the proteomic assay approach. Their results identified gelsolin, fibronectin, angiotensinogen, and haptoglobin as four candidate biomarkers. The best candidate biomarker identified was gelsolin with a characteristic value of 89% for lymph node positivity in OSCC samples. However, due to the limited sample size in the aforementioned study a long-term longitudinal study is needed to validate these novel biomarkers for clinical utility.[34]

Salivary biomarkers connote a very promising noninvasive approach to oral cancer detection and in monitoring the disease process and the therapeutic response increased attention has been placed on salivary biomarkers based on the convenient and noninvasive method of sample collections. In the last two decades, a greater number of research papers have been published that report on unstimulated salivary constituents and suggest that these constituents have a potential role in the field of oral cancer biomarkers. However, challenges in the salivary biomarker research have pointed toward the need to standardize saliva sample collection, improve processing and storage of the sample, and reduce the wide variability in cancerous and noncancerous individuals. Brinkmann *et al*. in 2011 studied salivary biomarkers in OSCC patients from Serbia and revealed that three salivary proteomic biomarkers and four salivary mRNA biomarkers are significantly associated with late stage OSCC. Salivary proteomic biomarkers such as interleukin 1b (IL-1B), interleukin 8 (IL-8), M2BP, and mRNA markers such as IL-8, S100P, SAT1, and IL-1B were observed with statistically significant values.[35]

Further development of discovered oral cancer biomarkers

Following the discovery of candidate oral cancer biomarkers, subsequent testing will involve analyzing and validating the original hypothesis and findings, following which additional information will be evaluated from the findings that can inform clinical decision-making using analytic validity, clinical validity, and clinical utility.[36]

In the process of developing candidate biomarkers preanalytic and analytic tests are conducted. Preanalytic validity refers to the handling of the sample that will be tested using the new assay. The results of using the new assay could be influenced by (i) time and storage conditions between sample collection and processing; (ii) type and duration of fixation or lack of fixation, and (iii) storage time and conditions following sample processing. Analytic validity refers to the evaluation of the technical aspects of the biomarker, which needs to meet specific criteria and determines the specificity and sensitivity of the assay.[37] Following the development of the analytic validity of the assay, the biomarker will be investigated for clinical validity. Clinical validity relates to the observation that the biomarker reliably divides the overall population of interest into two distinct groups those more likely to suffer an event or those less likely to suffer an event. The final process in the development of the candidate biomarker is that of investigating clinical utility with very high levels of evidence (LOE). Following this, the biomarker will be ready to be used in direct patient care. This process includes assessment of the effectiveness and the benefit-harm ratio of the biomarker. It is important to mention that very few tumor markers have established clinical utility despite the thousands of reported biomarkers in the literature.[38]

PROTOCOLS IN BIOMARKER REPORTING AND EVALUATION

The consistency of the biomarker biopsy specimens can be significantly altered during the collection, processing, and storage stages. This has led to biomarker reporting protocols being developed to reduce the challenges in experimental outcomes and scientific results, and also to ensure that all necessary information has been included. Standard protocols for biomarkers reporting are biospecimen reporting for improved study quality (BRISQ), reporting recommendations for tumor marker (REMARK), standards for reporting of diagnostic accuracy (STARD), and minimum information about a microarray experiment (MIAME). BRISQ and REMARK provide criteria for reporting the details of preanalytical and analytical issues related to potential prognostic factor studies in an organized and transparent fashion.[37,39] STARD for publishing diagnostic tests and MIAME provide guidelines for reporting microarray research.[40,41] A biomarker evaluation protocol has been developed to determine the clinical utility of discovered biomarkers. The American Society of Clinical Oncology Tumor Marker Guideline Committee proposed tumor marker utility grading system (TMUGS) to facilitate the critical evaluation of biomarkers. The highest LOE Level I of the TMUGS protocol requires evidence from a prospective clinical study to test the biomarker of interest or evidence from a meta-analysis or systematic review of well-conducted LOE II studies.[42] The Level II studies provide evidence about a biomarker from prospective clinical trials. The revised system of the critical evaluation protocol requires that prospective clinical trial studies be conducted at the highest LOE, namely LOE Level I.[43]

CHALLENGES OF BIOMARKERS IN CANCER STUDIES

The ideal cancer biomarker should be a unique indicator of malignancy and should create absolute true positivity of the malignancy type without any confounding factors between malignant tissue and nonmalignant tissue, and it should not match with any other malignancy type. It is also worth mentioning that the ideal cancer biomarker should limit false-positive tests and should produce valid and reliable results.[44]

Genetic assays have revealed that a greater numbers of genes are over-expressed in malignant tissues as compared with benign tissues or precursor lesions, and no transcripts or proteins have been identified to be uniquely elevated in cancer. Most of the candidate cancer biomarkers belong to pathways intrinsic to normal cells and tissues, such as cell proliferation, cell differentiation, apoptosis, angiogenesis, cell death, and inflammation. Thus, biomarkers expressed in the nucleus or cytoplasm is not accessible, and attention is therefore paid to the cell surface or secreted proteins. Furthermore, transcript proteins are expressed in relatively increased levels, and therefore fail as candidate biomarkers because of low-level expression.[45]

The cancer tissue is composed of transformed cells which result from mutational changes. On the establishment of malignancy, the inherited genetic instability of tumor cells could lead to the emergence of a subpopulation of cancer cells (termed as clones) that can relatively expand because of growth potential.[1] Further, during cancer progression, it is more likely that new clones will emerge through genetic and epigenetic alterations. This phenomenon is termed "clonal diversity."[46] Clonal diversity is a result of a spectrum of mutations and structural alterations occurring within cancers of a single histological type, resulting in a heterogeneous population of cells.[47] In the context of cancer biology clonal diversity, genetic or histological changes that are naturally occurring with aging further complicate cancer detection strategies.

Cancer tissues are complex tissues composed of malignant cells, nonmalignant cells, and inflammatory cells in a tumor microenvironment. It is most likely that the host immune response to the malignancy, and the interactions of malignant cells to surrounding stroma are not captured by biomarkers.[43,48] Thus, focusing on genetic mutations and structural alterations in malignant cells will yield limited predicting behavior. However, a great number of cancers take years or decades to show clonal diversity. This window appears to provide a greater period of opportunity for detection and eradication of transformed cells. However, to detect tumor metastasis, it is estimated that the tumor needs to be 5 mm in diameter. Detection of the tumor protein from a 5 mm diameter ovarian tumor diluted in a 5 L blood sample from an adult patient is well beyond the sensitivity of currently available technologies.[49]

Many biomarkers fail because of clonal diversity, genomic instability in cancer tissues, the heterogeneous nature of cancer tissue, or incorrect identification of the metastatic signature molecule. Further, complicating issues are overdetection and overtreatment strategies due to biomarker failure.[50]

Sample tissue selection plays a role in biomarker validity, lack of generalizability among various ethnic groups, organ site variation (OSCC vs. squamous cell carcinoma of the skin), and the lack of unique expression of genomic or proteomic component in cancer tissue. This is another challenging area in biomarker research. Therefore, successful development of biomarkers for oral cancer detection must be coupled with the uniqueness of the marker and other multiple factors mentioned above.

HUMAN PAPILLOMAVIRUS TYPE 16-RELATED ORAL CANCER BIOMARKERS AND INFLAMMATORY BIOMARKERS

Human papillomavirus type 16 (HPV 16) is recognized as a cause of most cancers of the cervix and a substantial portion of anogenital and oropharyngeal cancers. E6 and E7 oncoproteins have been correlated with the initiation of malignant transformation of the affected cervical tissues. Current study reports suggested that the HPV-16 E6 antibodies are present prior to diagnosis.[51] A biochemical assay to detect p16 levels in cervical cancer cells has been developed recently and statistical analysis of the results showed a high level of sensitivity. The study report revealed that p16 quantification could be a promising tool for cervical cancer screening.[52] The p16INK4a marker has been used in identifying the OSCC and resulted in the successful identification of HPV-related oral cancers.[53,54]

FUTURE RESEARCH DIRECTIONS

Future research should focus on identifying and categorizing oral cancer candidate biomarkers in the following areas: Screening, differential diagnosis, recurrence predictor, prognosis, therapeutic, and metastases. These candidate biomarkers will be great tools in determining clinical outcomes and developing dental public health strategies. The development of biomarkers targeting oral cancer drug therapy evaluation would greatly assist the determination of therapeutic efficacy. The recommendation is for researchers to use innovative research approaches that adhere to the principles of analytic validity, clinical validity, and clinical utility, and that follow the protocols of biomarker reporting and evaluation.

CONCLUSION

Understanding the steps, methodologies, and reporting and evaluation protocols will help new researchers to reduce bias in biomarker research. Further research to develop biomarkers is recommended to better understand the heterogeneous cell population of cancer tissue, and the host immune response to the cancer cell population. The research should focus on finding a distinction between biomarkers for cancer diagnosis, and therapeutic targets are recommended. Development of clinically valid candidate biomarkers, with greater clinical utility values, for oral cancer screening is highly recommended because early identification of oral cancer will help to reduce patient morbidity and mortality.

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Conflicts of interest

There are no conflicts of interest.

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