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NOVEL GENETIC MARKERS OF ORAL CANCER

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Resumen: El cáncer oral (CO) es uno de los 10 cánceres más comunes en el mundo y está representado por el carcinoma oral de células escamosas (COCE) en la mayoría de los casos. En la clínica dental, el diagnóstico de CO se basa en una combinación de examen clínico y análisis de una biopsia. La mayoría de las veces, la detección de CO se realiza en etapas avanzadas, lo que conduce a una disminución de la tasa de supervivencia. Por lo tanto, es esencial y necesario encontrar biomarcadores genéticos más confiables, como una técnica de diagnóstico más confiable para la detección de CO en etapas tempranas. Esta revisión de la literatura presenta estudios recientes sobre nuevos biomarcadores genéticos salivales como el ADN circulante tumoral (ADNc), las vesículas extracelulares (VE) y el microARN, que pueden aplicarse para la detección y el diagnóstico en estadios tempranos de la enfermedad. Además, también presenta un nuevo método de diagnóstico no invasivo, la "biopsia líquida", y se han discutido ventajas y desventajas. La información obtenida de 329 pacientes con cáncer oral (CO) incluidos en un total de 9 estudios poblacionales humanos, estudios retrospectivos y prospectivos, realizados en diferentes países, ha demostrado que el ctDNA, EVs y miRNAs son biomarcadores genéticos salivales que nos proporcionan datos útiles en el diagnóstico precoz de CO y por tanto se mejorará el pronóstico de la enfermedad. Por el contrario, esta revisión indica que las técnicas para analizar estos biomarcadores están disponibles, pero son costosas y se necesita más investigación para desarrollar protocolos estandarizados y reproducibles que puedan usarse en la clínica dental. Además, nuestros resultados indican que la biopsia líquida tiene varias aplicaciones prometedoras en la clínica dental en la evaluación de la CO; es indoloro, no invasivo, accesible, de bajo costo y su muy buena fuente para el análisis de biomarcadores genéticos. Aunque faltan protocolos estandarizados de aislamiento y evaluación para la biopsia líquida de saliva, estudios recientes sugieren que la saliva puede ser parte de los procesos de diagnóstico en el futuro.

Abstract: oral cancer (OC) is one of the 10 most common cancers in the world, and it is represented by oral squamous cell carcinoma (OSCC) in most cases. The diagnosis of OC, in the dental clinic, is based on a combination of clinical examination and biopsy analysis. Most of the time, detection of OC is done at advanced stages which leads to decreased survival rate. Thus, finding more reliable genetic biomarkers and diagnostic techniques for OC detection at early stages is essential and needed. This literature review presents recent studies regarding novel salivary genetic biomarkers such as tumor circulating DNA (ctDNA), extracellular vesicles (EVs) and microRNA, that can be applied for the detection and diagnosis in early stages of the disease. Furthermore, it also presents a new non-invasive diagnostic method, "liquid biopsy", and advantages and disadvantages have been discussed. Information obtained from 329 oral cancer (OC) patients included in a total of 9 human population studies, retrospective and prospective studies, performed in different countries have showed that ctDNA, EVs and miRNAs are salivary genetic biomarkers providing us with useful data in the early diagnosis of OC and therefore the prognosis of the disease will be improved. In contrast, this review indicates that techniques to measure the biomarkers analyzed are available but are expensive, and more research is needed to develop standardized and reproducible protocols that could be used in the dental clinic. Additionally, our results indicate that liquid biopsy has several promising applications at the dental clinic in the assessment of OC; its painless, non-invasive, accessible, low cost and its very good source for analysis of genetic biomarkers. Even though standardized isolation and evaluation protocols for saliva liquid biopsy are missing, recent studies suggest that saliva may be part of diagnosis processes in the future.

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1. Introduction

1.1. Oral cancer

Oral cancer is one of the 10 most common cancers worldwide. Oral cancer is a malignancy that can arise on any part of the oral cavity (**Table 1**). Oral cancer is a multifactorial disease that result of interaction between multiple genetic and environmental factors (e.g. tobacco, alcohol and virus such as human papilloma virus (HPV)). Of special relevance is the fact that alcohol and tobacco consumption are the most prominent environmental factors associated with 90% of all oral cancer patients (1), suggesting that education of the patients regarding these risk factors are primary responsibility of dentistry.

Oral cancer could be classified according to tumor location and histological characteristics (see **Table 1 and Table 2**). At least three clinical subtypes have been described: oral squamous cell carcinoma (OSCC), mucoepidermoid carcinoma (MC) and adenoid cystic carcinoma (ACC). The squamous cell carcinoma (OSSC) is a cancer of stratified squamous epithelium and is the most frequent type representing about 95% of oral cancers. The most common site for OSCC is the tongue and floor of the mouth (1).

TABLE 1. ANATOMICAL LOCATIONS OF THE ORAL CAVITY CANCER
Upper lip and Lower lip (philtrum outer, skin surface, inner surface of the mucosa)
Labial commissure
Mobile tongue (dorsal surface, margins and apex, ventral surface)
Floor of mouth (anterior, lateral)
Buccal mucosa
Retromolar region
Lower gum and Upper gum
Hard palate

TABLE 2: HISTOLOGICAL ASPECTS OF ORAL CANCER	
Tumor of epithelial origin	EPIDERMOID CARCINOMA AND MELANOMA
Tumor of mesenchymal origin	FIBROSARCOMA, MALIGNANT FIBROUS ISTOCITOMA, ANGIOSARCOMA, SARCOMA, RHABDOMYOSARCOMA, LIPOSARCOMA

The biological behavior of these tumors, shown in **Table 2**, is highly heterogenous and variations within the same histological type may be individual, characteristic for the tumor, but may determine prognosis and treatment at the same time as it is discussed below.

Another histological type of oral cancer, with prevalence of approximately 16%, is mucoepidermoid carcinoma (MC). Mucoepidermoid carcinoma (MC) is the most common occurring cancer in salivary glands. This malignant tumor affects both minor and major salivary glands, and it occurs in the parotid gland in 89.6% of all cases of major salivary glands malignancy (2). MC appears more in the posterior regions of the mandible than in maxilla and it affects more females than males with a ratio at 3:1 at age of 40 and 50 years old (2). Mucoepidermoid carcinoma can manifest in two variants, high-grade variant and low-grade variant (2). A rare occurring oral cancer is adenoid cystic carcinoma (ACC) which accounts for 1% of all head and neck cancers. ACC affects more the minor salivary glands; therefore, it's found in the palate in most cases. It is asymptomatic and it progresses slowly. It has also a female predominance like mucoepidermoid carcinoma (3)

Unfortunately, many of these types of oral cancer are detected in advanced stages causing the death of the patients. Creating awareness, discovery through screening, early diagnosis and when it's appropriate referral to medicine for treatment should be responsibility of

dentists. Thus, one of the most important steps in reducing the death rate from oral cancer is early diagnosis. Several authors have indicated the late detection of oral cancer results in poor prognosis in almost 50% of all cases. Therefore, an early diagnosis tool is needed to improve the outcome and increase the survival rate of patients with oral cancer. The early diagnosis of oral cancer can be inhibited/limited by several factors. Such factors can be an absence of symptoms of the premalignant lesions and OSCC in early stages, socioeconomic situation of the patients that prevents the accessibility to dental clinic and the current diagnostic methods and genetic markers that only detect the disease in advanced stages (4). Therefore, the dental community is the first line of defense in early detection of the disease and finding a biomarker for early diagnosis of these tumors, is of tremendous importance to reduce morbidity and mortality. Recent studies and investigations present a promising new diagnostic tool that can be used to study novel genetic biomarkers for early diagnosis of oral cancer. Such diagnostic tool is liquid biopsy of blood and saliva that contain genetic biomarkers such as circulating tumor DNA, microRNA and extracellular vesicles (5)

1.2. Statistics and epidemiology of oral cancer worldwide

Oral cancer is the 11th most common cancer worldwide with more than 350 thousand new cases and 177 thousand deaths in 2018. The incidence rate of oral cancer varies widely throughout the world, with an evident prevalence in South Asian countries (**Figure 1 and Table 3**) and it decreases in comparison with Western world. This high incidence is associated with environmental factors such as alcohol, tobacco use, and HPV in developed countries (6). There are more than 200 thousand new cases and of oral cancer in Asia (Figure 1 and Table 3), while the mortality is approximately 130 thousand in 2018, (**Figure 2 and Table 3**) (7). The most

prominent Asian country with highest oral cancer incidence is India, as a consequence of low socioeconomic status, which means a low probability of visiting the dentist regularly, and higher exposure of predisposing factors such as tobacco and alcohol (6).The incidence rate in Europe is decreased in comparison with Asia. European countries have around 61 thousand new cases (**Figure 1 and Table 3**) and 24 thousand mortality cases (**Figure 2 and Table 3**) in 2018 (7). The incidence rate and mortality rate decrease even more in the rest of the continents such as North America, South America and Africa (**Figure 1&2 and Table 3**). African countries have the lowest incidence rate but higher mortality rate than North and South America (**Figure 1&2 and Table 3**) (7).

FIGURE 1: PERCENTAGE OF INCIDENCE OF ORAL CANCER WORLDWIDE (7)

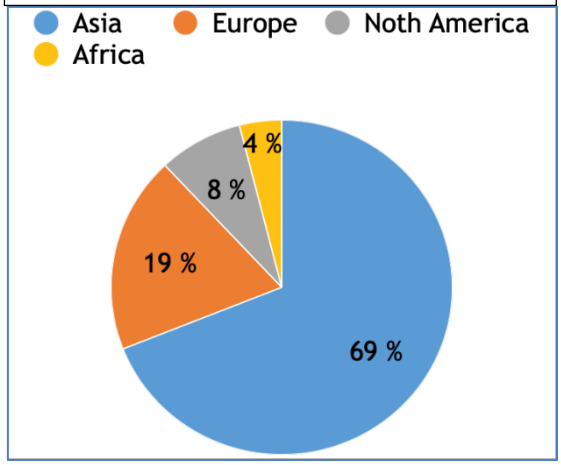


FIGURE 2: PERCENTAGE OF MORTALITY OF ORAL CANCER WORLDWIDE (7)

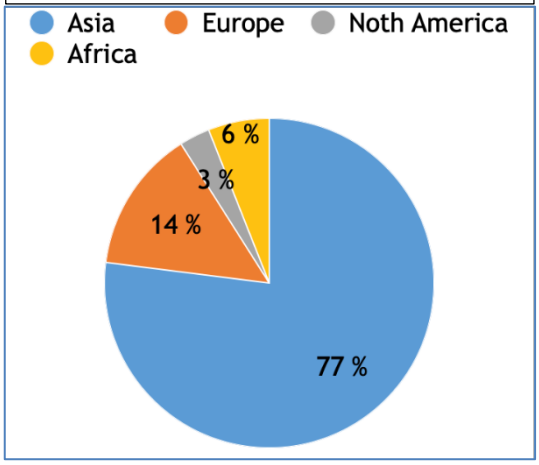


TABLE 3: INCIDENCE AND MORTALITY OF ORAL CANCER WORLDWIDE, IN NUMBERS IN 2018 (7)

Continents	Asia	Europe	North America	South America	Africa
Incidence	227906	61885	27112	19898	13613
Mortality	129939	24063	5198	7874	9314

1.3. Strategies in oral cancer prevention

Prevention of oral cancer plays a significant role in reducing mortality rate and increasing life expectancy were. Preventive measurements of oral cancer can be subcategorized into three categories: primary, secondary and tertiary prevention. As for the primary prevention, the purpose of this prevention is to increase the populations knowledge about oral cancer by providing educational sessions and programs about oral health and self-examination as well as awareness about risk factors. The secondary prevention includes screening tests, of the precancerous lesions, that provide early diagnosis of the disease by extracting tissue/serum/saliva from the diseased area in order to study the genetic biomarkers available. Genetic biomarkers help us diagnose any possible mutation on different tissues/cells of the oral cavity in order to treat the affected area as soon as possible before it progresses into malignant lesion (see **Table 4** below). While the tertiary prevention aims at reducing the risk of recurrencies (8). Not all oral cancer cases can be prevented, however, by avoiding certain risk factors, it might reduce risk of developing the malignant manifestation in the oral cavity. Predisposing factors such as alcohol and tobacco are involved greatly in the developing of oral malignancies. Therefore, avoiding alcohol and smoking is the first step in the right direction (9). Infections such as HPV infection is considered one of the oral cancer risk factors. It should be avoided by limiting oral sex and multiple sex partners. Some types of HPV infection can be avoided by vaccines (9). Reducing UV light exposure is also another important preventing factor of oral cancer. Ultraviolet radiation is considered a great risk factor to develop lips cancer, therefore using of sun cream and lip balm that contains a sun protection factor (SPF) is important during sun exposure (9). Frequent clinical examination of oral cavity is very

important in order to detect any precancerous lesions such as leukoplakia or erythroplakia, and to eliminate them before developing into malignant carcinoma.

In dentistry, an early screening test is required in high-risk patients. Therefore, genetic biomarkers for early detection of oral cancer can save lives (8). Some risk factors cannot be avoided, such as socio-economic status. People with low socio-economic status tend to have poor diet, they are not able to choose healthy diet due to poor income. On the other hand, these people tend to have high prevalence of alcohol consumption and tobacco use (8).

1.4. Early diagnosis of oral cancer

The diagnostic possibilities of oral cancer are based on knowledge of their etiology and pathogenesis. Current diagnosis methods to date have focused primarily on the clinical examination of the oral cavity such as leukoplakia and biopsy (4). Furthermore, molecular biology has also brought more recent knowledge about this disease (see **Table 4** below). An element of dentistry is the early diagnosis of oral cancer and monitoring of traditional parameters, which includes clinical examination of oral cavity and histological study of biopsy. Early diagnosis is a critical factor in increasing the survival rate, avoiding more aggressive procedures and improving the quality of life of the patient. Therefore, it's important to observe any change in the oral cavity that manifests as patch/plaque lesions such as leukoplakia (4). That is considered a premalignant lesion (10).

TABLE 4: GENETIC BIOMARKERS OF ORAL CANCER COULD BE USED FOR PREVENTION AND PROGNOSIS ACCORDING TO TIMELINE OF ORAL CANCER PROGRESSION			
Inherited/Acquired genetic factors	**Biological Onset of the disease**	**Disease Detection**	**Tumor observation and Tissue damage**
Genetic tests	Salivary and Blood biomarkers	Salivary and Blood biomarkers	Clinical examination and histopathological biopsy

In the precancerous stage, we clinically observe a painless thick white/red patch known as white leukoplakia or erythroplakia. Oral leukoplakia has a mean prevalence value of 2.6% and can be present in any part of the oral cavity and has two subtypes: homogenous and non-homogenous lesions. Homogenous lesions are clinically characterized by flat, thin and white lesions while non-homogenous lesions are nodular and verrucous leukoplakia. Depending on the risk factors, mentioned previously, (tobacco, alcohol) and the degree of dysplasia the dentist should choose the appropriate treatment approach to treat the oral leukoplakia. If the precancerous lesions are left un-treated or un-detected, they may develop into squamous cell carcinoma which is the most common type of oral cancer (10).

The most common diagnosis method for oral precancerous/cancerous lesion is a combination of clinical examination of oral cavity and histopathological study of biopsy, that will be explained below(11).

In order to make the clinical examination of oral cancer, we need to recognize the characteristic of the disease. The initial clinical appearance of cancer is normally a small ulcer that doesn't cure. We need to ask the patient if the lesion is painful or not. In the initial stage of oral cancer, usually the lesion is not painful, and if it starts hurting it means it has passed the connective tissue which results in bad prognosis (12).

The histopathological study consists of slides of samples, obtained from a biopsy, analyzed under a microscope to observe changes at cellular and molecular level (11). It's very important to do a biopsy of premalignant lesions or when we suspect oral cancer. There are two types of microscopical changes that we observe in the dysplasia of the lesion in a biopsy; architectural changes and cytological changes (13). Architectural change means irregular stratification of the cells, loss of polarity of basal cells, strange epithelial crest, and increased mitosis. Microscopical changes are cytological changes that mean abnormal variation of core size, core shape, cell size and cell shape. We will also observe hyperchromatism core of the cells and that they have dark/intense blue color, more than normal. Another important factor that we need to analyze under the microscope is the degree of the dysplasia. The degree of dysplasia determines if the lesion is turning into malignant oral cancer or it can be prevented. There are four stages of dysplasia that will be explained below (see **Table 5**) (13)

TABLE 5: STAGES OF DYSPLASIA IN ORAL CANCER (13)	
Mild Dysplasia	Changes in the basal third of the epithelium thickness
Moderate Dysplasia	Two thirds of the epithelium thickness is affected
Severe Dysplasia	More than 2/3 of the epithelium thickness is affected
Carcinoma in Situ	Full epithelium thickness is affected

The clinical examination and biopsy have their limitations in fact, disadvantages. These include, in particular:

1. They are an excellent indicator of history of the disease, however, we don’t have standardized long-term measurements.
2. The damage must be significant in order to provide information about the severity of the disease.

The tissue biopsy remains an aggressive and invasive technique to analyze the cancerous tissue and it doesn't always reveal the heterogenicity or behavior of the tumor. Sometimes, it's even difficult to perform a biopsy in a difficult accessible area such as cervical lymph nodes (14). Additionally, this traditional "gold standard" technique is performed through an incisional biopsy in most cases. The incisional biopsy requires obtaining a representative tissue that manifests the most variations of the lesion and it should be including a healthy tissue as well to be examined under a microscope. Considering that the oral cavity is moist and has limited access, tissue biopsy faces some challenges in obtaining a representative tissue (15). Other challenges and obstacles that a tissue biopsy has to overcome are difficulty in analyzing the tumor heterogeneity (cellular morphology, gene expression, metabolism, motility, proliferation and metastatic potential) and limitations in continuous sampling (16). As stated previously, tissue biopsy considers an aggressive and invasive method (15). However, some of the traditional analyzing technique are in fact less invasive such as oral brush biopsy, but this technique is not 100% reliable as the sensitivity of this test is only 43% (17). As a result of these disadvantages, laboratory molecular-biochemical approaches are increasingly used for early diagnosis of oral cancer in the clinic (**Table 4**).

One approach to solve this problem, in the diagnostic step of oral cancer, would be to improve the ability of dentists to detect relevant potentially malignant lesions or cancerous lesions at their earliest or most incipient stage. Such a goal could be achieved by implementing new ongoing science techniques, such as liquid biopsy of saliva and blood as a less-invasive technique with higher sensitivity (18). Another strategy would be laboratory molecular-biochemical approaches that could be used for the needs of early diagnosis and predicting the worsening of the disease with emphasis on their usefulness in routine outpatient practice.

This study will examine the role of new genetic biomarkers in oral cancer used in research and evaluate the literature specifically about circulating tumor DNA (ctDNA), microRNA and Extracellular vesicles (EV) that can be obtained from bodily fluids such saliva through a liquid biopsy to aid in the detection and diagnosis of cancerous and precancerous lesions (18).

2. Objectives:

2.1. Main objective: Literature review of novel genetic biomarkers involved in early diagnosis of oral cancer.

2.2. Specific objectives:

- To describe the application of circulating tumor DNA (ctDNA), extracellular vesicles (EVs) and microRNA as salivary genetic biomarkers in early diagnosis of oral cancer, as new diagnostic tools.
- To review the new diagnostic technique “salivary liquid biopsy” as a non-invasive diagnostic test.

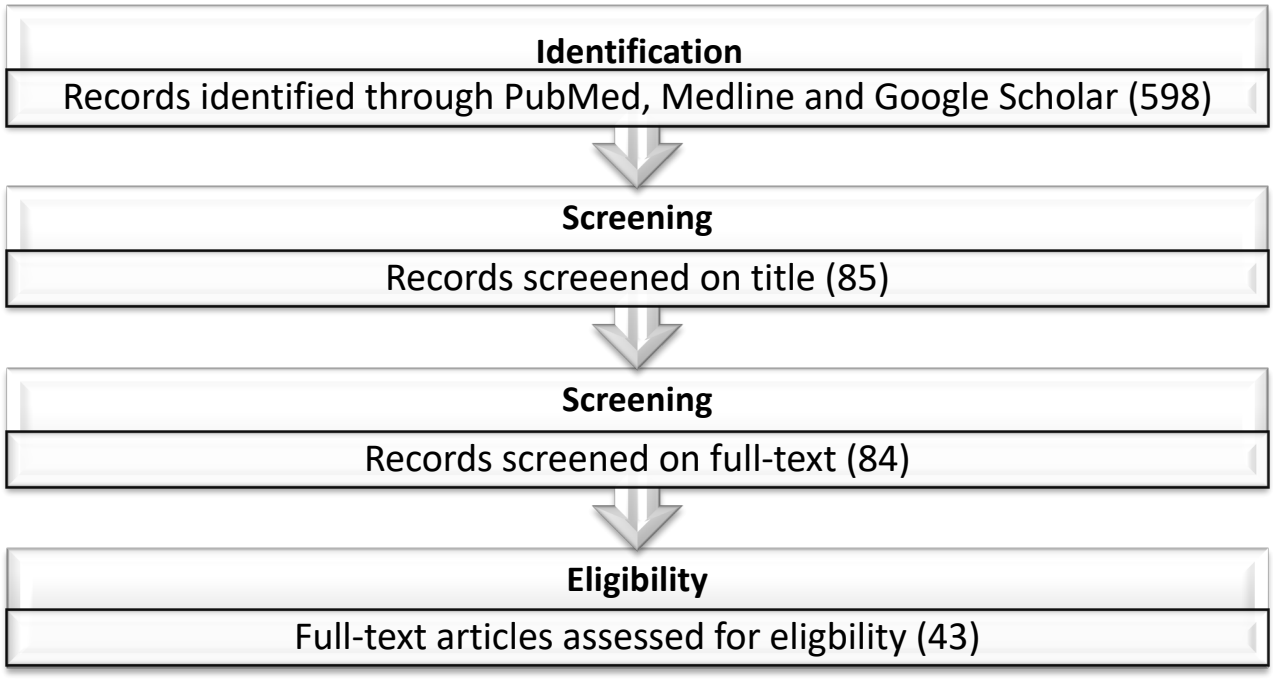
3. Materials and methods

The review is based on search of literatures in Medline, PubMed and Scholar Google. The search started in October 2020 using the following key words: “novel genetic markers”; “oral cancer”; “premalignant lesions”; “saliva”; “leukoplakia”; “salivary genetic markers”; “tumor markers in saliva”; “circulating tumor DNA”; “miRNA”; “extracellular vesicles”, leading to 598 articles. The screening was based on title leading to 85 articles and later we ended up with 84 articles after assessing the accessibility to full text and the quality of the texts. Finally, the

articles selected were 43 based on the eligibility of the texts, of which 9 of them were focused on human population studies. These 9 articles were used for the results, see **Table 6.**

The review presents different types of studies; retrospective and prospective follow-up studies performed on humans published between 2008-2020 on several genetic alterations observed in association with oral cancer. For the selection of the studies, the inclusion criteria applied were as follows: only English and Spanish language articles were chosen, and the studies were applied in humans (cohort of patients). Other studies tested in other parts of the human body were excluded. The selection of the articles is displayed in **Figure 3**.

FIGURE 3: STRATEGY USED DURING THE SELECTION OF THE RESEARCH OF THE ARTICLES USED IN THIS PAPER.



4. Results

Since OC disease is most frequently diagnosed at an advanced stage, finding genetic biomarkers for early diagnosis of this pathology is of tremendous importance for dentistry community in order to increase the probability of recovery and success and reduce mortality, and improve quality of life of the patients. Several biomarkers have been investigated so far. We provide a general overview of known genetic biomarkers circulating tumor DNA (ctDNA), extracellular vesicles (EVs) and microRNA, in patients suffering OC; with the idea of discussing the utility of these biomarkers in the early diagnosis of this disease. In **Table 6**, all the investigated diagnostic biomarkers with brief additional information of the studies are presented. In the following paragraphs, the biomarkers based on the type of studies (retrospective and retrospective) have been presented.

Research studies has demonstrated that saliva is a highly viable biofluid for diagnostic application in the dental clinic setting. Saliva includes various components, including DNA, RNA, proteins, metabolites and microbiota containing therefore specific biomarkers for early detection and diagnosis of OC. Saliva, as an inexhaustible biofluid, provides real-time data of the patient's health status with translational applications. Saliva collection is straightforward, easily accessible and repeatable, and non-invasive that doesn't require any extensive equipment and handling. In this review, we present current knowledge and future aspects of utilizing Liquid saliva biopsy as a reliable technique that could be used in the dental clinic to quantify and analyzed the genetic biomarkers described in this study.

4.1. Type of studies reviewed

The studies reviewed in this paper are 9 studies (see **Table 6**). These studies investigate the salivary biomarkers (ctDNA, miRNA and EVs) separately. They are retrospective (7 studies, (Viet and Schmidt et al,2008) (19), (Guerrero-Presto et al, 2011)(20), (Ferlazzo et al.,2017) (21), (Zlotogorski-Hurvitz et al, 2016) (22), (Liu et al, 2012) (23), (Kadhim Al-Malkey et al, 2015) (24) and (Zahran et al, 2015) (25)) and prospective (2 studies, (Zlotogorski-Hurvitz et al, 2019) (26), and (Yang et al, 2013) (27)) studies, in which 329 oral cancer patients were being studied, of which 218 had OSCC. Most of these studies used controls (healthy individuals) to obtain better outcome. The majority of the participants were middle-aged people of both genders. These studies were performed in different parts of the world, 1 study was performed in China (Yang et al, 2013), 2 studies in USA (Viet and Schmidt et al, 2008), (Guerrero-Presto et al, 2011), 2 studies in Israel (Zlotogorski-Hurvitz et al, 2016), (Zlotogorski-Hurvitz et al, 2019), 1 study in Italy (Ferlazzo et al.,2017), 1 study in Spain (Guerrero-Presto et al, 2011), one study in Taiwan (Liu et al, 2012), 1 study in Iraq (Kadhim Al-Malkey et al, 2015) and 1 study in Saudi Arabia (Zahran et al, 2015).

The sample collection in these studies was based on unstimulated saliva and the extraction technique of the genetic biomarkers was different depending on which genetic biomarker it concerns, see **Table 7**.

4.2. Circulating tumor DNA, a novel genetic biomarker that detect genetic and epigenetic alterations

Circulating tumor DNA (ctDNA) is fragmented DNA cells derived from tumor and found in bodily fluids. When the human body is under exposure to a pathological condition, necrotic

cells release debris and DNA/RNA molecules into body fluids such as blood, saliva, cerebrospinal fluid and urine (14)(28) (**Figure 4**). Physiologically, this debris and cell-derived molecules are taken care of by phagocytes, however in the presence of mutation this physiological mechanism is impaired. Instead of eliminating the debris and cell-derived molecules, the tumor circulating (ctDNA) accumulates in the biological fluids. This result in high amount of ctDNA in the body fluids of cancerous patients. Normally, the measurement of ctDNA is between 100-200 base pairs, however DNA molecules becomes larger, up to 400 base pairs, in presence of mutation. Recent studies demonstrate that ctDNA found in the cancer patients represents the genetic (point mutation, CNV (Copy Number Variation), chromosomal rearrangements) and epigenetic alterations in the tissue samples of cancerous lesions (**Figure 4).**

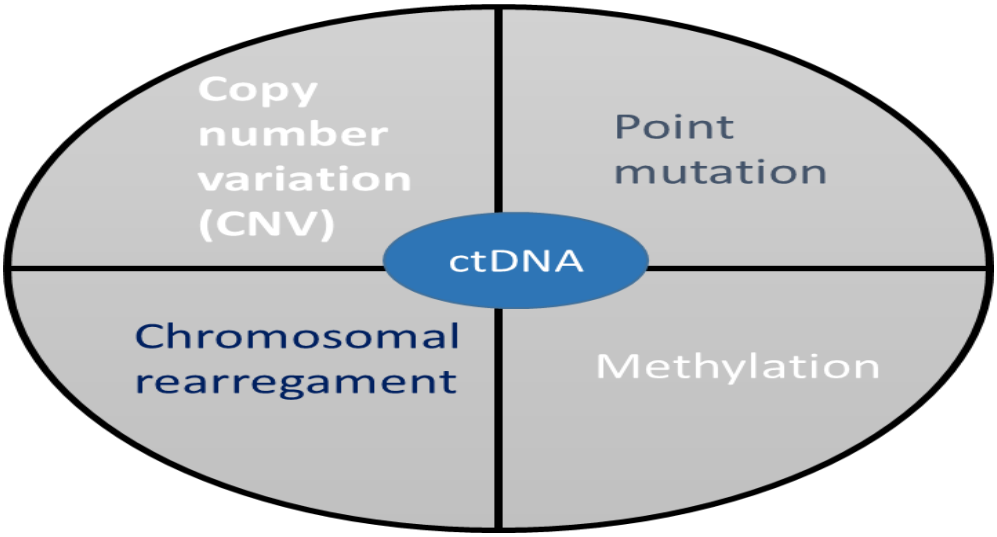


Figure 4: Cancerous cells release ctDNA into the saliva and blood through apoptosis, necrosis and secretion. From the primary tumor lesions, some aggressive ctDNA cells enter saliva and bloodstream. Quantifying ctDNA, a variety of genetic and epigenetic alterations or changes, can be analyzed providing information about tumor heterogeneity, diagnosis of cancer, monitoring of disease and improving target therapies. Figure adopted from (28)

Mainly, ctDNA is found in the blood fluid, however it is also present in salivary fluid with information about primary tumor or metastasis, this fact is the highly interest for dental clinic applications.

4.2.1. Circulating tumor DNA in Oral Cancer patients

As demonstrated in **Table 6**, several studies have applied salivary ctDNA in the diagnosis of oral cancer and OSCC in particular (Viet and Schmidt et al, 2008) (19), (Guerrero et al., 2011)(20) and (Ferlazzo et al., 2017)(21). All these three-studies studied salivary ctDNA obtained from both people with OSCC and from controls with healthy oral cavity, with and without smoking habits in both groups. 87 of the participants had OSCC that hadn't undergone cancer treatment yet. The studies were performed in different countries. The first study was performed retrospectively by Viet and Schmidt et al., 2008 (19) in USA. It consisted of 13 OSCC (M/F= 12/1, average age= 60.8 y/o). As for the control group, there were 10 healthy individuals participated (M/F= 8/2, average age= 45.5 y/o). Both cases and controls referred to alcohol habits. The purpose of this study was to perform methylation array analysis of 807 cancer-associated genes with the aim of defining highly methylated gene loci with diagnostic value as a biomarker. The methylation analysis array was performed on DNA extracted from preoperative and postoperative OSCC saliva and saliva from healthy individuals. Unstimulated saliva was collected in sterile cups, see **Table 7** for saliva analysis protocol. The result of this study indicates that hypermethylation of genes was found in preoperative saliva samples, but it was absent in postoperative and healthy saliva samples. A genetic classifier based on specifically methylated gene loci has been developed, and it can be used as a biomarker for OSCC early diagnosis, see **Table 6**.

Guerrero et al., 2011 (20) performed a study in two countries, USA and Spain. The study consisted of 16 OSCC patients and 19 healthy individuals. The purpose of the study was to study DNA hypermethylation of HOXA9 and NID2 in salivary ctDNA. References about the participants age, gender and alcohol/smoking habits were missing. Salivary samples were frozen in liquid nitrogen and stored in -80 grades, see **Table 7** for saliva analysis protocol. The result of this study suggests that DNA hypermethylation of HOXA9 and NID2 genes in salivary ctDNA has the power in distinguishing between healthy and OSCC patients. Also, it shows that the sensitivity of HOXA9 is 68% and NID2 71% while the specificity of them is 100% in OSCC patients (**Table 6**).

In the study performed by Ferlazzo et al.,2017 (21) in Italy, 58 (F and M, 50,2 +/-) OSCC patients were participating for the diagnosis of OSCC, of which 22 were smokers. As for the control group, 90 healthy individuals with the same age and gender, were participating too. Some of them were smokers. The purpose of this study was to assess the DNA methylation rate in the participants. For the saliva collection, saliva samples were collected with Oragene DNA Self-Collection kit, see **Table 7** for saliva analysis protocol. The result of this study states that the epigenetic alteration such as DNA methylation rate can be easily found in salivary ctDNA in the assessment of OSCC, which makes salivary ctDNA a promising diagnostic tool. An important finding in this study was that the rate of DNA methylation in OSCC patients was increased in respect to healthy individuals. In conclusion, ctDNA is considered as a powerful genetic biomarker in the diagnosis of OSCC as shown in **Table 6**.

4.3. Extracellular Vesicles in early detection and diagnosis of Oral Cancer

Extracellular vesicles (EVs) are lipid bilayer particles used as a communication mechanism intercellularly. Three EVs subtypes exist: microvesicles, exosomes and apoptotic bodies (**Figure 5**)**.** The exosomes are the ones found in bodily fluids such as plasma, saliva, urine cerebral spinal fluid and bronchial fluid. The ability of EVs to accumulate selectively contents such as DNAs, RNAs, miRNA and proteins makes it an additional source of biomarkers. One of the most analyzed vesicles in tumor formation and progression are exosomes and microvesicles. Recent studies state the role of extracellular vesicles in oral cancer as well as its role in transporting protein and nucleic acids, it's very important in the development of cancer. That means EVs are present in the tumor microenvironment. Additionally, studies have been able to identify exosomal markers and their presence in OSCC tissue cells as well as in metastatic tissue cells. As a result, exosomal markers of OSCC cells can be investigated and studied in order to determine a diagnosis, prognosis and evaluation of the therapy outcome, and even detection of possible recurrency (29) (30). EVs can be found in bodily fluids, as stated previously, likewise the other biomarkers presented in this paper. Salivary exosomes from a tumor sample have different characteristics and features than a healthy sample. This shows the possibility of using salivary exosomes in early detection and diagnosis of oral cancer in high-risk patients. Additionally, apart from salivary exosomes ability to diagnose OSCC, they also have the ability of giving data about the prognosis of the disease by pointing the existing of proteins related and associated with the cancer inflammatory response, transport of metals, cellular proliferation and therapy (18) (30).

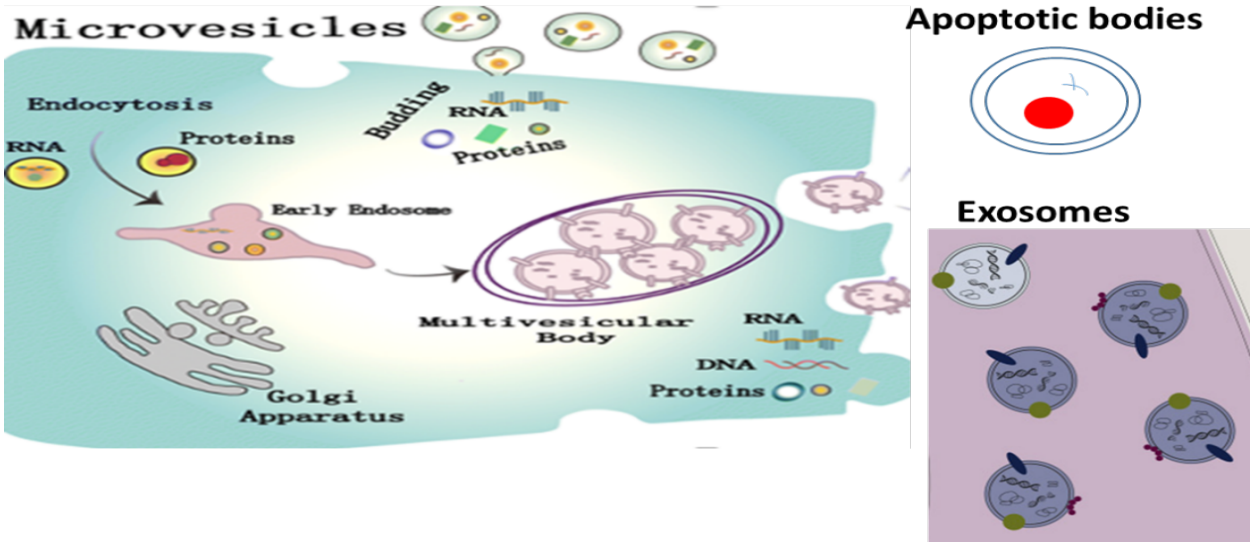


Figure 5: Exosomes, microvesicles and apoptotic bodies are examples of extracellular vesicles (EVs). Exosomes are intraluminal vesicles that are released when fusion occurs between multivesicular body and cell membrane through exocytosis. Microvesicles are formed when the cell membrane sheds into the extracellular space. Apoptotic bodies are the result of cells suffer apoptosis. Figure adopted from (30).

4.3.1. Extracellular Vesicles in Oral Cancer Patients

Nevertheless, a few studies have applied the salivary EVs in the management of OC, see **Table 6**. One of these studies of salivary EVs was performed by Zlotogorski-Hurvitz et al., 2016 (22) in Israel. In this study, salivary EVs from 36 oral cancer (OC) patients (F & M, average age= 61 y/o) and 25 healthy individuals (F & M, average age= 50 y/o) was analyzed. Saliva of 2-7 ml in OC and 5-20 ml in healthy individuals (HI) was collected into sterile cups, see **Table 7** for saliva analysis protocol. The purpose of this study was to analyze the expression of salivary exosomal markers; CD9, CD81 and CD63 through nanoparticle tracking analysis (NTA). The outcome of this study suggests that there are significant differences at morphological and molecular level in salivary EVs obtained from OC patients in respect to salivary EVs obtained from healthy individuals, meaning that NTA finding showed a significant higher concentration

of nanoparticles and greater nanoparticles size in OC salivary samples. The expression of CD81 and CD9 was low in OC salivary samples while the expression of CD63 was higher than in HI samples. This study states that the EVs are powerful genetic biomarkers that have the potential to be used in detection of oral cancer in early stages, even in the absence of clinical signs.

The second study was performed prospectively by Zlotogorski-Hurvitz et al., 2019 (26) in Israel, in a period of 24 months. It analyzed the salivary EVs in 21 oral cancer patients (F & M, age: between 38 and 81 y/o) and 13 healthy individuals, (HI), (F & M, age: between 28 and 52 y/o). Habits references weren't addressed in this study. Saliva was assessed in the same way as the previous study, see **Table 7**. The aim of this study was to determine the Fourier-transform infrared (FTIR) spectra of salivary exosomes and the expression of exosomal markers such as CD9, CD81 and CD63. The result of this study is identical to the previous one, meaning higher concentration of nanoparticles in OC as well as larger size of them. In comparison to healthy subjects, salivary EVs from OC patients demonstrated differences at morphological and molecular level, based on assessment of changes in the conformations of carbohydrates, proteins, lipids and nucleic acids in both OC and HI samples. It also indicates that the expression of CD63 was abundant in OC in respect to HI, while CD9 and CD81 were more prominent in HI. In conclusion, this study suggests the analysis of salivary EVs in the detection of oral cancer as they present a sensitivity of 100% and a specificity of 86% (see **Table 6**).

4.4. MicroRNAs in early detection and diagnosis of Oral Cancer

miRNAs are a group of non-coding RNA that play a significant role in the management of gene expression. miRNAs are single- strand molecules that can be cell-free miRNA in the body fluids or packed into EVs. miRNA has been investigated and studied to understand its role in the development or inhibition of tumor. Normally, miRNA's expression alteration is frequently tumor related. Therefore, using microRNA as an OC salivary biomarker has a lot of advantages in diagnosis of OC. The salivary miRNA expression in form of miR-125, miR-200a, miR-21, miR-145, miR-200, miR-93, miR-375 and miR-184 found in OSCC patients in comparison to healthy individuals, is a promising and reliable technique in early diagnosis of cancerous oral lesions and OSCC. Some of miRNA markers are even used as follow up markers of OC such as miR-130-5p. Additionally, miRNA is demonstrating as a reliable marker in detecting low- grade dysplasia. This means we can obtain information about possible transformation or progression of oral malignant leukoplakia into OSCC (29).

4.4.1. MicroRNAs in Oral Cancer patients

A study performed retrospectively by Liu et al., 2012 (23), see **Table 6**, in Taiwan consisted of 45 OSCC cases (M/F= 43/2, average age= 53, 13 drinkers, 39 smokers), 10 oral verrucous leukoplakia (OVL) individuals (M/F= 9/1, average age= 49, 3 drinkers, 9 smokers) and 24 healthy subjects (M/F= 23/1, average age= 51, 6 drinkers, 21 smokers). The objective of this study was to assess the level of salivary miRNA-31 in OSCC patients with quantitative reverse transcriptase- polymerase chain reaction (RT-PCR) in comparison with a previous study that was done using plasma miRNA-31. Saliva of 3-5ml was collected and centrifugated, see **Table 7** for saliva analysis protocol. The result of this study states that mi-RNA-31 was significant high in OSCC cases in respect to healthy subjects while there was no obvious difference in OVL

cases comparing to healthy individuals. The other important thing that this study shows is that salivary miRNA-31 demonstrate a higher sensitivity in diagnosing OSCC than plasma miRNA-31.

Another follow-up study was performed by Yang et al., 2013 (27) in Shanghai to analyze 8 selected deregulated miRNAs; miR-10b, miR-145, miR-99b, miR- 660, miR-197, miR-708, miR-181c and miR-30e. This study consisted of 45 subjects (F and M, middle aged) with low grade dysplasia (LGD). 10 of these subjects developed carcinoma in situ or OSCC after some time. Among the remaining 35 subjects with LGD, 5 were discarded due to low RNA quality and 12 were also discarded due to appearance of a new oral lesion. At the end, only 7 subjects with LGD were included in the study and 8 patients who developed oral cancer were also selected. Another 7 healthy subjects were also studied for comparison. 2ml saliva was collected and mixed with 5ml RNA Protect Saiva reagent preserved at room temperature for 24h before the extraction of RNA, see **Table 7** for saliva analysis protocol. As a result, they could find a specific miRNA aberrant profile of the miRNAs mentioned above in salivary samples obtained from LGD in comparing to healthy individuals, providing monitoring of precancerous lesions for early detection of OSCC.

Kadhim Al-Malkey et al., 2015 (24) performed a study analyzing miRNA-31 as well in Baghdad. This study consisted of 35 oral cancer (OC) cases and 20 healthy subjects. The participants in both groups were from both genders, with male predominance, average age of 52 y/o and a significant high use of alcohol and tobacco. Saliva samples of 5ml was collected and centrifugated, see **Table 7** for saliva analysis protocol. The result of this study suggests the use

of salivary genetic biomarker miRNA-31 in early detection of OC as it shows a significant high level in OC samples in respect to healthy samples.

The following three salivary miRNAs were studied and investigated in one of miRNA studies displayed in Table 6: miRNA-21, miRNA-184 and miRNA-145. This study of Zahran et al., 2015 (25) consists of 100 Arab participants. They divided the participants into 5 groups. The first group consisted of 20 healthy subjects, the second and third groups consisted of 40 patients with oral potentially malignant disease (PMD) with and without dysplasia, the fourth group consisted of 20 OSCC patients and the last groups consisted of 20 patients with recurrent aphthous stomatitis (RAS). The objective of this study was to evaluate if these three microRNA salivary biomarkers can be used as salivary diagnostic biomarkers in malignant transformation of oral lesions. Unstimulated saliva, 5ml, samples were collected after chewing gums, see **Table 7** for saliva analysis protocol. They found out that there was a significant increase of salivary miRNA-21 and miRNA-184 in OSCC and PMD patients in comparison to healthy subjects. However, the level of miRNA-145 was significantly decreased in these two groups. There was no significant difference in RAS group comparing to healthy subject. The sensitivity and specificity of miRNA-21 were 65% while miRNA-145 had a specificity of 70% and a sensitivity of 60%. The specificity of miRNA-184 was 75% and its sensitivity was 80%, making it of best diagnostic value among these three miRNAs.

4.5. Liquid biopsy as a novel technique for Oral Cancer

To evaluate disease status, liquid biopsies use “liquid” samples such as saliva, blood, urine, as well as other minimally invasive biological samples. The detecting of disease using biomarkers present in bodily fluids is one application of liquid biopsy technology (31). Liquid

biopsy is a new, promising analysis tool that seeks to deliver a useful information obtained from biofluids to minimize the use of tissue biopsy. Tissue biopsy has some obstacles that liquid biopsy can overcome (16). In a liquid biopsy we can obtain important and valuable means that can help in early detection of malignancies that don't show clinical signs. In liquid biopsy, whether its blood or saliva, some constituents can be investigated and studied in order to provide important information about cancerous diseases such as; circulating tumor DNA, microRNA and exosomes (16) (32) (**Figure 6).**

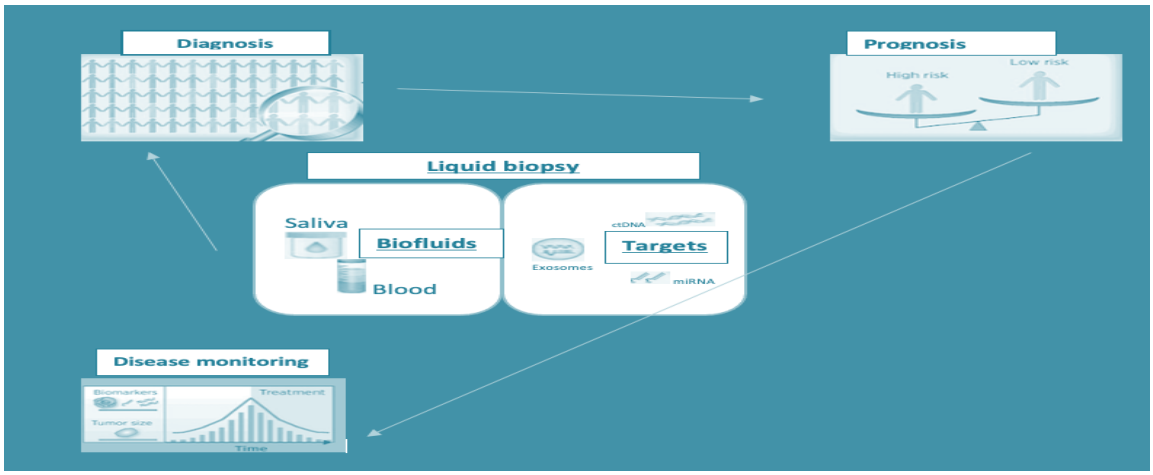


FIGURE 6: Liquid biopsy a technique with clinical uses. Saliva, blood and urine are examples of biofluids used in liquid biopsy. Cancer-derived subcellular elements such as ctDNA, miRNAs and EVs are found in these biofluids and can be used in diagnosis of oral cancer at its first stages. Figure adopted from (32).

In dentistry, saliva is the most investigated body fluid in the diagnosis of oral cancer. It has more better advantages over blood in being easily accessible, lower contamination of its contents (cells, DNA, RNA, and proteins) and easier to analyze comparing to blood. Additionally, saliva plays a key role in detection of oral cancer given the proximity of it to the potential premalignant lesions (14). Saliva is a biofluid with contents such as cytokines, DNA, RNA, circulating and tissue- derived cells and extracellular vesicles (EVs). These contents can

be used as biomarkers and diagnostic tools to detect OSCC in early stages to improve the prognosis. Studies show that liquid biopsy of salivary biomarkers in early diagnosis of OSCC are showing future promising uses in the clinic; its painless, accessible and low cost (18). An effective salivary component analysis includes an efficient procedure for the collection, processing and storage of the samples. All these aspects need to be stable between different selection and analysis points, especially when testing the same patient at different time points (16). Centrifugation is carried out for isolation of cells from saliva after collection of salivary samples. The supernatant is clearly produced after centrifugation, isolated from the pellet of cells from the entire saliva. Finally, the separated samples are stored at -80 °C after the addition of stabilizing agents (16).

TABLE 6: APPLICATION OF SALIVARY BIOMARKERS IN OSSC, BASED ON POPULATION STUDIES					
Author	**Technique**	**Biomarker**	**Cases and controls**	**Diagnostic tool (early or late)**	**Results**
(Viet and Schmidt et al., 2008) (19)	DNA extraction from saliva Methylation analysis array	ctDNA hypermethylation	13 OSCC 10 Controls	In early diagnosis of OSCC	Preoperative saliva samples were highly hypermethylated in respect to postoperative and healthy samples. Sensitivity of 62%-77% and specificity of 83%-100%.
(Guerrero-Preston et al., 2011) (20)	DNA extraction from saliva Quantiative Methylation Specific PCR	ctDNA hypermethylation	16 OSCC 19 Controls	In early detection of OSCC	Overexpression in DNA hypermethylation of HOXA9 and NID2 in OSCC versus healthy individuals. Sensitivity values of HOXA9 and NID2 are 68% and 71% while the specificity
(Ferlazzo et al., 2017) (21)	DNA extraction from saliva Formic acid for DNA methylation	ctDNA DNA methylation	58 OSCC 90 Controls	In early detection of OSCC	DNA methylation rate increases in OSCC samples in respect to healthy samples.
(Zlotogorski-Hurvitz et al., 2016) (22)	Exosomes extraction from saliva Atomic force microscopy (AFM)	EVs	36 OC 25 Controls	In high-risk patients without clear clinical signs of cancer	Molecular and morphological changes in OC exosomes versus healthy samples. Overexpression of CD63 and low expression of CD9 and CD81.
(Zlotogorski-Hurvitz et al., 2019) (26)	Exosomes extraction from saliva Fourier-transform infrared (FTIR) spectroscopy	EVs	21 OSCC 13 Controls	For early detection of lesions with potential malignant transformation	Molecular and morphological changes in OC exosomes versus healthy samples. A sensitivity of 100% and specificity of 86% in OSCC cases in early and late stages
(Liu et al., 2012) (23)	miRNA extraction from saliva qRT-PCR	miRNA-31	45 OSCC 24 Controls	For early diagnosis of OSCC	Significant overexpression of miRNA-31 in OSCC patients at all stages vs healthy samples. miR-31 was more abundant in saliva than plasma.

TABLE 6: APPLICATION OF SALIVARY BIOMARKERS IN OSSC, BASED ON POPULATION STUDIES (continuation)					
Author	**Technique**	**Biomarker**	**Cases and controls**	**Diagnostic tool (Early or late)**	**Results**
(Yang et al., 2013) (27)	miRNA extraction from saliva Taq-man low density array and qRT-PCR	miRNA aberrant profile miR-10b miR-145 miR-99b miR- 660 miR-197 miR-708 miR-181c miR-30e	45 OSCC 7 Controls	In diagnosis of precancerous stages	Overexpression of miRNAs was found in saliva samples obtained from leukoplakia lesions with low grade dysplasia.
(Kadhim Al-Malkey et al., 2015) (24)	miRNA extraction from saliva transcriptase-PCR (RT-PCR)	miRNA-31	35 OC 20 Controls	Early diagnosis of OC	miR-31 is overexpressed in OC patients than healthy. miR-31 is more abundant in saliva than plasma.
(Zahran et al., 2015) (25)	miRNA extraction from saliva qRT-PCR	miRNA-21 miRNA-184 miRNA-145	20 OSCC 40 PMD 20 RAS 20 Controls	In early detection of OSCC, miRNA-184 in particular	High expression of Salivary miRNA-21 and miRNA-184 and low expression of miRNA-145 in OC samples compared with saliva the healthy individuals. Sensitivity: miRNA-21 (65%), miRNA-184 (80%), miRNA-145 (60%) Specificity: miRNA-21(65%), miRNA-184(75%), miRNA-145(70%)

TABLE 7: PROTOCOL OF SALIVA SAMPLES	
Studies	**Saliva analysis technique**
(Viet and Schmidt et al, 2008)	Saliva of 7,5ml was collected and stored at -80 grades. The postoperative saliva sample was collected 4 weeks after surgery. DNA extraction was done using iPrep Chargeswitch Buccal Cell kit; Invitrogen. Then the samples were analyzed using GoldenGate Methylation Array (Illumina).
(Guerrero-Preston et al, 2011)	Saliva samples were frozen in liquid nitrogen and stored in -80 grades. Later, the samples were centrifugated leading to the isolation of DNA from the pellets. HumanMethylatuion27 BeadChip and Quantitative Methylation Specific PCR were used to identify methylation in OSCC samples.
(Ferlazzo et al,2017)	Saliva samples were collected with Oragene DNA Self-Collection kit and was transported to the laboratory for DNA extraction. DNA was purified using a specific DNA kit and then hydrolyzed with 90% formic acid for the assessment of DNA methylation.
(Zlotogorski-Hurvitz et al, 2016)	Saliva of 2-7 ml in OC and 5-20 ml in healthy individuals was collected into sterile cups and was examined by nanoparticle tracking analysis (NTA). After ultracentrifugation, exosomal pellets of both groups (cases and controls) were assessed by transmission electron microscopy and atomic force microscopy (AFM). Analyzing of the exosomal markers expression was performed by enzyme-linked immunosorbent assay (ELISA) and western blotting (WB).
(Zlotogorski-Hurvitz et al, 2019)	Saliva of 2-7 ml in OC and 5-20 ml in healthy individuals was collected into sterile cups and was examined by nanoparticle tracking analysis (NTA). After ultracentrifugation, exosomal pellets of both groups (cases and controls) were assessed by transmission electron microscopy and atomic force microscopy (AFM). Analyzing of the exosomal markers expression was performed by enzyme-linked immunosorbent assay (ELISA) and western blotting (WB).
(Liu et al, 2012)	Saliva of 3-5ml was collected and centrifugated. The supernatant was kept at -80 degrees and then centrifugated at 1000 revolutions per minute for 5mins to eliminate possible contamination. The measurement of mi-RNA-31 was performed using qRT-PCR.
(Yang et al, 2013)	2ml saliva was collected and mixed with 5ml RNA Protect Saiva reagent preserved at room temperature for 24h before the extraction of RNA. For the extraction of RNA, TRIzol reagent (Invitrogen) was using and later on TaqMan low density array (TLDA) qRT-PCR system (Applied Biosystems) were applied for the analysis of miRNA expression.
(Kadhim Al-Malkey et al, 2015)	Saliva samples of 5ml was collected and centrifugated at 3000xg for 15min following another centrifugation at 12000xg for 10min. RNA was extracted by use of AccZol kit. Before analyzing miRNA-31 with RT-PCR, saliva samples were purified of DNA with DNase enzyme
(Zahran et al, 2015)	5ml, samples were collected after chewing gums for 30 mins and the RNA was extracted from the saliva using microRNA isolation kit (Qiagen, UL). Then the analysis of miRNA was done using qRT-PCR (Applied Biosystems)

5. Discussion

Early diagnosis of oral cancer (OC) plays a significant role in both survival rate and treatment success. As mentioned previously, most OC cases are detected too late which leads to higher mortality rate (18). Finding a biomarker that has the potential to detect OC at early stages means reduction in morbidity and mortality rate (33). Recently, some of salivary contents that can help in early diagnosis of OC have been identified. Such biomarkers are ctDNA, microRNA and EVs (18).

Oral cancer is one of the 10 most common cancers worldwide and OSCC accounts for 95% of all oral cancer cases affecting the head and neck zone, as stated previously (1). Despite the ongoing and continuous scientific effort in improving the outcome of OSCC, the prognosis is still poor due to diagnostic delay. The conventional cancer markers and screening strategies are not adequate for the successful management of OSCC, given the intra-tumoral and inter-tumoral heterogeneity and complex behavior with modification over time on the molecular profile, novel biomarkers and novel strategies are desperately needed (29).

ctDNA is a very useful genetic biomarker in detection of OSCC, see studies in **Table 6**. The studies of ctDNA indicate that there is hypermethylation of cancerous salivary samples in respect to healthy samples, providing useful information in the early diagnosis of OC. Cristaldi et al, 2019 states that information obtained from ctDNA found in salivary fluid is more accurate and sensitive than the one found in the bloodstream due to low level of contamination. In fact, recent studies demonstrate that the sensitivity of salivary ctDNA is higher. Detection of ctDNA in early stages of OSCC is found to be 100% and 95% in advanced stages. One of the epigenetic alterations found in salivary ctDNA is gene promoter

methylation, which has been investigated in several studies of oral cancer. Studying the methylation rate of genes that are part of the cell cycle, apoptosis and proliferation provides different methylation rates between oral cancer samples and healthy samples. Thus, salivary ctDNA methylation rate can be a great genetic biomarker in the diagnosis of oral cancer (29)**.** Despite its ability to carry information about the DNA genetic and epigenetic alterations, intratumoral heterogeneity and its potential in detection OSCC in early stages of the disease, ctDNA has to overcome some challenges in order to be implemented in the dental clinics (34). First, isolation methods and detection techniques of ctDNA need to be improved. The ctDNA sample can be contaminated due to the presence of ctDNA from non-tumoral cells. In addition, there is high number of bias due to inter-patient variability (age, gender, diet and smoking), intra-patient variability and tumoral heterogeneity. In order to reach higher specificity of diagnosis, we need higher coverage platform of ctDNA alteration detection (35) (**Table 8**). Regarding the results obtained from the salivary ctDNA studies we can indicate that ctDNA is a promising genetic biomarker in the assessment of OSCC. However, the sensitivity and specificity are still not high enough to be used in the clinical practice. The studies shown in **Table 6** include small samples of participants. Therefore, a larger sample should be studied in the future for better outcome.

Another genetic biomarker that is being reviewed in this paper is extracellular vesicles, EVs, see **Table 6**. The studies of EVs state that there are morphological and molecular changes in cancerous salivary samples in comparison to healthy samples, meaning that EVs are very useful in detecting OC at its early stages. EVs help in detection of low-expression biomarkers that are hard to be detected in saliva. Additionally, its morphology and composition (lipids, proteins, DNA and miRNAs) provide a very important informative source for detection of oral

cancer. However, the application of EVs in OSCC management has obstacles and limitations. These limitations are associated with the complicated and expensive isolation and analysis methods, see **Table 8**. Currently, some of the isolation techniques that are being used are: ultracentrifugation and ExoQuick-TC. The first one, ultracentrifugation, is widely used isolation technique and provides the least contaminated EV pellet, however it's a long and complex process that needs a large number of samples. On the other hand, ExoQuick-TC doesn't require a big sample, but the contamination risk is higher with this methodology (36) (37). Aqueous two-phase system is another isolation technique that has been developed by (38). It provides better isolation features and better purity than the above-mentioned techniques. Although, these techniques are being used in the laboratories on a daily basis, they are still very expensive and time-consuming in the clinics (39). Finally, validated and standardized protocols are needed in order to develop an easy and low-cost clinical techniques (25). The results obtained from salivary EVs studies, shown in **Table 6**, are very optimistic and demonstrate that salivary EVs can be reliable biomarkers in early diagnosis of OC. Nevertheless, the studies are few and they studied a small sample of patients.

As mentioned previously miRNAs are great genetic biomarkers in early detection of oral cancer, see Table 6. The studies that analyzed microRNAs show that there is overexpression of miRNAs in salivary cancerous samples compared to healthy salivary samples, suggesting that miRNAs are very useful salivary genetic biomarkers in early diagnosis of OC. Nevertheless, they face limitations likewise the other salivary biomarkers when it comes to standardized protocols for the isolation and analysis of miRNAs (40). Another factor that may affect the miRNAs specificity and sensitivity is the inter-patient variability (age and inflammation), see **Table 8**. Many studies indicate that miRNAs interfere in the age regulation processes and as

known age is a variable that continuously influence the OSCC analysis (41). Another inter-patient factor is inflammation. As a result of cancer development, inflammation arises as a typical condition of this disease and it affects the expression of miRNAs. Alteration of miRNAs expression doesn't always associate with cancer development but it can be due to the body response as a consequence of the cancer disease, disturbing the miRNA expression and lowering the reproducibility of data (42). The salivary miRNAs studies present in this paper include comparatively a large sample of patients, but still more number or patients are needed to determine the validity of these biomarkers.

Liquid biopsy is currently under clinical investigation as a promising tool in molecular diagnosis of OSCC. Liquid biopsy of plasma, saliva and urine is still under research. As stated previously, salivary liquid biopsy of ctDNA, EVs and MiRNAs in the diagnosis of OSCC provides higher sensitivity and specificity. Due to its accessibility, ease of management and natural proximity with OSCC cells, "saliva is considered one of the most indicative body fluids for liquid biopsy in OSCC" (29). Given that saliva contains the genetic biomarkers ctDNA, EVs and MiRNAs derived from OSCC where genetic and epigenetic alterations can be provided easily, saliva is considered as a valuable means for diagnosis of oral cancer in early and late stages. For the diagnosis of OSCC, the preferred biomarkers seem to be the salivary genetic biomarkers since they are very supportive in the management of oral cancer (29).

All proposed biomarkers in this paper have their advantages and disadvantages. Some of them lack the required sensitivity and specificity to be utilized in the dental clinic. Additionally, the reviewed studies have small samples and most of them are retrospective studies. Therefore, larger and prospective studies are needed to be performed in the future in order to obtain

more accurate assessment of the utility of these biomarkers in early diagnosis of oral cancer. As mentioned by several authors, these biomarkers should be tested in populations at high risk of developing oral cancer (18).

TABLE 8: ADVANTAGES AND DISADVANTAGES OF SALIVARY BIOMARKERS REVIEWED IN THIS STUDY

Salivary Biomarkers	Advantages	Disadvantages
ctDNA	• ctDNA contains genetic and epigenetic modifications of cancer DNA • DNA alterations found in ctDNA are of great use in cancer diagnosis • ctDNA presents the tumor heterogenicity	• Inter- and intra- patient variability • Expensive isolation and detection techniques • Absence of standardized and reproducible protocols • Little coverage platform of ctDNA
EVs	• Evs allow detection of low-expression biomarkers in saliva • The morphology and composition (lipids, proteins and nucleic acids) of EVs provide a useful diagnosis of oral cancer	• Expensive and complicated isolation • Absence of standardized and reproducible protocols
miRNAs	• The altered expression in miRNA provides useful information for cancer diagnosis	• Expensive detection and analysis techniques • Inter-patient variability • Absence of standardized and reproducible protocols

6. Conclusion

- A lot of research is still ongoing to apply salivary biomarkers such as circulating tumor DNA (ctDNA), microRNAs and extracellular vesicles (EVs), in the dental clinic for early detection of OSCC.
- All the studies reviewed in this paper indicate that these salivary genetic biomarkers (ctDNA, miRNA and EVs) can be used in early diagnosis of oral cancer, some of them such as EVs and some miRNAs markers (miR-10b, miR-145, miR-99b, miR- 660, miR-197, miR-708, miR-181c, miR-30e) even proved to be very useful in the diagnosis of lesions with potential of malignant transformation.
- The use of ctDNA, miRNA and EVs as genetic salivary biomarkers have some disadvantages linked with technical problems associate to isolation and lack of standardized protocols and the required sensitivity and specificity in order to be utilized in the dental clinic. Additionally, the reviewed studies have small patient samples, therefore the utility of these biomarkers should be tested in populations at high risk of developing oral cancer
- Future studies on application of ctDNA, MiRNAs and EVs as salivary biomarkers in OSCC clinical routine can help in establishing strategies in early diagnosis of precancerous lesions.
- The use of salivary liquid biopsy in the dental clinic for the diagnosis of OC can be accurate in the future, as its inexpensive, non-invasive and very good tool for analysis of the salivary genetic biomarkers, despite the lack of standardized isolation and evaluation protocols for liquid biopsy.

7. Responsibility

This project has clear interest for dentistry, medicine and society. This work review new genetic biomarkers that can be used to detect and diagnose oral cancer at its early stages. This represents a part of personal medicine that works towards improving the health of the patients at high risk of suffering from oral cancer. The new salivary genetic biomarkers (ctDNA, EVs and microRNA) are very useful in the early diagnosis of oral cancer, leading to a better prognosis of the disease and a better quality of life of the patient. The use of these biomarkers in the future, in dental clinic, could lead to better treatments, avoiding unnecessary treatments saving costs and lives.

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9. Annexes

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Histological and molecular aspects of oral squamous cell carcinoma (Review)

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Abstract. Oral squamous cell carcinoma (OSCC) represents 95% of all forms of head and neck cancer, and over the last decade its incidence has increased by 50%. Oral carcinogenesis is a multistage process, which simultaneously involves precancerous lesions, invasion and metastasis. Degradation of the cell cycle and the proliferation of malignant cells results in the loss of control mechanisms that ensure the normal function of tissues. The aim of the current review is to present the histopathological features of OSCC, including potentially malignant changes, the international classification of tumors, the tumor invasion front and tumor biomarkers (Ki-67, p53, homeobox genes and collagen type IV), as well as the tumor microenvironment and function of cancer-associated fibroblasts in the most common type of oral cancer that is encountered by dental surgeons. In OSCC, associations have been identified between the proliferation, basal lamina degradation and connective tissue modulation. Therefore, the comparison of these factors with the survival time of OSCC patients from the histopathological diagnosis is of interest.

Contents

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Key words: mouth neoplasms, oral squamous cell carcinoma, oral cancer, p53, Ki-67, collagen type IV

1. Introduction

Head and neck cancer is one of the 10 most common types of cancer worldwide, afflicting >500,000 individuals each year. Oral cancer is considered to be a preventable condition, due to the possibility of early detection and treatment (1). Oral squamous cell carcinoma (OSCC) represents 95% of all forms of head and neck cancer, and during the past decade its incidence has increased by 50% (2,3). Snuff and alcohol consumption are associated with 90% of patients that exhibit oral cancer (1) and the two factors appear to have a synergistic effect (4).

The majority of OSCC are diagnosed at a late phase (5), in stages III or IV (6,7), which markedly decreases the chances of survival and leads to a significant deterioration in patient quality of life.

Despite the currently available therapeutic strategies, which include the excision of malignant tissue and combination of radiotherapy and chemotherapy, the five-year survival rate is only 53% (3). In addition, a high percentage of patients have a poor response to therapy and high recurrence rates (8).

The purpose of the current review was to present the histological and molecular characteristics of the most common type of oral cancer encountered by dental surgeons.

2. Histology

In general, cancers, including OSCC, emerge from the accumulation of genetic changes and epigenetic anomalies in the signaling pathways that are associated with cancer, resulting in phenotypes that facilitate OSCC development. This process was summarized by Hanahan and Weinberg in 'Hallmarks of Cancer' (9).

OSCC is a malignant neoplasm derived from the stratified squamous epithelium of the oral mucosa (10). Its pathogenesis is multifactorial, associated with cigarette smoke, alcohol (11) and snuff, as well as the papilloma virus, among others (12). The malignant neoplasm occurs at various sites, the most frequent being the lip, lateral edges of the tongue (Fig. 1A) (13) and floor of the oral cavity. The incidence of OSCC increases with age, with the majority of OSCC occuring in patients >40 years (14).

OSCC is characterized by histopathological and clinical manifestations. All carcinogenesis evolves from initial

ACTA OTORHINOLARYNGOLOGICA ITALICA 2013;33:286-288

CASE REPORT

Mucoepidermoid carcinoma of the tonsil: a very rare presentation

Carcinoma mucoepidermoide della tonsilla: una presentazione molto rara

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SUMMARY

Mucoepidermoid carcinoma is the most common malignant salivary gland tumour. However, short series or individual case reports have identified this tumour in the maxilla, mandible, breast tissue and thymus. Mucoepidermoid carcinoma originates from minor salivary glands, and it is therefore surprising that it is not more commonly seen in the tonsil. To date, we believe there has been only one previously reported case in the world literature of mucoepidermoid carcinoma occurring in the tonsil [1]. We present a very rare case of mucoepidermoid carcinoma arising from within the structure of the palatine tonsil, rather from the adjacent pharyngeal wall, together with a short review of the literature.

KEY WORDS: Palatine tonsil • Mucoepidermoid carcinoma

RIASSUNTO

Il carcinoma mucoepidermoide rappresenta la neoplasia maligna più comune delle ghiandole salivari. Tuttavia numerosi studi fanno riferimento a casi isolati in cui questo tipo di tumore origina dal mascellare, dalla mandibola, dalla ghiandola mammaria e dal timo. Poiché il carcinoma mucoepidermoide origina solitamente nelle ghiandole salivari, l'origine dalla tonsilla palatina è considerata alquanto insolita. Fino ad oggi riteniamo sia stato precedentemente riportato in letteratura solo un caso di carcinoma mucoepidermoide nella tonsilla [1]. Presentiamo un caso molto raro di carcinoma muco epidermoide che originante dalla tonsilla palatina piuttosto che dalla adiacente parete faringea, insieme a una revisione dei casi riportati in letteratura.

PAROLE CHIAVE: *Tonsilla palatina • Carcinoma mucoepidermoide*

Acta Otorhinolaryngol Ital 2013;33:286-288

Introduction

Mucoepidermoid carcinoma is the most common malignant salivary gland tumour [2-4]. It has been reported in all ages with peak incidence at the 4th and 5th decades, with females affected more than males in a 3:1 ratio. It is the most frequent malignant salivary gland neoplasm in children [5]. In the major salivary glands, 89.6% of cases present in the parotid [6]. Mucoepidermoid carcinoma demonstrates a broad spectrum of aggressiveness, which can be predicted by microscopic grading.

High-grade tumours are highly aggressive and regional lymph node spread is common. The low-grade variant usually demonstrates a favourable outcome, but it is important to note that metastasis may also be present [7]. Distant metastasis is rare, but case reports of metastases to the lungs, brain, ovary and peritoneum have been reported [8]. Histologically, the tumour is composed of mucous, basaloid, intermediate and epidermoid cells. We present an unusual case of mucoepidermoid carcinoma arising in the tonsil.

Case report

A 48-year-old male presented with an asymptomatic lump in the neck at level II. The lump was progressively increasing in size over 4 weeks. Intra-oral examination and flexible nasoendoscopy was normal. A fine-needle aspiration cytology specimen showed malignant cells with no obvious architecture to determine the tissue of origin. Ultrasound detected the presence of a suspected necrotic node. A CT scan of the neck and chest confirmed a right-sided pathologic node at level II. No other abnormalities were evident. Examination under anaesthesia (EUA) was performed, which was suggestive of a bulky mass within the ipsilateral tonsil, and tonsillectomy was performed. Biopsy confirmed a high-grade mucoepidermoid carcinoma arising from entirely within the substance of the ipsilateral tonsil, and not from the adjacent pharyngeal wall. Management involved complete tonsillectomy with ipsilateral neck dissection level II-IV. Histology of the resected specimen confirmed metastatic

PanAfrican Medical Journal

Open Access

Case report

Adenoid cystic carcinoma of the palate: case report and review of literature

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Key words: Cribriform variant, palate, perineural invasion, salivary gland

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Abstract

Adenoid Cystic Carcinoma (ACC) is a rare tumor constitutes for less than 1% of head and neck malignancies and 10% of all salivary gland tumors. Palate is the most common site to be involved in the oral cavity followed by parotid gland and submandibular gland. They are usually asymptomatic, slow growing, characteristically shows infiltrative growth and perineural invasion. This paper reports a case of Adenoid Cystic Carcinoma in a 35 year old female man reported with a swelling on the left side of palate involving the hard and soft palate since 8 months which was diagnosed histopathologically and review of literature of the peculiar clinical, and histopathological features.

Pan African Medical Journal. 2016; 24:106 doi:10.11604/pamj.2016.24.106.8596

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Early Diagnosis of Oral Cancer

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Survival rates for oral cancer are very poor, at approximately 50% overall, and have not improved markedly in recent decades despite advances in therapeutic interventions. Detecting oral cancer at an early stage is believed to be the most effective means of reducing rates of death, morbidity and disfigurement from this disease. Tobacco and alcohol consumption and pre-malign lesions are the most common aetiological factors. The proportion of patients presenting with oral cancer at an advanced stage is troubling. Early diagnosis is the most effective way of reducing the individual burden of the disease, decreasing morbidity and mortality and improving quality of life. For early diagnosis, healthcare providers should perform oral cancer examinations as part of their patient care regime, and need to be knowledgeable about early signs of oral carcinoma. Oral cancer awareness among the public should also be improved.

KEY WORDS: Oral cancer; Early diagnosis; Cancer awareness; Review

Introduction

Oral cancer is a global health problem with increasing incidence and mortality rates; more than 500 000 patients are estimated to have oral cancer worldwide.[1] Oral cavity squamous cell carcinoma (SCC) accounts for 90 – 94% of oral cancers, but various malignancies, such as salivary gland malignancies, soft and hard tissue sarcomas and metastatic cancers, also occur.[2,3] Survival rates for oral cancer are very poor, at approximately 50% overall, and have not improved markedly in the last few decades despite advances in therapeutic interventions.[4,5] It is now well established that early diagnosis of oral malignancies is an effective way of improving the clinical outcome for patients.[5] Detecting oral cancer at an early stage, when lesions are small or localized, is believed to be the most effective means to reduce death, morbidity and disfigurement from this disease.[6]

Risk factors

The consumption of tobacco and alcohol is strongly associated with the subsequent emergence of oral tumours.[7 – 12] In the USA, 74% of the risk of oral cancer can be attributed to tobacco and alcohol use, particularly when these substances are consumed heavily.[13] A minority of patients develop a cancer in the apparent absence of one or both of these risk factors.[14] In a Kentucky population, Hodge *et al.*[15] reported a rate of 3.4% for oral cancers in non-users of tobacco. Rich and Radden[16] found that

Critical Reviews in Oral Biology & Medicine

Liquid Biopsy in Head and Neck Cancer: Promises and Challenges

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T. Nonaka[1,2] and D.T.W. Wong[1,2]

Abstract

Head and neck cancer is the sixth most common cancer worldwide. It remains one of the leading causes of death, and its early detection is crucial. Liquid biopsy has emerged as a promising tool for detecting and monitoring the disease status of patients with early and advanced cancers. Circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and exosomal miRNAs have received enormous attention because of their apparent clinical implications. Analyses of these circulating biomarkers have paved the way for novel therapeutic approaches and precision medicine. A growing number of reports have implicated the use of circulating biomarkers for detection, treatment planning, response monitoring, and prognosis assessment. Although these new biomarkers can provide a wide range of possible clinical applications, no validated circulating biomarkers have yet been integrated into clinical practice for head and neck cancer. In this review, we summarize the current knowledge of circulating biomarkers in this field, focusing on their feasibility, limitations, and key areas of clinical applications. We also highlight recent advances in salivary diagnostics and their potential application in head and neck cancer.

Keywords: biomarker, circulating tumor DNA, circulating tumor cell, exosomal miRNA, salivary diagnostics, saliva-exosomics

Introduction

Head and neck cancer is the sixth leading malignancy worldwide (Jemal et al. 2011). The predominant histological type is squamous cell carcinoma (SCC) that mainly occurs in the oral cavity, oropharynx, hypopharynx, and larynx. Despite advanced surgery and therapeutic strategies, the overall survival of head and neck cancer patients has remained unchanged for decades. Traditional cancer-screening techniques such as imaging and protein biomarkers are not sufficient for early detection. The Cancer Genome Atlas Network recently provided a comprehensive catalog of somatic genomic alterations in 279 head and neck SCCs (HNSCCs) to understand the molecular basis, thus accelerating the development of novel strategies for diagnosis and targeted therapies (The Cancer Genome Atlas Network 2015). Liquid biopsy has been increasingly considered as an option for molecular characterization and detection of cancer as it can provide real-time information about cancer in a minimally invasive manner. Circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and exosomal miRNAs are emerging biomarkers that can be applied to cancer detection, treatment planning, and response monitoring (Siravegna et al. 2017). Notably, ctDNA and exosomal miRNAs have been shown to be present in multiple body fluids, including saliva, and are very promising biomarkers for cancer (Weber et al. 2010). In this review, we summarize the current knowledge about circulating biomarkers (ctDNA, CTCs, and exosomal miRNAs) and their potential clinical applications in head and neck cancer.

Circulating Tumor DNA and Circulating Tumor Cells

Early Detection

ctDNA mainly originates from apoptotic or necrotic tumor cells and contains the mutations present in the tumor (Fig. 1). Somatic mutations are tumor specific, and evaluation of these unique genetic changes offers the potential for better diagnostic accuracy. Several studies have demonstrated a high concordance of mutational profiles between plasma ctDNA and matched tumor samples in lung cancer (Newman et al. 2014), breast cancer (Beaver et al. 2014; Bettegowda et al. 2014), and colorectal cancer (Diehl et al. 2008; Thierry et al. 2014).

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Prevalence of Oral Cancer in India

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Abstract

Aim: To review about the oral cancer in India

Objective: To understand about the prevalence, management, cause, symptoms, diagnosis and management of oral cancer in detail

Background: Oral cancer is defined as uncontrollable growth of cells seen in the oral cavity. It appears as a growth or sore in the mouth that does not cure. Oral cancer includes cancers of the lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx.Squamous cell carcinoma is the most common type of oral cancer.

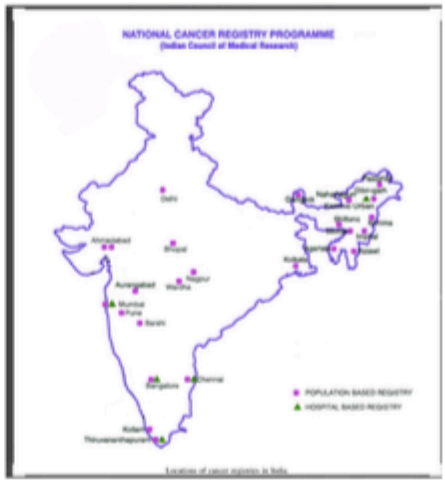
Reason : In the present century the prevalence of oral cancer is more due to excessive consumption of alcohol, tobacco chewing, smoking. Men face twice the risk of developing oral cancer when compared to women.

Key Words- Oral cancer, tobacco, alcohol consumption, poor oral hygiene, low economic status, diet

INTRODUCTION

Cancers are the most commonest cause of death in adults[1]. Oral cancer is any malignant neoplasm which is found on the lip, floor of the mouth, cheek lining, gingiva, palate or in the tongue. Oral cancer is among the top three types of cancers in India[2]. Severe alcoholism,use of tobacco like cigarettes, smokeless tobacco, betel nut chewing and human papilloma virus(HPV) are the most common risk factors for oral cancer[3,4]. Oral cancer may also occur due to poor dental care and poor diet[5].The incidence of oral cancer is highest in India, south and Southeast Asian countries. In India, 90 -95% of the oral cancers is squamous cell carcinoma [6]. The international agency for research on cancer has predicted that India's incidence of cancer will increase from 1 million in 2012 to more than 1.7 million in 2035. This indicates that the death rate because of cancer will also increase from 680000 to 1-2 million in the same period[7]. A case control study from India demonstrates that oral cancer is interrelated with low income. Low social economic class is interrelated with factors like nutrition, health care, living condition and risk behaviors which contributes to the development of oral cancer[8]. In many low-income and middle-income countries, including India, most of the population does not have access to a well organized and well regulated cancer care system. A diagnosis of cancer often leads to high personal health expenditures. Such expenditures can push entire families below the poverty line and may threaten social stability[9]. No significant advancement in the treatment of oral cancer has been found in recent years, though the present treatments improve the quality of life of oral cancer patients but the overall survival rate of 5 years has not improved in the past decades.

Here we review published data about the prevalence on oral cancer in India. We also discuss about the cause, symptoms, diagnosis and management of oral cancer in brief.



BURDEN OF ORAL CANCER IN INDIA

In India, 20 per 100000 population are affected by oral cancer which accounts for about 30% of all types of cancer[10]. Over 5 people in India die every hour everyday because of oral cancer and the same number of people die from cancer in oropharynx and hypo pharynx[11]. CANCER registration is not compulsory in India, so the true incidence and mortality may be higher, as many cases are unrecorded and loses follow up[12]. None of the national registry provides cancer incidence or mortality data for India. However, the National Cancer Registry Program provides population-based data from a selected network of 28 cancer registries located across the country[9]. A number of studies use data from urban and rural cancer registries established at the national regional level. Urban registries includes Delhi ,Mumbai and Chennai and rural registries include Barshi ,Dindigul, Manipuri ,Karunaga-pally , Eranakulam ,Srikakulam and Bhavnagar cancer is of significant public health importance

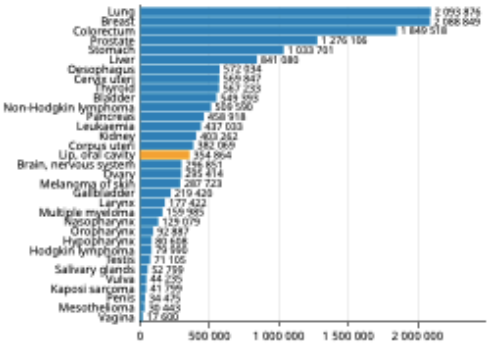
International Agency for Research on Cancer

World Health Organization

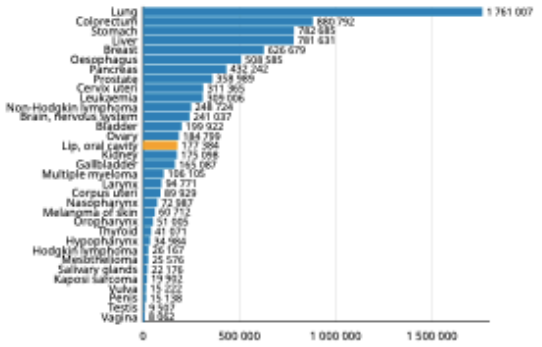
Lip, oral cavity

Source: Globocan 2018

Number of new cases in 2018, both sexes, all ages



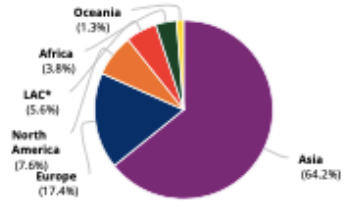
Number of deaths in 2018, both sexes, all ages



Cancer incidence and mortality statistics worldwide and by region

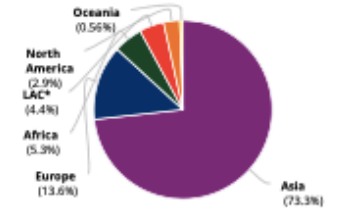
	Incidence						Mortality					
	Both sexes		Males		Females		Both sexes		Males		Females	
	New cases	Cum. risk 0-74 (%)	New cases	Cum. risk 0-74 (%)	New cases	Cum. risk 0-74 (%)	Deaths	Cum. risk 0-74 (%)	Deaths	Cum. risk 0-74 (%)	Deaths	Cum. risk 0-74 (%)
Eastern Africa	5 088	0.27	2 686	0.31	2 402	0.25	4 195	0.24	2 289	0.28	1 906	0.21
Middle Africa	1 526	0.16	845	0.20	681	0.13	1 140	0.14	653	0.18	487	0.10
Northern Africa	3 125	0.17	1 779	0.21	1 346	0.13	1 007	0.05	546	0.06	461	0.04
Southern Africa	1 556	0.34	1 012	0.53	544	0.19	1 011	0.23	682	0.38	329	0.11
Western Africa	2 318	0.13	1 154	0.13	1 164	0.13	1 961	0.12	1 002	0.12	959	0.12
Caribbean	2 288	0.47	1 603	0.72	685	0.24	757	0.13	554	0.22	203	0.05
Central America	2 655	0.16	1 192	0.16	1 463	0.16	937	0.06	500	0.07	437	0.04
South America	14 955	0.34	9 959	0.51	4 996	0.18	6 180	0.14	4 449	0.23	1 731	0.06
North America	27 112	0.51	18 652	0.77	8 460	0.28	5 198	0.08	3 509	0.13	1 689	0.04
Eastern Asia	47 532	0.20	31 074	0.28	16 458	0.13	21 062	0.08	13 752	0.11	7 310	0.05
South-Eastern Asia	16 818	0.28	10 234	0.37	6 584	0.20	8 542	0.14	5 327	0.19	3 215	0.10
South-Central Asia	159 750	0.98	118 716	1.43	41 034	0.53	98 851	0.63	67 330	0.84	31 521	0.42
Western Asia	3 806	0.19	2 411	0.25	1 395	0.13	1 484	0.07	849	0.09	635	0.06
Central and Eastern Europe	22 706	0.54	16 805	0.96	5 901	0.21	12 101	0.29	9 593	0.55	2 508	0.08
Western Europe	18 755	0.60	12 210	0.85	6 545	0.37	5 490	0.16	3 767	0.24	1 723	0.07
Southern Europe	11 604	0.40	7 587	0.61	4 017	0.21	3 847	0.12	2 549	0.19	1 298	0.05
Northern Europe	8 820	0.54	5 579	0.75	3 241	0.34	2 625	0.14	1 711	0.21	914	0.07
Australia and New Zealand	3 209	0.75	2 179	1.08	1 030	0.42	520	0.10	320	0.15	200	0.06
Melanesia	1 200	1.95	711	2.63	489	1.38	462	0.81	300	1.17	162	0.51
Polynesia	20	0.35	16	0.56	4	0.14	10	0.15	9	0.30	1	0
Micronesia	21	0.43	16	0.75	5	0.11	4	0.10	2	0.21	2	0
Low HDI	10 970	0.23	6 003	0.26	4 967	0.20	8 177	0.18	4 522	0.21	3 655	0.16
Medium HDI	171 753	0.77	125 891	1.12	45 862	0.42	104 924	0.48	71 221	0.66	33 703	0.31
High HDI	64 552	0.22	41 347	0.30	23 205	0.15	29 423	0.10	19 644	0.14	9 779	0.06
Very high HDI	107 465	0.50	73 086	0.77	34 379	0.26	34 830	0.15	24 284	0.24	10 546	0.06
World	354 864	0.46	246 420	0.66	108 444	0.26	177 384	0.23	119 693	0.32	57 691	0.14

Incidence, both sexes



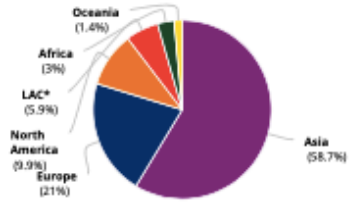
Population	Number
Asia	227 906
Europe	61 885
North America	27 112
*Latin America and the Caribbean	19 898
Africa	13 613
Oceania	4 450
Total	354 864

Mortality, both sexes



Population	Number
Asia	129 939
Europe	24 063
Africa	9 314
*Latin America and the Caribbean	7 874
North America	5 198
Oceania	996
Total	177 384

5-year prevalence, both sexes



Population	Number
Asia	536 185
Europe	192 094
North America	90 258
*Latin America and the Caribbean	54 182
Africa	27 560
Oceania	13 235
Total	913 514

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Review

Preventive measures in oral cancer: An overview

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ABSTRACT

Worldwide oral cancer is creating an alarming situation and it's a matter of global concern as it is the 11th most common carcinoma around the globe. After cardiovascular ailments, cancer is the next biggest killer. Approximately 90% of the total oral malignancies are squamous cell carcinomas. The etiological base of oral cancer is tobacco intake, smoking, smokeless tobacco (snuff or chewing tobacco), alcohol and areca nut intake, excessive sunlight exposure, reverse end smoking and Human Papilloma Virus (HPV). The treatment measures for oral cancer are very costly and affordability is low. So, taking preventive measures at the first place itself is of immense importance. Preventive measure is a multidisciplinary approach involving co-ordinated efforts from all the sectors of the society. The preventive measures are categorised into primary, secondary and tertiary measures. Along with the various screening tests employed to detect oral cancer the review focuses on biomarkers, melatonin, tea constituents, polyphenols, chemoprevention, Chios mastic gum extract, Poly (ADP-ribose) Polymerase 1 (PARP1) targeted optical imaging agent, and their role in oral cancer prevention and control. The review gives a brief outline on the preventive measures to be adopted to help prevent oral cancer and improve the quality of life.

1. Introduction

Oral cancer is rising day by day. Squamous cell carcinomas account for 90% [1]. Invasive squamous cell carcinoma means that the cancer cells have spread to deeper parts of the oral cavity [2]. 75% of oral cancers are related to lifestyle choices. The distribution of oral cancer is approximately 32% in buccal mucosa, 22% in tongue, 11% in lower lip, 11% in palate, 8% in vestibule, 5% in alveolus, 5% in floor of the mouth, and 3% in gingiva [3]. The occurrence of oral cancer are high in countries like India, Taiwan, Sri Lanka, Pakistan and Bangladesh [4]. Approximately 25% of all upcoming cases are oral cancer in these countries and it is seen majorly in men [4]. The International Agency for Research on Cancer has recently stated that smokeless tobacco can be considered as a cause of oral cancer [5]. The risk factors are many including smoking, betel nut, tobacco chewing, drinking, poor nutrition, HPV virus, mouth washes with a high alcohol content, poor oral hygiene, immune system suppression, age, gender, *etc.* Other risk factors include genetic factors, mate drinking and chronic trauma [6,7]. But there are strong synergistic effects if the person is both a smoker and a drinker [8]. Tobacco smoke includes more than 4000 chemicals and 60 of them are found to be carcinogens which include nicotine, methoxy methyl furfural, arsenic, and methanol [9]. Alcohol increases the activation of procarcinogens & behaves like a solvent for entry of harmful carcinogens into the body cells. Once the tumor attains a finite size, pain in the area develops and medical assistance comes into action [10]. As they are asymptomatic at the earlier stages, detection of oral cancer becomes difficult. Diagnosis at the earliest stage is therefore very important for increasing the rates of patient survival. The survival rates are approximately 80–90% when detected at the earliest [10].

The delay in detection leads to higher chances of mortality. This poor survival rate has not improved despite several treatment options available. Hence the major goal is to focus on preventive measures at the first place so that mortality due to oral cancer is reduced and is in control.

Screening methods employed is for early detection and prediction of oral cancer which includes OralCDx, Vizilite, VizilitePlus, Micolux/DL, Orascoptic DK, and VELscope [11]. These help to locate the oral cancer and help in diagnosis. Salivary biomarkers like L-phenylalanine, angiogenetic marker *i.e.* Cluster of differentiation factor 34 (CD34), Genomic biomarkers such as integrin α3 and integrin β4, Cloning of an acidic laccase gene 2 which is a proteomic biomarker aid in early diagnosis, monitoring and differentiation of oral cancer [12]. Favourable agents for chemoprevention of oral cancer include β-carotene, retinoids, N-acetyl cysteine, NSAID's, vitamin-E, retinoids, and curcumin [13,14]. Melatonin which is secreted by the pineal gland also plays an important role as it has protective action against oral cancer, has anti-

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Causes of oral cancer – an appraisal of controversies

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VERIFIABLE CPD PAPER

IN BRIEF

- Provides evidence-based information on important and relevant risk factors for oral cancer.
- Enables the dentist to advise their clients on harm reduction.
- Provides an opinion on debated controversies.

OPINION

Major risk factors for oral cancer are cigarette smoking and alcohol misuse. Among Asian populations, regular use of betel quid (with or without added tobacco) increases oral cancer risks. Dentists should be aware of some emerging risk factors for oral, and particularly oropharyngeal cancer such as the role of the human papillomavirus infection (HPV). Decreases in risk could be achieved by encouraging high fruit and vegetable consumption. Some controversies related to the aetiology of this disease also need clarification. The objective of this paper is to provide an opinion on these debated controversies.

INTRODUCTION

Oral cancer, defined as cancers of lip, tongue and mouth (ICD 10: C 01-06), is a serious and growing problem in many parts of the globe including Europe. Oral and oropharyngeal cancer (ICD 10: C01-06, C09-10, C14) grouped together is the sixth most common cancer in the world. The areas characterised by high incidence are in South Asia, Pacific regions, Latin America and in parts of central and eastern Europe. A recent review provides up to date information on the global epidemiology.[1]

In this context it is important for United Kingdom (UK) dental practitioners to be aware of some cancer statistics and figures for the UK. There were 5,325 new cases diagnosed in 2006. Since the 1980s, the numbers of incident oral cancers reported to the UK cancer registries have been rising every year and more recent data suggest a rise of 41.2% over a period of ten years. The Cancer Research (UK)[2] figures issued in August 2009 confirmed a further steep rise in the latest figures, while other tobacco-associated cancers, eg Lung cancer, have declined. In fact, based on these crude data (age unadjusted), no other cancer site has shown such a rapid rise in incidence in the past quarter of a century.

Oral cancer to a large extent is a self-induced disease.[3] In order to plan preventive measures it is important to understand the risk factors associated with the disease. The major risk factors are well known, have been reviewed recently[4] and will not be described in detail in this paper. However, there are some emerging risk factors for oral cancer that dentists should be aware of, and some controversies related to the aetiology of this disease that need clarification.

The objective of this paper is to provide an opinion on these debated controversies. Several factors that have been often cited as likely to be associated with oral cancer, namely heredity and familial risk, marijuana (cannabis) smoking, khat chewing, medicinal nicotine use, HIV infection and alcohol containing mouthwashes, have not been adequately validated as having sufficient evidence to be linked with oral cancer. It is important to clear some myths about the disease causation so that dentists can, with some confidence, discuss only the important and relevant risks with their patients.

MAJOR RISK FACTORS – TOBACCO, ALCOHOL AND BETEL QUID

Major risk factors for oral cancer in the UK population are cigarette smoking and alcohol misuse. There are several key epidemiological studies from many countries that confirm the associated risk with these two lifestyle habits. While all forms of smoking (cigarette or cigar) have equal excess risks, there is no clear evidence that specific alcoholic drinks (wine, beer, spirits) have different effects on oral cancer. The most prevalent alcoholic beverage in a given population would be the one with the highest risk in that population.

Smokeless tobacco (ST) use also significantly increases the risk of oral cancer.[5] The sale of ST is banned in the UK so the public have no access to this form of tobacco. However, chewing tobacco is available on sale mostly mixed with betel quid (areca nut). Betel quid is carcinogenic to humans (both with and without added tobacco)[6] and is an important risk factor among people with this habit in the Asian ethnic minorities residing the UK.[7] For this reason, being South Asian (ethnicity but not race) could be considered a risk factor.

The estimated elevated risks from these different agents and exposures (among smokers, regular users of alcohol and betel quid; adjusted for each other) compared to non-users are reported consistently from many populations. The elevated risks are confirmed by several meta-analyses or systematic reviews, providing proof of significant risks of these lifestyles for oral cavity cancers.[8-10] In the UK there is evidence that the increasing incidence of oral cancer, especially affecting younger people, is associated with increased intake of alcohol.[11] The synergistic effect on the

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EDITORIAL

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Oral Leukoplakia – An Update

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ABSTRACT

The main purpose of this paper was to assess the current state of science on oral leukoplakia. Although it is considered a potentially malignant disorder the overall malignant progression of oral leukoplakia is of the order of 5% and even more. Nowadays there are no currently accepted markers to distinguish those that may progress to cancer from those that may not. The current golden standard is considered the presence of epithelial dysplasia on the tissue biopsy of the lesion. Proliferative verrucous leukoplakia is a rare form of OL which has multiple recurrences, is refractory to treatment and has malignant transformation in a short period. It is considered a true premalignant lesion. The management of oral leukoplakia varies from a "wait and see" attitude and topical chemopreventive agents to complete surgical removal.

Keywords: oral leukoplakia, potentially malignant disorder

INTRODUCTION

It has been reported that oral squamous cell carcinoma is associated with the presence of potentially malignant disorders in 15-48% cases (1). Oral leukoplakia (OL) is the most frequent potentially malignant disorder of oral mucosa. Although OL is mentioned in clinical reviews since 1969 (2), it was first defined by World Health Organization in 1978 (3) as a white patch or plaque which cannot otherwise be characterized clinically or pathologically as any other disease. Since then until now, the meaning of oral leukoplakia is not very much changed. In 1994 (4), after an international symposium held in Uppsala, Sweden in the definition, was added that oral leukoplakia is not associated with any physical or chemical cause, excepting smoking and it can become cancer. In 2007 it was decided that the name of leukoplakia should be limited only to a clinical diagnosis defined by exclusion of other white lesions such as oral lichen planus, white sponge nevus, nicotine stomatitis, leukoedema etc (5). In 2012 van der Waal (6) proposed a new definition which seems more oportune as it includes the histological confirmation "A predominantly white lesion or plaque of questionable behavior having excluded, clinically and histopathologically, any other definable white disease or disorder". This one hasn't been assessed yet by WHO but it has good chances for acceptance.

Incidence. Demographic distribution

The pooled estimated prevalence rate of oral leukoplakia in 2003 varied between 1.7 to 2.7% in general population (7). For this estimated rate, the author- Stefano Petti, in a meta-analysis including 23 primary studies from all over the world published in the period 1986-

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Review Article

A Clinicopathological Study of Various Oral Cancer Diagnostic Techniques

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Abstract

Oral cancer is one of the most commonly occurring malignant tumors in the head and neck regions with high incident rate and mortality rate in the developed countries than in the developing countries. Generally, the survival rate of cancer patients may increase when diagnosed at early stage, followed by prompt treatment and therapy. Recently, cancer diagnosis and therapy design for a specific cancer patient have been performed with the advanced computer-aided techniques. The responses of the cancer therapy could be continuously monitored to ensure the effectiveness of the treatment process that hardly requires diagnostic result as quick as possible to improve the quality and patient care. This paper gives an overview of oral cancer occurrence, different types, and various diagnostic techniques. In addition, a brief introduction is given to various stages of immunoanalysis including tissue image preparation, whole slide imaging, and microscopic image analysis.

Keywords: *Biomarker, histopathology, immunohistochemistry, microscopic image analysis, whole-slide imaging*

Introduction

Oral cancer is a frequently occurring and is the sixth most common cancer worldwide. Oral cancer is considered to be one of the most challenging malignancies of the head and neck regions that reportedly affect more than half million people every year worldwide.[1,2] Especially oral squamous cell carcinoma (OSCC) accounts for 85% of all oral cancer which affects its site or origin and can spread to cervical node, lungs, liver, and bones. During the year 2012, three million oral cancer patients (both sexes) have been identified, of which 145,000 cases were fatal. The incident rates of oral cancer are higher in the developed countries than in the developing countries. In India, the incident rate of oral cancer is 12.6% per one million populations and remains the most common cancer in other Asian countries. In addition, the incident rate remains higher in some of the developed countries, namely, Australia, New Zealand, Germany, Poland, Denmark, Scotland, and the USA. The 5-year survival rate of early stage oral cancer patients is approximately 82%, and the advanced stage is about 20%. However, the 5-year survival rate increased in developed countries such as the USA from 1983 to 2006. At the same time, a negative trend is noticed in some countries such as Brazil, Egypt, Japan, UK, and the Netherlands where the number of causality has increased due to oral cancer.[1]

The main risk factors associated with the development of oral cancer and potentially malignant lesions (PML) are smoking, consuming alcohol, and betel quid.[3] Early diagnosis of OSCC followed by prompt treatment can offer the best chance for cure and improve the survival rate of patients. Unlike others, the malignant portion of the oral cavity can be easily analyzed visually and/or different types of noninvasive method can also be used as a tool to increase the accuracy of physical examination. In spite of all, detection of OSCC is still an important and challenging problem for clinicians due to the fact that many lesions are asymptomatic at an early

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Review Article

Warning Signs and Symptoms of Oral Cancer and its Differential Diagnosis

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ABSTRACT
Oral Cancer (Oral Squamous Cell Carcinoma; OSCC) contributes to one of the important causes of mortality in human beings. These impending malignancies however may present clinically with warning signs and symptoms which is often neglected due to the lack of awareness in the population. Though there are developments in diagnosing early stage of oral cancer,the detection rate is continued to be minimal. Research proved a strong association between early diagnosis and better prognosis in these malignancies. Hence, knowledge regarding these warning indicators will certainly facilitate the clinicians to discover early cancer and provide an overall benefit pertaining to treatment in these patients.This review aims to highlight the early signs and symptoms of OSCC and their significance in improved prognosis of the patients. A Medline-PubMed search was conducted of the literature over the past years using the keywords: "oral squamous cell carcinoma", "warning signs", "Early symptom", "clinical presentation", "white lesion", "ulcer", "erythroplakia", and "abnormal growth". A total of 58 articles were reviewed, of which 11 were literature reviews, 15 were original studies and 32 were case reports. Appropriate Asssesment and analysis were performed to identify prompt warning signs and symptoms of OSCC.The key findings of all the early presentation were Eloborated with its clinical significance. In addition,this paper also helps to identify particular type of oral malignancies with their common clinical presentation reaching its emphasis based on the warning signs and symptoms.The importance of re-evaluation and biopsy to diagnose/eliminate malignant lesion in doubtful clinical situation is also highlighted.

Key words: Oral cancer, Warning signs, Symptoms, Persistent ulcer, White/red patch.

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INTRODUCTION

Oral and maxillofacial cancer (malignancy) contribute to one of the primary reasons of mortality worldwide. According to histological data, oral squamous cell carcinoma accounts for majority of all oral cancer. Dentists add to the crucial group of health care professionals in diagnosing oral cancers by observing essential warning signs and symptoms on examination of the oral cavity and confirming with suitable diagnostic aids. Thus, pertaining to the oral region, a "*sign*" refers to an objective evidence of a oral condition detected by a dentists during the examination of the oral cavity while a "*symptom*" refers to a subjective evidence of disease or a patient's oral condition. Few signs may go unnoticed by the patient although they remain meaningful and significant to the health-care provider in assisting the diagnosis of medical condition(s) responsible for the patient's symptoms. Among all the dental signs and symptoms detected on regular history and examination of the patients, the warning sign points to the intimation, threat, or sign of an impending danger of the underlying disease. Usually, at the molecular level, the cells undergo multistep process which includes initiation, proliferation and progression to become cancer cells during the pathogenesis of oral cancer. The clinical features also depend upon the etiopathological behavior of the individual tumors.

In this article, warning signs and symptoms of oral cancer (Table 1,2) from the review of the available literature has been highlighted which might be first signal of undiscovered malignancies. These signs and symptoms adds clue to the clinicians that aid in early diagnosis, timely evaluation and improved prognosis for the patients from an oncological perception.The other associated oral malignancies with the warning sign and symptoms are also highlighted (Table 3) which would be helpful for differential diagnosis in essential clinical situation.

WARNING SIGNS AND SYMPTOMS OF ORAL CANCER AND ASSOCIATED MAXILLOFACIAL MALIGNANCIES

Non Healing Ulcer [Figure 1]

Ulcerations in the mouth may exhibit from a simple to highly complex variations suggestive of oral malignancy. In other words, patients present with this chief complaint of *"ulceration"* depicting to the epithelium and connective tissue damage with the existence of an obvious central crater caused by oedema or proliferation in the surrounding tissue.[1] Health care professional must clearly grasp the distinction between malignant/premalignant lesions from the group of reactive lesions persisting for more than two weeks following the removal of etiological factors. These lesions become more suspicious when it increases in its intensity and turns non responsive to the ongoing treatment. Therefore, appropriate diagnostic procedures (i.e., gold standard biopsy in addition to other non-invasive chairside procedures of the lesion) are essential diagnostic

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Review Article

Epithelial Dysplasia in Oral Cavity

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This article has Continuous Medical Education (CME) credit for Iranian physicians and paramedics. They may earn CME credit by reading this article and answering the questions on page 491.

Abstract

Among oral lesions, we encounter a series of malignant epithelial lesions that go through clinical and histopathologic processes in order to be diagnosed. Identifying these processes along with the etiology knowledge of these lesions is very important in prevention and early treatments. Dysplasia is the step preceding the formation of squamous cell carcinoma in lesions which have the potential to undergo dysplasia. Identification of etiological factors, clinical and histopathologic methods has been the topic of many articles. This article, reviews various articles presenting oral cavity dysplasia, new clinical methods of identifying lesions, and the immunohistochemical research which proposes various markers for providing more precise identification of such lesions. This article also briefly analyzes new treatment methods such as tissue engineering.

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Keywords • Dysplasia • Review • Iran

Introduction

In oral mucosa we might encounter some epithelial or mesenchymal lesions. Based on epidemiologic research, the epithelial lesion could be the cause of most malignancies. Clinicians need to know the procedure of turning a malignant lesion to carcinoma.[1,2] Awareness of epidemiology of oral cancers, especially squamous cell carcinoma, may provide effective and appropriate treatment plans, mortality reduction and enhanced life quality. Squamous Cell Carcinoma (SCC) is the highest prevalence lesion among other oral malignancies in epidemiologic studies.

Idris et al. in Sudan reported 66.5% prevalence of SCC among malignant lesions.[3] In 2007, Razavi et al. claimed that epithelial malignancies were the most prevalent lesions (63%) and reported 54.5% SCC prevalence in Isfahan.[4] As some scientists believe that early diagnoses of lesions are important in both prevention and therapeutic procedures of oral cancers, many clinical, histological and cytological studies have been carried out. While few focused on clinical evaluation of different lesions diagnoses methods, few others recommend modern cytological method used in genecology. Despite the availability of such modern methods, pathologists claim that microscopic view of biopsies makes higher specificity and sensitivity in diagnosis.[5-7]

At first, Definition of Few Terms

Precancerous Lesion; refers to a tissue with benign morphological change having high potential to turn into malignance.

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Molecular Cytogenetics

REVIEW Open Access

Head and neck cancer: searching for genomic and epigenetic biomarkers in body fluids – the state of art

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Abstract

Head and neck squamous cell carcinoma (HNSCC) affects multiple sites of the upper aerodigestive tract and exhibited high incidence and mortality worldwide, being frequently diagnosed at advanced stage. Early detection of HNSCC plays a crucial role in a successful therapy. In the last years, the survival rates of these tumors have not improved significantly due to the late diagnosis and the lack of precise disease biomarkers and targeted therapies. The introduction in the clinical practice of body fluids to detect and analyze circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and exosomes provides a minimally or non-invasive method also called as liquid biopsy for diagnostic and prognostic biomarkers detection, representing a shift of paradigm in precision medicine through the revolution in the way to perform HNSCC diagnosis and to screen high risk population. Despite the use of body fluids being an emergent and up-to date issue to early diagnosis HNSCC and their recurrences, no strategy has yet proven to be consistently effective and able to be translated to clinical application in the routine clinical management of these patients. In this review we will discuss the recent discoveries using blood and saliva to identify biomarkers for the early detection and prognosis of HNSCC.

Keywords: Body fluids, Cell-free DNA, Circulating tumor DNA, Exosomes, Head and neck cancer

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide with an annual incidence of around 600 000 new cases, mostly diagnosed as locally advanced disease [1]. This carcinoma is a heterogeneous disease at clinical and molecular level, encompassing several tumors from hypopharynx, oropharynx, lip, oral cavity, nasopharynx, and larynx. This tumors group presents different epidemiology, etiology and molecular alterations that drive carcinogenesis and, consequently distinct therapy responses. The traditional risk factors related to the pathogenesis of HNSCC are smoking and excessive alcohol comsumption, being also infection with high-risk human papillomaviruses (HPVs) associated to a rising number of these tumours, especially at the oropharynx in younger patients [2]. Human papillomavirus-related oropharyngeal cancer (HPV+) exhibited not only better response to treatment but also better survival, being generally associated to a good prognosis when compared to HPV-negative [3, 4], which lead to the adaptation of the eighth edition of the HNSCC tumour-node-metastasis (TNM) staging in order to include $p16^{INK4A}$ immunostaining as a surrogate for HPV status. The HPV-positive cancer incidence is rising, while HPV-negative cancers incidence is decreasing [5]. The five-year overall survival rate of HNSCC patients is almost unchanged in the last decades, remaining around 50%, even with the improvements in the treatment (i.e surgery, radiotherapy, chemotherapy and novel targeted therapies), mainly due to the advanced clinical tumor stage at the diagnosis and the treatment failure associated to frequent recurrencies [6]. The HNSCC treatment selection is based in some clinical-pathological parameters, such as the tumor anatomic location and tumor stage; however, these patients with similar clinic-pathological characteristics may differ in their clinical outcome, justifying the tumor's biologic

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Review Article

Biopsy of Oral Lesion -A Review Article

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ABSTRACT:

Biopsy is the removal of tissue from a living person for microscopic examination to confirm or to establish the diagnosis of a disease The purpose of this article is to review those skills, to discuss new developments in this area, and to highlight some of the potential pitfalls that may occur in taking a biopsy and methods available to avoid them. We feel it will be of value to both general dental practitioners and junior hospital staff. Problems related to specific areas will be covered including apical lesions and those associated with the dental hard tissues.

Key words: Biopsy , Dental hard tissues, FNAC

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INTRODUCTION:

Biopsy is the removal of tissue from a living person for microscopic examination to confirm or to establish the diagnosis of a disease.

The term was coined by Ernst Henry, a French dermatologist in 1879. This approach is used for all tissues of the body, including those of the oral cavity, where a wide spectrum of disease processes may present. Proper management of an oral mucosal lesion begins with diagnosis, and the gold standard for diagnosing disease, oral or otherwise, is tissue biopsy[2]. The oral environment, which is moist and confined, poses challenges for collecting a viable tissue sample that will be suitable for diagnosis. These challenges are further compounded by the myriad of biopsy techniques and devices now available. The dental clinician should be aware of the various biopsy techniques that are available for the oral tissues, as well as the challenges specific to these tissue. Whatever the method used, however, the aim is to provide a suitably representative sample for the clinician to interpret, while minimising preoperative discomfort for the patient. An unsuitable, unrepresentative sample is of no use to the clinician or most importantly the patient who would be ill served by an unnecessary repeat procedure (fig 1)[1].

Rovin has made several observations on biopsy decisions.[3]

1. Any lesion that persists for more than two weeks with no apparent etiological basis.
2. Any inflammatory lesion that does not respond to local treatment after 10 to 14 days that is, after removing local irritant.

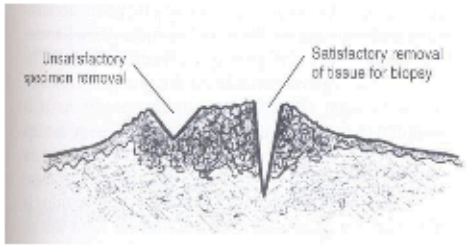

Figure 1

3. Persistent hyperkeratotic changes in surface tissues.
4. Any persistent tumescence either visible or palpable beneath relatively normal tissue.
5. Inflammatory changes of unknown cause, that persists for long periods.
6. Lesions that interfere with local function. Eg. Fibroma.
7. Bone lesion not superficially identified by clinical and radiographic findings.
8. Any lesion that has characteristics of malignancy.
   - Erythroplasia: Lesion is totally red or has a speckled red and white appearance.
   - Ulceration: an ulcerated lesion
   - Duration: Lesion has persisted more than 2 weeks.
   - Growth rate: Lesion exhibits rapid growth.
   - Bleeding: Lesion bleed on gentle manipulation.
   - Induration: Lesion and surrounding tissue is firm to touch.

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Saliva Liquid Biopsy for Point-of-Care Applications

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Saliva is a non-invasive biofluid, which is easy to collect, transport, and store. Because of its accessibility and connection to systemic diseases, saliva is one of the best candidates for the advancement of point-of-care medicine, where individuals are able to easily monitor their health status by using portable convenient tools such as smartphones. There are a variety of scenarios with which saliva can be used: studies have been conducted on using saliva to measure stress hormones, enzyme levels, developmental disease biomarkers, and even cancer mutations. If validated biomarkers were combined with high-quality detection tools, saliva would open up a new frontier in high-quality healthcare, allowing physicians and patients to work together for real-time health monitoring and high-impact personalized preventative medicine. One of the most exciting emerging frontiers of saliva is liquid biopsy, which is a non-invasive means to assess the presence and characteristics of cancer in a patient. This article will review current basic knowledge of biomarkers, review their relation to different diseases and conditions, and explore liquid biopsy for point-of-care applications.

Keywords: saliva, liquid biopsy, point-of-care, biomarker, cancer

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INTRODUCTION

In the era of new diagnostic methods and treatment options, patient care is rapidly changing. There are many new paradigms in the evolution of modern healthcare: the White House has advocated for precision medicine, which tailors individualized treatment to the patient (1). Early detection is another emerging paradigm, which seeks to decrease patient morbidity and mortality by detecting disease at a phase where it is easily treatable. Early detection usually improves the success of treatment, prevents complications, and enhances patient prognosis. This is highlighted in common diseases affecting large populations such as cardiovascular diseases, diabetes mellitus, and various malignancies, as a recent review discusses (2). Precision medicine and early detection merge together with a third major paradigm: point-of-care diagnostics. Point-of-care diagnostics is a field of investigation that explores technologies that allows patients and health providers to gain actionable medical information rapidly and conveniently. Point-of-care diagnostics seeks to achieve "bed-side" diagnosis, removing the time delay that is caused by the conventional workflow of collecting samples and transporting them to a central lab for testing. The paradigm of point-of-care diagnostics joined to precision medicine and early detection paint a compelling vision of the future: one where doctors and patients can use small and portable devices to rapidly assess a patient's health status, catching diseases extremely early and allowing ultracustomized treatment based on a patient's personal characteristics.

One of the most critical questions that must be answered in point-of-care personalized medicine, however, is the question of which biomarkers to use for health monitoring. A biomarker is defined as a measurable, objective indicator of an individual's normal and abnormal physiological

ORIGINAL ARTICLE

The sensitivity and specificity of computerized brush biopsy and scalpel biopsy in diagnosing oral premalignant lesions: A comparative study

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ABSTRACT

Background: The diagnosis of oral malignancy and epithelial dysplasia has traditionally been based upon histopathological evaluation of full thickness biopsy from lesional tissue. As many studies had shown that incisional biopsy could cause progression of the tumors, many alternative methods of collection of samples had been tested. Oral brush biopsy is a transepithelial biopsy where it collects cells from basal cell layer noninvasively. **Aim:** To assess the diagnostic accuracy of brush biopsy when compared to histopathology in a group of patients with features of potentially malignancy. **Materials and Methods**: In the present study, 60 cases of clinically diagnosed leukoplakia are selected and subjected to histopathology and brush biopsy. **Results and Conclusion:** Results showed that of 16 dysplasia cases confirmed by histopathology, only 12 were positively reported in oral brush biopsy. In 44 cases, the reports are same for histopathology and brush biopsy. The sensitivity of oral brush biopsy is 43.5% and specificity is 81.25% with a positive predictive value of 58.3%. Oral brush biopsy with molecular markers like tenascin and keratins can be an accurate diagnostic test.

Key words: Brush biopsy, leukoplakia, sensitivity, specificity

INTRODUCTION

Detection of cancer in the early asymptomatic stage improves the cure rates and quality of life of the patient by minimizing extensive, debilitating treatments and can be conservatively managed with minimal surgical morbidity and 100% survival.[1] Early cancerous lesions are asymptomatic and vary in clinical presentations as they do not have ulcerations, indurations, elevations, bleeding, and cervical adenopathy as in case of advanced cancers.[1]

The significance of evaluation of leukoplakic lesions, which is the most common precursor of oral cancer, aids in prognostic implications. Leukoplakia has varied clinical appearance without any symptoms. But, it may show severe dysplasia, carcinoma *in-situ*, or frank carcinoma. The cytological examination had failed to diagnose cases of dysplasia or malignancy accurately as that of the histopathology. In the oral cavity, the cytology has been of limited use due to the superficial cells collected and the keratin layer that is present. As a result, deeper epithelial abnormalities are not detected.[2] In earlier days, cotton swabs were used for collection of smears and this was followed by sponge, wooden, or metal spatulas, where only superficial layer cells are collected.[3]

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The basis for development of newer techniques for collection of cells is, dysplasia starts in basal layers (stratum germinatum) and extends to all the layers of the epithelium.[4] In order to collect the basal layer cells in a new transepithelial, noninvasive technique was developed by the Oral CDx laboratories. In this technique, the smears obtained contain cells from all the layers of the epithelium and has improved diagnostic applications for mass screening campaigns, without the need for surgery and surgically trained personnel for taking the biopsy. The concept developed in western countries has been established and its use in South Indian population has not been validated. This confirmatory study intends to validate the use of this novel technology in the study population from South India.

MATERIALS AND METHODS

The present study is done on 60 patients who are referred

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Salivary biomarkers for detection of oral squamous cell carcinoma – current state and recent advances

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Abstract

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity. Detection of OSCC is currently based on thorough clinical oral examination combined with biopsy for histological analysis. Most cases of OSCC are not detected until the cancer has developed into advanced stages; thus, a reliable early stage diagnostic marker is needed. This literature review presents an overview of the status of current advances in salivary diagnostics for OSCC. Though many protein and mRNA salivary biomarkers have been identified that can detect OSCC with high sensitivity and specificity, the most discernable findings occur with the use of multiple markers. Studies that incorporate proteomic, transcriptomic, and potentially additional "omics", including methylomics, need to be initiated to bring technology to clinical applications and allow the best use of saliva in diagnosing OSCC.

Keywords

salivary diagnostics; salivary biomarker; oral fluid diagnostics; oral squamous cell carcinoma; oral cancer; salivaomics; transcriptomics; proteomics; microbiomics; methylomics; metabolomics; exosomes

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Conflict of Interest

Dr. Maha Yakob received a grant from NIH.
Dr. Laurel Fuentes received a grant from NIH.
Dr. Marilene B. Wang and Dr. Elliot Abemayor each declare no potential conflicts of interest relevant to this article.
Dr. David T.W. Wong is co-founder of RNAmeTRIX Inc., a molecular diagnostic company. He holds equity in RNAmeTRIX, and serves as a company Director and Scientific Advisor. The University of California also holds equity in RNAmeTRIX. Intellectual property that David Wong invented and which was patented by the University of California has been licensed to RNAmeTRIX. Additionally, he is a paid consultant to PeriRx.

Compliance with Ethics Guidelines

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Methylation Array Analysis of Preoperative and Postoperative Saliva DNA in Oral Cancer Patients

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Abstract

Purpose: **To perform methylation array analysis of 807 cancer-associated genes using tissue and saliva of oral squamous cell carcinoma (OSCC) patients with the objective of identifying highly methylated gene loci that hold diagnostic and predictive value as a biomarker.**
Experimental Design: **We did the methylation array on DNA extracted from preoperative saliva, postoperative saliva, and tissue of 13 patients with OSCC, and saliva of 10 normal subjects. We identified sites that were highly methylated in the tissue and preoperative saliva samples but not methylated in the postoperative saliva samples or in normal subjects.**
Results: **High quality DNA was obtained and the methylation array was successfully run on all samples. We identified significant differences in methylation patterns between the preoperative and postoperative saliva from cancer patients. We established a gene classifier consisting of 41 gene loci from 34 genes that showed methylation in preoperative saliva and tissue but were not methylated in postoperative saliva or normal subjects. Gene panels of 4 to 10 genes were constructed from genes in the classifier. The panels had a sensitivity of 62% to 77% and a specificity of 83% to 100% for OSCC.**
Conclusions: **We report methylation array analysis of 807 cancer-associated genes in the saliva of oral cancer patients before and after oral cancer resection. Our methylation biomarker approach shows the proof of principle that methylation array analysis of saliva can produce a set of cancer-related genes that are specific and can be used as a composite biomarker for the early detection of oral cancer.** (Cancer Epidemiol Biomarkers Prev 2008;17(12):3603–11)

Introduction

Biomarker detection within biological fluids shows promise for the early diagnosis of cancer. In particular, evaluation of fluids approximating the cancer has significant clinical applicability. For example, sputum analysis has been used to detect lung carcinoma (1). In similar fashion, saliva is the proximal fluid for head and neck squamous cell carcinoma (SCC). The cellular and fluid content of whole saliva, which includes protein, genetic, and epigenetic changes, has been studied in head and neck SCC. Promoter hypermethylation is an epigenetic change that involves the addition of methyl groups to cytosine residues in the context of a CpG dinucleotide. This usually occurs in the promoter region of a gene, which contains a high density of CpG dinucleotides, termed CpG islands. The methyl group will interfere with transcriptional proteins resulting in long-term silencing of that gene. Promoter hypermethylation is a critical step in oral carcinogenesis and has a number of significant advantages over genetic and protein diagnostic markers. Epigenetic silencing events (i.e., promoter hypermethylation) are more frequent mechanisms of gene silencing than genetic changes, making it a more attractive marker than detecting a genetic mutation or measuring gene expression. It is one of the earliest events in oral carcinogenesis, preceding protein expression level changes. In fact, promoter hypermethylation is a more frequent mechanism in gene silencing than genetic mutation (2). Because DNA methylation leads to gene silencing (a negative biological event), protein is not produced and immunohistochemistry or ELISA cannot be used in a clinical setting. For a diagnostic test to be implemented clinically, the test must measure a positive event. Therefore, by analyzing for DNA methylation, we can turn a negative biological event into a positive clinical test. Previous studies analyzing promoter hypermethylation have looked at a panel of 2 to 20 genes to establish the sensitivity of detection of head and neck cancer (3-9). In these studies, genes have been chosen based on their known role in head and neck carcinogenesis. The specificity and sensitivity of this approach has not yielded a gene panel that is viable in a clinical setting. Moreover, current techniques to measure promoter methylation, which include combined bisulfite restriction analysis (10), quantitative methylation-specific PCR (MSPCR; refs. 11, 12) and pyrosequencing (13), are inadequate for genome-wide methylation analysis because the labor required for such an analysis with one of these techniques is prohibitive. However, once a gene panel consisting of a manageable number of genes has been developed using a genome-wide approach, one of the above technical approaches could be implemented in a clinical laboratory on a routine basis. We sought to discover genes that have not been previously studied in head and neck cancer that might hold significant diagnostic value. We targeted our discovery approach by analyzing the matched preoperative saliva, postoperative saliva, and tissue of a specific head and neck cancer

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NID2 and HOXA9 promoter hypermethylation as biomarkers for prevention and early detection in Oral Cavity Squamous Cell Carcinoma tissues and saliva

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Abstract

Differentially methylated oral squamous cell carcinoma (OSCC) biomarkers, identified *in-vitro* and validated in well-characterized surgical specimens, have shown poor clinical correlation in cohorts with different risk profiles.

To overcome this lack of relevance we used the HumanMethylation27 BeadChip, publicly available methylation and expression array data, and Quantitative Methylation Specific PCR to uncover differential methylation in OSCC clinical samples with heterogeneous risk profiles.

A two stage-design consisting of Discovery and Prevalence screens was used to identify differential promoter methylation and deregulated pathways in patients diagnosed with OSCC and head and neck squamous cell carcinoma.

Promoter methylation of *KIF1A* ($\kappa = 0.64$), *HOXA9* ($\kappa = 0.60$), *NID2* ($\kappa = 0.60$), and *EDNRB* ($\kappa = 0.60$) had a moderate to substantial agreement with clinical diagnosis in the Discovery screen.

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Article

Influence of MTHFR Genetic Background on p16 and MGMT Methylation in Oral Squamous Cell Cancer

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Abstract: Genetic polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) enzyme may influence DNA methylation. Alterations in DNA methylation patterns of genes involved in the regulation of the cell cycle, DNA repair, cell adherence and metastasis process are known to contribute to cancer development. In this study, the influence of the MTHFR C677T and A1298C gene polymorphisms on global DNA methylation and site-specific methylation on *p16* and O^6-methylguanine-DNA methyltransferase (*MGMT*) gene promoters was investigated in patients with oral squamous cell cancer (OSCC). To this aim, methylation studies were carried out by using genomic DNA isolated from saliva samples of 58 OSCC patients and 90 healthy controls. The frequency of the CT/AC and TT/AA genotypes was significantly higher in patients than in controls. Whereas no difference in global DNA methylation levels was observed between patients and controls, a higher frequency of methylation at both *p16* and *MGMT* gene promoters was detected in patients compared with controls. A significant association between *MTHFR* gene polymorphisms and *p16* and *MGMT* gene promoter methylation was found. The frequency of *p16* and *MGMT* methylation was around 60% in patients with either the CT/AC or TT/AA genotype. Our results suggest that hypermethylation of cancer-related genes may be affected by *MTHFR* polymorphisms.

Keywords: oral squamous cell cancer; *MTHFR* polymorphisms; global DNA methylation; *p16* promoter methylation; *MGMT* promoter methylation

1. Introduction

Head and neck cancer (HNC), representing the sixth most common cancer worldwide [1,2], encompasses a heterogeneous group of aggressive epithelial malignancies, more than 90% of which are squamous cell carcinomas (SCC).

Oral SCC (OSCC) is one of the most common types of HNC, with a considerable incidence of new cases every year. OSCC more frequently affects men than women (M:F = 2:1). The probability of developing OSCC increases with the period of exposure to risk factors, represented by a diet low in fresh fruits and vegetables, poor vitamin intake, alcohol consumption, and abuse of tobacco smoking [3,4]. Moreover, infection with high-risk human papillomavirus genotypes has also recently been implicated in the etiopathogenesis of OSCC [5].

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ORIGINAL ARTICLE – CANCER RESEARCH

Morphological and molecular features of oral fluid-derived exosomes: oral cancer patients versus healthy individuals

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Abstract

Purpose Oral cancer (OC) patients are at high risk to develop recurrent disease or secondary primary cancers with no available biomarkers to detect these events until a visible lesion is readily present and diagnosed by biopsy. Exosomes secreted by cancer cells are involved in tumor growth, invasion and metastasis. We aimed to determine morphological and molecular differences between oral fluid (OF)-derived exosomes of OC patients and those isolated from healthy individuals (HI).

Methods OF from OC patients ($n = 36$) and HI ($n = 25$) was initially assessed by nanoparticle tracking analysis (NTA). Following ultracentrifugation, exosomal pellets of OC patients and HI were morphologically examined by transmission electron microscopy and atomic force microscopy (AFM). Enzyme-linked immunosorbent assay (ELISA) and western blotting (WB) were used to analyze the expression of exosomal markers—CD9, CD81 and CD63.

Results NTA showed that OC samples of OF had a significantly higher concentration of nanoparticles/ml ($p = 0.01$) and modal nanoparticle size ($p = 0.002$) compared to HI. The difference in size was structurally highlighted by AFM three-dimensional images applied on exosomal pellets. ELISA and WB showed differential expression of exosomal markers in OC exosomes compared to HI: lower expression of CD81 and CD9 in contrast to a higher expression of CD63 (~53 kDa).

Conclusions OF-derived exosomes from OC patients differ both morphologically and molecularly from exosomes present in HI. This study is a baseline that provides a starting point for finding exosomal biomarkers for early detection of malignant changes in high-risk patients without overt clinical signs/lesions.

Keywords Oral cancer · Oral fluid-derived exosomes · Nanoparticle tracking analysis · Atomic force microscopy · Tetraspanins

This work was performed in partial fulfillment of the requirements for a Ph.D. degree of Ayelet Zlotogorski-Hurvitz, Sackler Faculty of Medicine, Tel Aviv University, Israel

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Introduction

Head and neck squamous cell carcinoma is the seventh most common malignancy worldwide with an annual incidence of >600,000, of which about one-half are located

EXPLOITING SALIVARY *miR-31* AS A CLINICAL BIOMARKER OF ORAL SQUAMOUS CELL CARCINOMA

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Abstract: *Background.* Oral carcinoma is an important malignancy throughout the world. MicroRNAs (miRNAs) are endogenously expressed, non-coding RNAs that regulate post-transcriptional levels of targeted mRNAs. *MiRNA-31(miR-31)* is significantly upregulated in oral carcinoma tissues and plays oncogenic roles in oral carcinogenesis.

Methods. We analyzed the levels of *miR-31* in saliva of patients with oral carcinoma ($n = 45$), oral verrucous leukoplakia ($n = 10$), and control healthy individuals ($n = 24$) by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Results. Salivary *miR-31* was significantly increased in patients with oral carcinoma at all clinical stages, including very small tumors. However, our preliminary analysis showed no increase of salivary *miR-31*in patients with oral verrucous leukoplakia relative to controls. The *miR-31* was more abundant in saliva than in plasma, suggesting salivary *miR-31* was a more sensitive marker for oral malignancy. After excision of oral carcinoma, salivary *miR-31* was remarkably reduced, indicating that most of the upregulated salivary *miR-31* came from tumor tissues.

Conclusion. Our results point to a potential application of salivary *miR-31* as a biomarker for early detection and postoperative follow-up of oral carcinoma. © 2011 Wiley Periodicals, Inc. *Head Neck* **34:** 219–224, 2012

Keywords: biomarker; carcinoma; *miR-31*; oral; saliva

Oral squamous cell carcinoma (OSCC) is 1 of the most prevalent carcinomas throughout the world.[1] It usually results from multistep carcinogenesis. Multiple lesions at different neoplastic stages may simultaneously manifest over large areas of the carcinogen-exposed mucosa, a phenomenon called field cancerization.[2] This clinical behavior might be associated with tumor recurrence and poses a major challenge for early detection and postoperative follow-up of OSCC. Thus, more sensitive and specific biomarkers are urgently needed for patients with oral cancerous and oral precancerous lesions. Unlike other kinds of body fluid, saliva, in which oral tissues are continually immersed, may provide more direct information regarding the disease status of the oral mucosa. Therefore, a search for novel biomarkers in the secretome of saliva may benefit patients with oral cancer. Many specific salivary molecules have been suggested to be of use in the diagnosis and prognostic prediction for patients with OSCC.[3,4]

MicroRNAs (miRNAs) are short non-coding RNAs, which regulate translation and degradation of target mRNAs.[5] Increasing evidence has suggested important roles for various miRNAs in carcinogenesis.[5,6] A single miRNA is able to target multiple mRNAs and, thereby, potentially affect several important cellular pathways involved in tumorigenic processes. Therefore, the signature patterns of representative miRNAs may hold meaningful diagnostic value or serve as predictors of therapeutic efficacy in cancer.[5,6]

Several miRNAs have been implicated in the tumorigenesis of cancers including OSCC.[1,7–9] MiRNA-31 *(miR-31)* was found upregulated in a wide variety of neoplasms including head and neck cancer, hepatocellular carcinoma, and colorectal carcinoma, and this miRNA seemed oncogenic for these neoplasms.[10–16] Our previous studies showed that *miR-31* was markedly upregulated in OSCC tissues and mediated oral oncogenesis by regulating hypoxia pathways in oral cancer cells through targeting a molecule inhibiting hypoxia inducing factor.[1] *MiR-31* in plasma was significantly elevated in patients with OSCC. The plasma *miR-31* in patients was remarkably reduced after tumor resection suggesting that this marker is tumor-associated.[17] A recent study showed that *miR-125a* and *miR-200a* were significantly decreased in the saliva of patients with OSCC, compared with those of healthy individuals.[18] Saliva is a convenient specimen because it can be obtained in a noninvasive

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Original Research Article

Expression Analysis of Salivary Microrna-31 in Oral Cancer Patients

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Keywords

Oral Carcinoma, Saliva, MicroRNA-31, Taqman real time-qPCR

A B S T R A C T

Oral carcinoma is the 6th most common cancer in the world. Micro RNAs are small non-coding single-stranded RNAs. They have been shown to be capable of altering mRNA expression; thus some are oncogenic or tumor suppressive in nature. The salivary microRNA-31 has been proposed as a sensitive marker for oral malignancy since it was abundant in saliva more than in plasma. A total of 55 whole saliva samples were collected from 35 cases diagnosed with OC their ages and gender matched with 20 healthy subjects. Taq ManqRT PCR was performed for RNA samples. Mean age was 52.23+13.73 years in cases (range: 17-70 years) with male predominance represented 69%. Risk of smoking and alcoholism was highly significant. The median fold change of miR-31was significantly higher in patients group than in control group, 19.634 versus 1.962 ($P<0.001$). However, the correlation between age of patients and miR-31 fold change was non-significant negative correlation ($r = -0.236$, $P>0.05$). Median miR-31 fold change was 19.63 in smokers and 21.12 in drinkers. Salivary miR-31 appeared to have significantly elevated in OC patients which point to its potential application as a biomarker for early detection and postoperative follow-up.

Introduction

Oral carcinoma (OC) is one of the most prevalent malignancies worldwide, approximately 263,900 new cases and 128,000 deaths in 2008 (Jemal *et al*., 2011). Approximately 90% of all oral malignancies are oral squamous cell carcinoma (OSCC) and represents about 3.5% of all malignant tumors in the western societies (Ferlay *et al*., 2010). The Established etiological factors are cigarette smoking and heavy alcohol abuse; however, a growing group of patients, including young adults and women, have no known tobacco or alcohol exposure have been emerged, therefore; possible viral etiologic factors such as oncogenic human papilloma virus (HPV) have been proposed (Rosebush *et al*., 2011). The low survival rates and morbidity can be attributed to the late diagnosis (Peacock *et al*.,2008). Hence, several new trends have been emerging that have successfully addressed this problem among which salivary RNAs are noteworthy (Li *et al*., 2004). The 5-years survival rate for OSCC has remained around 50%, one of

ORAL DISEASES

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ORIGINAL ARTICLE

Salivary microRNAs in oral cancer

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OBJECTIVE: This study investigated the use of three salivary microRNAs (miRNA-21, miRNA-184, and miRNA-145) as possible markers for malignant transformation in oral mucosal lesions.

MATERIALS AND METHODS: Salivary whole unstimulated samples were collected from a study group of 100 subjects, consisting of 20 clinically healthy controls, 40 patients with oral potentially malignant disorders (PMDs) [20 with dysplastic lesions and 20 without dysplasia], 20 with biopsy-confirmed oral squamous cell carcinoma (OSCC), and 20 with recurrent aphthous stomatitis (RAS) as disease controls. Total RNA was isolated and purified from saliva samples using the microRNA Isolation Kit (Qiagen, UL). miRNA expression analysis was performed using qRT-PCR (Applied Biosystems).

RESULTS: There was a highly significant increase in salivary miRNA-21 and miRNA-184 in OSCC and PMD (with and without dysplasia) when compared to healthy and disease controls ($P < 0.001$). Conversely, miRNA-145 levels showed a highly significant decrease in OSCC and PMD overall ($P < 0.001$). RAS cases showed no significant difference from normal controls in any measured miRNA ($P > 0.05$). The only microRNA to discriminate between OSCC and PMD with dysplasia was miRNA-184. When receiver operating characteristic curves were designed for the three miRNAs, cutoff points delineating the occurrence of malignant change were a fourfold increase in miRNA-21 with specificity 65% and sensitivity 65%, a 0.6 decrease in miRNA-145, with specificity 70% and sensitivity 60%, and a threefold increase of miRNA-184, with specificity 75% and sensitivity 80%. Calculating the area under the curve revealed that miRNA-184 was the only one among the studied miRNAs that provided good diagnostic value.

CONCLUSION: Salivary determination of the miRNAs tested might furnish a noninvasive, rapid adjunctive aid for revealing malignant transformation in oral mucosal lesions, particularly miRNA-184.

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Keywords: oral cancer; microRNA; miRNA-21; miRNA-145; miRNA-184; miRNA salivary biomarkers; potentially malignant disorders; oral malignant transformation

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Introduction

Oral squamous cell carcinoma (OSCC) is the 6th most frequent cancer worldwide (Jemal *et al*, 2011). The poor prognosis has led to efforts to try to clarify the mechanisms underlying the high invasiveness and to investigate new diagnostic and therapeutic strategies (Yanamoto *et al*, 2002; Kawakita *et al*, 2013). Diagnostic and prognostic biomarkers have been sought, and as deregulation of microRNAs (miRNAs) has been shown to correlate with various tumor characteristics and prognosis in some cancers, including those affecting the oral cavity (Calin and Croce, 2006; Wu *et al*, 2011; Chen *et al*, 2013), this was considered to be a fruitful area to explore.

Like other cancers, oral carcinogenesis involves gradual accumulation of both genetic and epigenetic changes, leading to gain of function in certain oncogenes and loss of function in some tumor suppressor genes (Leemans *et al*, 2011). miRNAs are small noncoding RNAs which function in mRNA silencing and posttranscriptional regulation of gene expression. miRNAs are key regulators (Grosshans and Slack, 2002) transcribed by RNA polymerase II or RNA polymerase III as a part of an intron of mRNA or as an independent gene unit.

Both whole and supernatant saliva of healthy controls contain many miRNAs which enter the oral cavity through various sources, including the salivary glands, gingival crevicular fluid, and from desquamated oral epithelial cells (Park *et al*, 2006). Most salivary miRNAs are partially degraded (Park *et al*, 2007) and maintain stability in saliva

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ORIGINAL ARTICLE – CANCER RESEARCH

FTIR-based spectrum of salivary exosomes coupled with computational-aided discriminating analysis in the diagnosis of oral cancer

Ayelet Zlotogorski-Hurvitz[1,2] · Ben Zion Dekel[3] · Dov Malonek[3] · Ran Yahalom[4] · Marilena Vered[1,5]

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Abstract

Purpose To determine the Fourier-transform infrared (FTIR) spectra of salivary exosomes from oral cancer (OC) patients and healthy individuals (HI) and to assess its diagnostic potential using computational-aided models.

Methods Whole saliva samples were collected from 21 OC patients and 13 HI. Exosomes were pelleted using differential centrifugation (12,000*g*, 120,000*g*). The mid-infrared (IR) absorbance spectra (900–5000 cm^{-1} range) were measured using MIR8025 Oriel Fourier-transform IR equipped with a PIKE MIRacle ZnSe attenuated total reflectance attachment. Machine learning techniques, utilized to build discrimination models for the absorbance data of OC and HI, included the principal component analysis–linear discriminant analysis (PCA–LDA) and support vector machine (SVM) classification. Sensitivity, specificity and the area under the receiver operating characteristic curve were calculated.

Results IR spectra of OC were consistently different from HI at 1072 cm^{-1} (nucleic acids), 2924 cm^{-1} and 2854 cm^{-1} (membranous lipids), and 1543 cm^{-1} (transmembrane proteins). The PCA–LDA discrimination model correctly classified the samples with a sensitivity of 100%, specificity of 89% and accuracy of 95%, and the SVM showed a training accuracy of 100% and a cross-validation accuracy of 89%.

Conclusion We showed the specific IR spectral signature for OC salivary exosomes, which was accurately differentiated from HI exosomes based on detecting subtle changes in the conformations of proteins, lipids and nucleic acids using optimized artificial neural networks with small data sets. This non-invasive method should be further investigated for diagnosis of oral cancer at its very early stages or in oral lesions with potential for malignant transformation.

Keywords Oral cancer · Saliva · Exosomes · Fourier-transform infrared (FTIR) · Machine learning · Diagnosis

Introduction

Oral cancer (OC), referring to the main variant of squamous cell carcinoma, is now assessed to have a global incidence of over 300,000 new annual cases with a trend to further increase in younger patients and in developing countries (Ferlay et al. 2015; Shield et al. 2017). The 5-year prognosis has not substantially changed for more than 4 decades and stands as low as 50%. One of the caveats associated with OC is the ability to identify early changes in the oral lining epithelium, mainly in those patients at high risk to develop OC.

Tissue biopsy and light microscopy are still the gold-standard diagnostic tools. However, these are performed when lesions (cancerous or pre-cancerous) are visible and already contain substantial genetic changes. Moreover, tissue biopsies provide information that is limited to a portion of a tumor and at a specific time-point. Liquid biopsy, based on

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BMC Cancer

RESEARCH ARTICLE Open Access

Progress risk assessment of oral premalignant lesions with saliva miRNA analysis

Ya Yang[1], Yue-xiu Li[2], Xi Yang[3], Long Jiang[1], Zuo-jun Zhou[1] and Ya-qin Zhu[1*]

Abstract

Background: Oral cancer develops through multi-stages: from normal to mild (low grade) dysplasia (LGD), moderate dysplasia, and severe (high grade) dysplasia (HGD), to carcinoma *in situ* (CIS) and finally invasive oral squamous cell carcinomas (OSCC). Clinical and histological assessments are not reliable in predicting which precursor lesions will progress. The aim of this study was to assess the potential of a noninvasive approach to assess progress risk of oral precancerous lesions.

Methods: We first used microRNA microarray to profile progressing LGD oral premaligant lesions (OPLs) from non-progressing LGD OPLs in order to explore the possible microRNAs deregulated in low grade OPLs which later progressed to HGD or OSCC. We then used RT-qPCR to detect miRNA targets from the microarray results in saliva samples of these patients.

Results: We identified a specific miRNA signature that is aberrantly expressed in progressing oral LGD leukoplakias. Similar expression patterns were detected in saliva samples from these patients.

Conclusions: These results show promise for using saliva miRNA signature for monitoring of cancer precursor lesions and early detection of disease progression.

Keywords: Oral leukoplakia, Malignant transformation, Risk assessment, miRNAs, Salivary biomarker

Background

Oral squamous cell carcinomas (OSCCs) are among the most common types of head and neck cancers and are a major cause of significant morbidity. It was reported that 16–62% of OSCCs develop from premalignant lesions [1], which often presents clinically as white or red mucosal patches called leukoplakia and erythroplakia. Early detection of cancer development from oral premalignant lesions (OPLs) plays a crucial role in successful therapy. Currently risk of progression in oral leukoplakia is typically determined based on clinical assessment and histopathological evaluation of biopsied material. High grade dysplasia (HGD) and carcinoma *in situ* (CIS) are considered to have a high risk for progression to invasive disease. In contrast, most of the low grade dysplasias (LGDs) remain unchanged for years or even resolve over time [2]. But a small proportion of these LGDs may progress to carcinomas [3]. Clinical and histological characteristics cannot be used to separate "progressing" and "non-progressing" LGDs [4]. There is therefore an urgent need to find predictive biomarkers that can aid in defining progression likelihood of LGDs, which represent the majority of diagnosed OPLs.

miRNAs are an abundant class of small 18–25 nucleotides long single-stranded non-coding RNA. These non-coding RNAs participate in a variety of biologic processes including development, differentiation, apoptosis and proliferation through regulating its target genes [5-8]. Notably, a single miRNA is capable of regulating the translation of a multitude of genes [9]. And they are remarkably stable both in saliva samples and in tissue samples [10,11], which offers a great advantage over other classes of biomarkers and also an extremely important characteristic in clinical settings. The control of gene expression by miRNAs is a process seen in virtually all cancer cells. Recently, a bundle of studies have showed that miRNAs might behave as cancer 'drivers'

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REVIEW

Incorporating blood-based liquid biopsy information into cancer staging: time for a TNMB system?

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Tissue biopsy is the standard diagnostic procedure for cancer. Biopsy may also provide material for genotyping, which can assist in the diagnosis and selection of targeted therapies but may fall short in cases of inadequate sampling, particularly from highly heterogeneous tumors. Traditional tissue biopsy suffers greater limitations in its prognostic capability over the course of disease, most obviously as an invasive procedure with potential complications, but also with respect to probable tumor clonal evolution and metastasis over time from initial biopsy evaluation. Recent work highlights circulating tumor DNA (ctDNA) present in the blood as a supplemental, or perhaps an alternative, source of DNA to identify the clinically relevant cancer mutational landscape. Indeed, this noninvasive approach may facilitate repeated monitoring of disease progression and treatment response, serving as a means to guide targeted therapies based on detected actionable mutations in patients with advanced or metastatic solid tumors. Notably, ctDNA is heralding a revolution in the range of genomic profiling and molecular mechanisms to be utilized in the battle against cancer. This review will discuss the biology of ctDNA, current methods of detection and potential applications of this information in tumor diagnosis, treatment, and disease prognosis. Conventional classification of tumors to describe cancer stage follow the TNM notation system, heavily weighting local tumor extent (T), lymph node invasion (N), and detectable metastasis (M). With recent advancements in genomics and bioinformatics, it is conceivable that routine analysis of ctDNA from liquid biopsy (B) may make cancer diagnosis, treatment, and prognosis more accurate for individual patients. We put forward the futuristic concept of TNMB tumor classification, opening a new horizon for precision medicine with the hope of creating better outcomes for cancer patients.

Key words: liquid biopsy, noninvasive, circulating tumor DNA, cancer, cancer staging

Introduction

Malignant tumors are highly heterogeneous at multiple levels [1, 2]. Histologically, tumor tissues may exhibit remarkable variation in morphology and cellular composition within different regions of the same tumor, as well as among different tumors from the same primary site [3, 4]. In the case of metastatic cancer, metastases to regional lymph nodes and at distant sites present further divergence [5]. These heterogeneities may not be fully represented in morphology-based pathological classifications from biopsy of the primary tumor site at diagnosis. More recently, genomic analyses along with molecular characterization of cancers have helped reveal the foundation for these differences [6–8]. Complex relationships with the local tumor environment, particularly immune cells, may alter disease progression. Indeed, there is little doubt that cancer displays dynamic evolution

frontiers in Physiology

REVIEW
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Salivary Biomarkers for Oral Squamous Cell Carcinoma Diagnosis and Follow-Up: Current Status and Perspectives

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Oral cancer is the sixth most common cancer type in the world, and 90% of it is represented by oral squamous cell carcinoma (OSCC). Despite progress in preventive and therapeutic strategies, delay in OSCC diagnosis remains one of the major causes of high morbidity and mortality; indeed the majority of OSCC has been lately identified in the advanced clinical stage (i.e., III or IV). Moreover, after primary treatment, recurrences and/or metastases are found in more than half of the patients (80% of cases within the first 2 years) and the 5-year survival rate is still lower than 50%, resulting in a serious issue for public health. Currently, histological investigation represents the "gold standard" of OSCC diagnosis; however, recent studies have evaluated the potential use of non-invasive methods, such as "liquid biopsy," for the detection of diagnostic and prognostic biomarkers in body fluids of oral cancer patients. Saliva is a biofluid containing factors such as cytokines, DNA and RNA molecules, circulating and tissue-derived cells, and extracellular vesicles (EVs) that may be used as biomarkers; their analysis may give us useful information to do early diagnosis of OSCC and improve the prognosis. Therefore, the aim of this review is reporting the most recent data on saliva biomarker detection in saliva liquid biopsy from oral cancer patients, with particular attention to circulating tumor DNA (ctDNA), EVs, and microRNAs (miRNAs). Our results highlight that saliva liquid biopsy has several promising clinical uses in OSCC management; it is painless, accessible, and low cost and represents a very helpful source of diagnostic and prognostic biomarker detection. Even if standardized protocols for isolation, characterization, and evaluation are needed, recent data suggest that saliva may be successfully included in future clinical diagnostic processes, with a considerable impact on early treatment strategies and a favorable outcome.

Keywords: liquid biopsy, salivary biomarkers, circulating tumor DNA, extracellular vesicles, microRNAs, early diagnosis, prognosis, oral squamous cell carcinoma

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Journal of Experimental & Clinical Cancer Research

REVIEW Open Access

The roles of extracellular vesicles in the development, microenvironment, anticancer drug resistance, and therapy of head and neck squamous cell carcinoma

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is one of the main malignant tumours affecting human health, mainly due to delayed diagnosis and high invasiveness. Extracellular vehicles (EVs) are membranous vesicles released by cells into the extracellular matrix that carry important signalling molecules and stably and widely exist in various body fluids, such as plasma, saliva, cerebrospinal fluid, breast milk, urine, semen, lymphatic fluid, synovial fluid, amniotic fluid, and sputum. EVs transport almost all types of bioactive molecules (DNA, mRNAs, microRNAs (miRNAs), proteins, metabolites, and even pharmacological compounds). These "cargoes" can act on recipient cells, reshaping the surrounding microenvironment and altering distant targets, ultimately affecting their biological behaviour. The extensive exploration of EVs has deepened our comprehensive understanding of HNSCC biology. In this review, we not only summarized the effect of HNSCC-derived EVs on the tumour microenvironment but also described the role of microenvironment-derived EVs in HNSCC and discussed how the "mutual dialogue" between the tumour and microenvironment mediates the growth, metastasis, angiogenesis, immune escape, and drug resistance of tumours. Finally, the clinical application of EVS in HNSCC was assessed.

Keywords: Head and neck squamous cell carcinoma, Extracellular vesicles, EXOs, Tumour microenvironment, Drug resistance

Background

HNSCC is the sixth most common cancer worldwide [1]. Approximately 10 % of HNSCC patients are initially diagnosed with metastatic disease, and approximately half of them will relapse even if treated early [2, 3]. The head and neck region includes the oral cavity, larynx, and pharynx, and all structures are covered with squamous epithelium. Therefore, up to 90 % of head and neck tumours are squamous cell carcinomas [4]. Tobacco use, alcohol consumption, human papillomavirus (HPV) infection and some genetic alterations are risk factors in the development of HNSCC [5–7]. Despite many innovations in HNSCC treatment strategies and molecular targeted drugs, the overall 5-year survival rate is still only approximately 60 % [8, 9]. Therefore, the molecular mechanism of tumorigenesis and the screening of accurate biological markers are major challenges and opportunities for further elucidation of HNSCC.

The tumour microenvironment is composed of stromal cells, endothelial cells, immune cells and other complex components. EVs and EXOs (EXOs) are well

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CURRENT OPINION

Liquid Biopsy Applications in the Clinic

Dake Chen[1] · Tao Xu[2] · Shubin Wang[3] · Howard Chan[4] · Tao Yu[4] · Yu Zhu[3] · Jian Chen[5]

Abstract
The global liquid biopsy industry is expected to exceed $US5 billion by 2023. One application of liquid biopsy technology is the diagnosis of disease using biomarkers found in blood, urine, stool, saliva, and other biological samples from patients. These biomarkers could be DNA, RNA, protein, or even a cell. More recently, the use of cell-free DNA from plasma is emerging as an important minimally invasive tool for clinical diagnosis. The development of technology has increased the diversity of its application. Here, we discuss how liquid biopsies have been used in the clinic, and how personalized medicine are likely to use liquid biopsies in the near future.

Key Points

The focus of precision oncology is increasingly turning to liquid biopsy because it is minimally invasive and can be repeated at multiple time points to monitor disease progression.

Liquid biopsy biomarkers in blood include cell-free DNA, cell-free RNA, circulating tumor cells, and exosomes, among others.

Despite the many advantages of liquid biopsy technology in clinical applications, a number of challenges remain, such as the requirement for extremely high sensitivity and accuracy.

Dake Chen and Tao Xu contributed equally to this work.

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1 Liquid Biopsies

Liquid biopsies involve the use of "liquid" samples such as blood, urine, saliva, stool, and other minimally invasive biological samples to determine disease status. According to RNCOS market research, the global liquid biopsy market is expected to exceed $US5 billion by 2023 [1]. Human blood contains cell-free DNA (cfDNA) and RNA (cfRNA), proteins, cells, and exosomes that originate from all tissues, including cancers [2]. Advances in technology have enabled the detection of these biomarkers from human blood samples. This article comments on the role of all these biomarkers, specifically the use of cfDNA in disease diagnosis.

1.1 Circulating Tumor Cells as a Biomarker

Circulating tumor cells (CTCs) are tumor cells in the blood that originate from a solid tumor. The idea that tumor cells were secreted into the blood was first hypothesized by Ashworth in 1869 [3], but the clinical application of CTCs was not appreciated until much later [4]. The primary barrier to using them is that there can be as few as one CTC per 1×10^9 blood cells in patients with metastatic cancer [5]. CTCs are considered to be the main source of metastases [6]. Evidence showed that their number in the blood could be used as a prognostic biomarker, since levels correlated with reduced progression-free and overall survival [6, 7]. However, since CTC numbers vary according to tumor type, their clinical utility is complicated. In addition, CTCs can be difficult to isolate because of their various properties, including size, clustering capability, and varying cell surface markers [8]. As of 2018, the only US FDA-approved CTC quantification platform is the CELLSEARCH® CTC

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Review

Clinical Cancer Research

Emerging Role of MicroRNAs as Liquid Biopsy Biomarkers in Gastrointestinal Cancers

Kunitoshi Shigeyasu, Shusuke Toden, Timothy J. Zumwalt, Yoshinaga Okugawa, and Ajay Goel

Abstract

Cancer has emerged as a leading cause of mortality worldwide, claiming more than 8 million lives annually. Gastrointestinal cancers account for about 35% of these mortalities. Recent advances in diagnostic and treatment strategies have reduced mortality among patients with gastrointestinal cancer, yet a significant number of patients still develop late-stage cancer, where treatment options are inadequate. Emerging interests in "liquid biopsies" have encouraged investigators to identify and develop clinically relevant noninvasive genomic and epigenomic signatures that can be exploited as biomarkers capable of detecting premalignant and early-stage cancers. In this context, microRNAs (miRNA), which are small, noncoding RNAs that are frequently dysregulated in cancers, have emerged as promising entities for such diagnostic purposes. Even though the future looks promising, current approaches for detecting miRNAs in blood and other biofluids remain inadequate. This review summarizes existing efforts to exploit circulating miRNAs as cancer biomarkers and evaluates their potential and challenges as liquid biopsy–based biomarkers for gastrointestinal cancers. *Clin Cancer Res; 23(10); 2391–9. ©2017 AACR.*

Introduction

Gastrointestinal cancers occur primarily in the liver, stomach, colorectum, esophagus, and pancreas and account for about 35% of global cancer-related mortalities (1). Recent advances in surgical and endoscopic procedures have significantly improved the survival of patients with early-stage disease. However, the inherently low frequency of some of these cancers, the invasive nature of screening procedures, and the high costs associated with such modalities have resulted in poor compliance for the current generation of screening assays. Although noninvasive screening tests such as fecal immunochemical tests (FIT) are available for screening patients with colorectal cancer, their efficacy remains limited because of low sensitivity and specificity (2) and inability to detect other types of cancers within the gastrointestinal tract. Consequently, inadequate screening modalities for patients with gastrointestinal cancers highlight the imperative need for further research on this important clinically relevant issue.

Within the context of cancer, particularly gastrointestinal malignancies, epigenetic alterations, together with genetic events, have emerged as key drivers of disease development and progression (3). The term "epigenetic" broadly encompasses all heritable changes in gene expression that do not involve a permanent change in the DNA sequence. In cancer, the most well-investigated epigenetic alterations include aberrant DNA methylation, histone modifications, and dysregulated expression of noncoding RNAs (ncRNA; ref. 4). Epigenetic alterations manifest far more frequently than genetic mutations and often appear in early stages of tumorigenesis (5). These alterations are dynamic in nature and potentially reversible, and, hence, have shown promise as attractive substrates for developing disease biomarkers and serve as therapeutic targets in human cancers (5). To date, miRNAs remain the most studied epigenetic alteration in circulation, both as diagnostic and as prognostic cancer biomarkers. In contrast, DNA methylation has been preferentially assessed in tissues, primarily due to the limitation that significant volume of serum/plasma is needed to obtain adequate amounts of DNA for methylation analysis. Furthermore, the assessment of posttranslational histone modifications in the serum is quite limited. Over the last decade, several important studies have evaluated the potential of miRNAs as "liquid biopsy" biomarkers, and, therefore, now is perhaps the appropriate time to objectively assess their true potential as cancer biomarkers.

Among ncRNAs, dysregulated expression of miRNAs has been most widely studied over the last decade, and they appear to be promising diagnostic biomarkers for a variety of human cancers, including gastrointestinal malignancies (6). A large number of these small ncRNAs have been quite well characterized for their biological function in cancer and their ability to regulate the expression of protein-coding genes. From a clinical standpoint, dysregulated expression of miRNAs has been readily detected in a variety of biological fluids in patients with cancer, highlighting the stability of miRNAs in these biofluids and providing a rationale for developing them as liquid biopsy biomarkers. This review summarizes current efforts for implementing specific

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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frontiers in Oncology

REVIEW
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A Literature Review of the Potential Diagnostic Biomarkers of Head and Neck Neoplasms

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Head and neck neoplasms have a poor prognosis because of their late diagnosis. Finding a biomarker to detect these tumors in an early phase could improve the prognosis and survival rate. This literature review provides an overview of biomarkers, covering the different -omics fields to diagnose head and neck neoplasms in the early phase. To date, not a single biomarker, nor a panel of biomarkers for the detection of head and neck tumors has been detected with clinical applicability. Limitations for the clinical implementation of the investigated biomarkers are mainly the heterogeneity of the study groups (e.g., small population in which the biomarker was tested, and/or only including high-risk populations) and a low sensitivity and/or specificity of the biomarkers under study. Further research on biomarkers to diagnose head and neck neoplasms in an early stage, is therefore needed.

Keywords: head and neck neoplasms, biomarker, genomics, proteomics, metabolomics, volatomics, microbiomics, radiomics

INTRODUCTION

Head and neck cancers account for 5% of all malignant tumors and are responsible for about 600,000 new cases and 300,000 deaths in the world annually. About 50% of the patients fail to achieve cure and cancer relapse occurs despite intensive combined treatment (1, 2). To date, there is no adequate biomarker available for the diagnosis of head and neck cancer. However, it is expected that an earlier detection could improve the patient's outcome stage (3–7). In this review, we provide a general overview of biomarkers that were investigated to diagnose head and neck neoplasms in an early phase. Besides, we go into detail on the restrictions of these candidate biomarkers in the clinical practice.

Head and neck neoplasms are defined as benign, premalignant and malignant tumors above the clavicles, with exception of tumors of the brain, and spinal cord and esophagus (2). This includes tumors of the paranasal sinus, the nasal cavity, the

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BASIC SCIENCE REVIEW

WILEY

Circulating tumor DNA as a biomarker and liquid biopsy in head and neck squamous cell carcinoma

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Abstract

The use of circulating biochemical molecular markers in head and neck cancer holds the promise of improved diagnostics, treatment planning, and posttreatment surveillance. In this review, we provide an introduction for the head and neck surgeon of the basic science, current evidence, and future applications of circulating tumor DNA (ctDNA) as a biomarker and liquid biopsy to detect tumor genetic heterogeneity in patients with head and neck squamous cell carcinoma (HNSCC).

KEYWORDS
biomarker, circulating tumor DNA (ctDNA), head and neck cancer, liquid biopsy

1 | INTRODUCTION

Head and neck cancer continues to carry a significant global burden of disease.[1,2] Of concern is the increasing incidence and mortality of head and neck cancer, with a greater rise in developing countries and a contrasting increase of oropharyngeal cancer in developed countries.[1,2] Although several

Paul Nankivell and Hisham Mehanna contributed equally to this study as senior authors.

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Review

Clinical Cancer Research

Circulating Tumor Cells and Circulating Tumor DNA: Challenges and Opportunities on the Path to Clinical Utility

Michail Ignatiadis[1], Mark Lee[2], and Stefanie S. Jeffrey[3]

Abstract

Recent technological advances have enabled the detection and detailed characterization of circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) in blood samples from patients with cancer. Often referred to as a "liquid biopsy," CTCs and ctDNA are expected to provide real-time monitoring of tumor evolution and therapeutic efficacy, with the potential for improved cancer diagnosis and treatment. In this review, we focus on these opportunities as well as the challenges that should be addressed so that these tools may eventually be implemented into routine clinical care. *Clin Cancer Res; 21(21); 4786–800. ©2015 AACR.*

Disclosure of Potential Conflicts of Interest

M. Ignatiadis is the principal investigator for the Treat CTC trial, which is supported by grants from Janssen Diagnostics and Roche. M. Lee was an employee of Boreal Genomics and Genomic Health. S.S. Jeffrey is an inventor of intellectual property related to the MagSweeper device for rare cell capture that is owned by Stanford University and licensed to Illumina. The Jeffrey Laboratory has a research collaboration with Vortex BioSciences to help optimize and validate specific applications for Vortex technology that is administered by Stanford University. No other potential conflicts of interest were disclosed.

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Learning Objectives

Upon completion of this activity, the participant should have a better understanding of the technologies used to detect and characterize circulating tumor cells (CTC) and circulating tumor DNA (ctDNA), different strategies of testing their clinical utility, and the potential future applications of CTCs and ctDNA in the field of precision medicine.

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Introduction

Next-generation sequencing (NGS) studies performed in bulk primary tumor specimens have demonstrated extensive interpatient (1) and, more importantly, intrapatient (2) heterogeneity. Recently, single-cell analyses of primary breast tumors have provided higher-resolution evidence of intratumor heterogeneity (3) with the finding of substantial clonal diversity and subclonal heterogeneity, such that no two individual tumor cells are genetically identical. Beyond spatial heterogeneity, solid tumors also exhibit temporal heterogeneity, evolving over time under selection pressure from treatment (4, 5). Thus, there is an increased appreciation that the management of metastatic disease should rely on analysis of contemporary tumor tissue rather than on the primary tumor diagnosed years ago (6). However, obtaining serial samples of metastatic tissue is impractical and complicated by spatial heterogeneity and sampling bias. Analysis of circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) thus holds appeal and promise for noninvasive real-time assessment of tumor molecular profiles during the course of disease. Evaluation of CTCs and ctDNA may enable more sensitive monitoring of treatment efficacy and thereby guide drug selection, even potentially in the adjuvant setting where no such tools exist today.

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Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids

Exosomes are small vesicles secreted by most cell types in culture. Exosomes form intracellularly by inward budding of the limiting membrane of endocytic compartments, leading to vesicle-containing endosomes, called multivesicular bodies (MVBs). MVBs eventually fuse with the plasma membrane, thus releasing their internal vesicles (i.e., exosomes) into the extracellular medium. What, then, may the physiological function of exosomes be? On one hand, exosome secretion could be a function per se, e.g., exosome secretion by reticulocytes allows the elimination of proteins such as transferrin receptor or integrins, which are useless in differentiated red blood cells (Pan et al., 1985; Vidal et al., 1997). On the other hand, exosomes could be involved in intercellular communication, allowing exchange of proteins and lipids between the exosome-producing cells and target cells. Such a function has been exemplified in the immune system where exosomes allow exchange of antigen or major histocompatibility complex (MHC)-peptide complexes between antigen-bearing cells and antigen-presenting cells (e.g., dendritic cells; Wolfers et al., 2001; Andre et al., 2002, 2004; Théry et al., 2002). Nevertheless, the physiological functions of exosomes remain a matter of debate.

The purpose of this unit is to give simple and reliable methods for purifying and characterizing exosomes. Cell culture supernatants (conditioned media; CM) contain several types of shed membrane fragments and vesicles; therefore, before performing any functional analysis, it is critical to ensure that the purified vesicles are exosomes and not other contaminating material. The first part of this unit describes the most common protocols used to purify exosomes from cell culture conditioned media or from physiological fluids, and the second part describes different methods for characterizing and assessing the purity of the isolated exosomes.

Exosomes have been successfully purified from cell culture conditioned medium or bodily fluids. Support Protocols 1 and 2 provide all details and precautions to take in collecting materials from which exosomes will be purified. Starting from this material, the original and most commonly used protocol for exosome purification (Raposo et al., 1996) is described in Basic Protocol 1. It involves several centrifugation and ultracentrifugation steps. In some cases, the first centrifugation steps can be replaced by a single filtration step: this option is described in an Alternate Protocol. A slightly modified version of Basic Protocol 1, designed for purifying exosomes from viscous fluids (e.g., plasma) is described in Basic Protocol 2. An extra purification step that provides extremely pure exosomes can be added to these protocols, and is described in Support Protocol 3. A different purification procedure, involving trapping exosomes on beads bearing an antibody specific for exosomal surface molecules, has more recently been described (Clayton et al., 2001) and is provided in Basic Protocol 3. It is easy to use and useful for rough characterization of exosomes, but it is not intended for purification of large amounts of exosomes. An additional new method for purifying exosomes by ultrafiltration instead of ultracentrifugation is not described in this unit. This method employs ultrafiltration cartridges and pumps and is especially useful for purifying exosomes from large volumes (>1 liter) of conditioned medium. It is suitable for clinical applications of purified exosomes, but it is not the easiest option for laboratory applications. Interested readers should see Lamparski et al. (2002) for details.

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ABSTRACT

ExoQuick-TC™ (EQ), a chemically-based agent designed to precipitate exosomes, was calibrated for use on saliva collected from healthy individuals. The morphological and molecular features of the precipitations were compared to those of the classical physically-based method - ultracentrifugation (UC). Electron microscopy and immune-electron microscopy with anti-CD63 showed vesicular nanoparticles surrounded by bi-layered membrane, compatible with exosomes, in EQ, similarly to UC. Atomic force microscopy highlighted larger, irregularly-shaped/aggregated EQ nanoparticles that contrasted the single round-shaped UC nanoparticles. ELISA (performed on 0.5 ml saliva) revealed a tendency for a higher expression of the specific exosomal markers (CD63, CD9, CD81) in EQ than UC (p>0.05). ELISA for epithelial growth factor receptor, a non-exosomal-related marker, showed a significantly higher concentration in EQ than UC (p=0.04). Western blotting of equal total-protein concentration revealed bands of CD63, CD9 and CD81 in both types of preparations, although they were less pronounced in EQ compared to UC. This may be related to a higher fraction of non-exosomal proteins in EQ compared to UC. In conclusion, EQ is suitable and efficient for precipitation of salivary exosomes from small volumes of saliva however it tends to be associated with considerably more biological impurities, (i.e., non-exosomal-related proteins/microvesicles) compared with UC.

Keywords

saliva, extracellular vesicles, exosomes, ultracentrifugation, ExoQuick, isolation

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RNA in Salivary Extracellular Vesicles as a Possible Tool for Systemic Disease Diagnosis

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Abstract

Saliva contains biological information as blood and is recognized as a valuable diagnostic medium for their noninvasiveness. Although "-omics" researches have tried to investigate saliva, the origin and significance of its contents are not clear, and its usage is largely confined to oral disease in the diagnostic and prognostic field. In an attempt to broaden the applicability of saliva and to find systemic disease-derived RNA in saliva, we made mouse models that had human melanoma and isolated extracellular vesicles (EVs) from their saliva by an aqueous 2-phase system (ATPS), then identified and evaluated their expression of human melan-A RNA, which is associated with melanoma on skin. With ATPS, EVs were isolated efficiently and stably while taking less time compared to isolation by ultracentrifugation. When ATPS was used to isolate EVs from saliva, the mean ± SD percentage of EVs recovered from initial EVs was 38.22% ± 18.55% by the number of particles, and the mean ± SD percentage of RNA recovered from the initial amount was 60.33% ± 5.34%. RNAs within isolated EVs were analyzed subsequently by reverse transcription quantitative polymerase chain reaction and polymerase chain reaction from saliva and plasma. In melanoma mice, amplification of human melan-A was identified from saliva and plasma, even though a relative amount of normalized melan-A was lower than that of plasma. These results present a possibility that RNAs derived from systemic disease are transferred into saliva from blood in EVs. Also, they suggest that saliva could be exploited in obtaining information about systemic disease, not only about oral disease, by examining RNAs in EVs from saliva instead of blood.

Keywords: saliva, salivary diagnostics, biomarkers, mRNA, cell-derived microparticles, exosomes

Introduction

Saliva bathes oral structures, such as teeth, gingiva, and oral mucosa. The components of saliva are mainly produced by salivary glands, but gingival crevicular fluid and oropharyngeal mucosae also contribute to its composition (Proctor 2016). Systemic circulation also affects saliva by leakage from capillaries that surround salivary glands and the gingival sulcus (Haeckel and Hänecke 1996).

Molecules from blood can be transported into saliva by ultrafiltration, transudation, or selective transport depending on their physicochemical character, size, and presence of transporters (Chiappin et al. 2007). Based on their characteristics, molecules are carried into saliva by different mechanisms and are differently represented in saliva. For example, lipophilic molecules such as steroid hormones cross over plasma membranes by diffusion, and the active level of the hormone is well represented in saliva (Kumar et al. 2005).

However, RNAs of systemic origin have not been proven for their existence in saliva. Since RNAs have been found in body fluids despite the presence of nucleases, it is considered that extracellular RNAs exist in saliva by binding to ribonucleoproteins or being protected in extracellular vesicles (EVs) (Zhou et al. 2008; Tzimagiorgis et al. 2011; Redzic et al. 2014). Notably, EVs have been proposed to cross over an epithelial barrier such as blood-brain barrier by transcytosis (Schneider and Simons 2013; Yang et al. 2015), so they may play a role in transporting RNAs of systemic origin from blood into saliva.

Here we examine saliva to try to identify RNA released by a systemic disease. We do this by isolating EVs and analyzing RNAs from EVs in saliva and plasma. We make mouse models with human melanoma, then isolate EVs from their saliva by an aqueous 2-phase system (ATPS) (Sherbet and Lakshmi 1981; Asenjo and Andrews 2012). When ATPS was composed of polyethylene glycol (PEG) and dextran (DEX), most EVs moved into the DEX phase after centrifugation (Kim et al. 2015; Shin et al. 2015; Park et al. 2016). Also, EVs were recovered from cell-free saliva more easily and efficiently than by conventional isolation methods such as ultracentrifugation (U/C) (Thery et al. 2006).

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A supplemental appendix to this article is available online.

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Mini-Reviews

Circulating MicroRNA Biomarker Studies: Pitfalls and Potential Solutions

Kenneth W. Witwer[1*]

BACKGROUND: Circulating microRNAs have been proposed as disease biomarkers that may aid in risk assessment, diagnosis, prognosis, and monitoring of treatment response. The perceived opportunity has loomed particularly large in neoplastic disease, where alterations in cancer cells are thought to be reflected in the extracellular space as affected cells release upregulated miRNAs or fail to release apparently downregulated species. Despite the promise of miRNA biomarkers, evaluation of the diagnostic specificity and reproducibility of reported markers suggests that realizing this promise remains a work in progress.

CONTENTS: This review examines issues of diagnostic specificity and reproducibility that have afflicted circulating miRNA studies. Surveying the breast cancer literature as an example, few miRNAs are reported consistently. Furthermore, it is posited that the assumptions underlying models of direct contributions of diseased tissue to biofluid miRNA profiles may not hold. Suggestions for improving diagnostic specificity and reliability are provided.

SUMMARY: To maximize the likelihood of return on investment as miRNAs continue to be evaluated as specific and clinically useful markers, a focus is needed on miRNAs found in specific carriers, such as extracellular vesicles. Alternative sampling techniques should be developed, and nonblood biofluids should be considered. Careful optimization and standardization of preanalytical and analytical methods is needed to ensure that future results, positive or negative, are reliable.

MicroRNAs (miRNAs)[2] were found in 2007 among the cellular RNAs exported into the extracellular space in vesicles *(1)*, which were also reported to deliver RNA cargo to recipient cells. This compelling in vitro finding was quickly followed in 2008 by a report that extracellular RNA was detectable in bodily fluids and potentially useful as a biomarker. Chim et al. described in *Clinical Chemistry* that placental miRNAs circulated in protected fashion in the maternal blood *(2)*. Highly stable, these miRNAs were advanced as potential tools for pregnancy monitoring.

Additional confirmation of circulating miRNAs was provided over the course of 2008 by independent laboratories. Although other circulating nucleic acids as well as miRNAs within cells and tissues had been under investigation for some time, these reports gave the first indications that circulating miRNAs might serve as cancer biomarkers. Lawrie et al. found increased miRs-21, -155, and -210 in sera of diffuse large B-cell lymphoma patients *(3)*. Tewari's group published an association of increased serum miR-141 with prostate cancer *(4)*. A subsequent publication by Chen et al. measured increased miR-25 and miR-223 in serum of lung cancer cases *(5)*. The positive reception of these and later reports was accompanied by enthusiasm that the "liquid biopsy" might obviate the need for more invasive testing and also allow early warning of oncogenesis.

miRNA Biology and Biomarker Suitability

miRNAs are short RNA molecules. The average mature miRNA is 21 or 22 nucleotides in length and is processed from a hairpin precursor. When incorporated into the RNA-induced silencing complex, a miRNA may bind to target sequences in other RNA molecules with some degree of complementarity (for a comprehensive review of miRNAs and their function, see *(6)*). mRNAs that are targeted by miRNAs—and their protein products—are often modestly downregulated. Several thousand human miRNAs have been reported or predicted. Only a small fraction of these miRNAs are sufficiently abundant in any given cell type to exert posttranslational regulation. For example, a single miRNA, miR-122, comprises the majority of miRNA copies in the hepatocyte.

The twin bases for the attractiveness of extracellular miRNA as biomarkers are stability and dysregulation in the diseased cell. While bound to Argonaute proteins, miRNAs are stable in the extracellular environment after release from cells, whether as unprotected ribonucleopro-

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[2] Nonstandard abbreviations: miRNA, microRNA; BC, breast cancer; qPCR, quantitative PCR; EV, extracellular vesicle; CNS, central nervous system; EpCAM, epithelial cell adhesion molecule.

Method

Open Access

A novel and universal method for microRNA RT-qPCR data normalization

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Abstract

Gene expression analysis of microRNA molecules is becoming increasingly important. In this study we assess the use of the mean expression value of all expressed microRNAs in a given sample as a normalization factor for microRNA real-time quantitative PCR data and compare its performance to the currently adopted approach. We demonstrate that the mean expression value outperforms the current normalization strategy in terms of better reduction of technical variation and more accurate appreciation of biological changes.

Background

MicroRNAs (miRNAs) are an important class of gene regulators, acting on several aspects of cellular function such as differentiation, cell cycle control and stemness. Not surprisingly, deregulated miRNA expression has been implicated in a wide variety of diseases, including cancer [1]. Moreover, miRNA expression profiling of different tumor entities resulted in the identification of miRNA signatures correlating with patient diagnosis, prognosis and response to treatment [2]. Despite the small size of miRNA molecules, several technologies have been developed that enable high-throughput and sensitive miRNA profiling, such as microarrays [3-8], real-time quantitative PCR (RT-qPCR) [9,10] and bead-based flow cytometry [2]. In terms of accuracy and specificity, RT-qPCR has become the method of choice for measuring gene expression levels, both for coding and non-coding RNAs. However, the accuracy of the results is largely dependent on proper data normalization. As numerous variables inherent to an RT-qPCR experiment need to be controlled for in order to differentiate experimentally induced variation from true biological changes, the use of multiple reference genes is generally accepted as the gold standard for RT-qPCR data normalization [11]. Typically, a set of candidate reference genes is evaluated in a pilot experiment with representative samples from the experimental condition(s). Ideally these candidate reference genes belong to different functional classes, significantly reducing the possibility of confounding co-regulation. In case of miRNA profiling, only few candidate reference miRNAs have been reported [12]. Generally, other small non-coding RNAs are used for normalization. These include both small nuclear RNAs (for example, U6) and small nucleolar RNAs (for example, U24, U26).

Strategies for normalization of high-dimensional expression profiling experiments (using, for example, microarray technology, but recently also transcriptome sequencing) generally take advantage of the huge amount of data generated and often use (almost) all available data points. These strategies range from a straightforward approach based on the mean or median expression value to more complex algorithms such as

Method

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Review

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microRNAs: Emerging players in oral cancers and inflammatory disorders

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Abstract

Association of oral diseases and disorders with altered microRNA profiles is firmly recognized. These evidences support the potential use of microRNAs as therapeutic tools for diagnosis, prognosis, and treatment of various diseases. In this review, we highlight the association of altered microRNA signatures in oral cancers and oral inflammatory diseases. Advances in our ability to detect microRNAs in human sera and saliva further highlight their clinical value as potential biomarkers. We have discussed key mechanisms underlying microRNA dysregulation in pathological conditions. The use of microRNAs in diagnostics and their potential therapeutic value in the treatment of oral diseases are reviewed.

Keywords

MicroRNA, oral cancer, inflammatory disorders, oral pathology

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Introduction

It has been recognized that oral health and genetics are closely interlinked.[1] Studying the multitude of disorders affecting craniofacial tissues can provide scientists with insight on the role of inflammation in infection and pain, the consequences of depressed immunity, and the changes that can arise from a mutated gene.[2] Recently, there has been a focus on regulation of these inflammatory and cancer pathways by microRNA (miRNA) in innate and adaptive immunity.[3–6] In humans, miRNA expression has been shown to affect the pathobiology of oral diseases such as oral squamous cell carcinoma (OSCC),[7] Sjögren's syndrome (SS),[8] and periodontitis.[9] Recent studies have highlighted the differences in miRNA expression patterns between healthy and inflamed periodontal tissues, which suggest the possible involvement of miRNAs in the regulation of periodontal disease.[10,11] This review highlights a largely obscure and unexplored zone: expression of miRNAs in the hosts' immuno-inflammatory responses and their potential role in the pathophysiology of oral cancer and other oral diseases. Emerging lines of evidence indicate a correlation between oral inflammation and cancer progression. Therefore, identification of biomolecules that can serve as biomarkers for the diagnosis/prognosis of these oral diseases can be of high therapeutic value. Given the capability of miRNA to simultaneously modulate expression of hundreds of target genes, an in-depth study of the role of miRNAs in oral disease can facilitate the understanding of the immune system and efforts taken to achieve a state of homeostasis. We will discuss the probable mechanisms of altered miRNA expression leading to pathology. Finally, the therapeutic potential of miRNAs will be highlighted.

MicroRNA biogenesis

MiRNAs are small, non-coding, single-stranded, ~22-base nucleotide sequences, which bind approximately 60% of all genes.[12,13] It has been suggested that the human genome may encode over 2500 miRNAs, which have emerged as

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