Review

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Oesophageal squamous cell carcinoma (ESCC): Advances through omics technologies, towards ESCC salivaomics

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Summary **Oesophageal Squamous Cell Carcinoma (ESCC) is one of the two main subtypes of oesophageal cancer, affecting mainly populations in Asia. Though there have been great efforts to develop methods for a better prognosis, there is still a limitation in the staging of this affection. As a result, ESCC is detected at advances stages, when the interventions on the patient do not have such a positive outcome, leading in many cases to recurrence and to a very low 5-year survival rate, causing high mortality. A way to decrease the number of deaths is the use of biomarkers that can trace the advance of the disease at early stages, when surgical or chemotherapeutic methodologies would have a greater effect on the evolution of the subject. The new high throughput omics technologies offer an unprecedented chance to screen for thousands of molecules at the same time, from which a new set of biomarkers could be developed. One of the most convenient types of samples is saliva, an accessible body fluid that has the advantage of being non-invasive for the patient, being easy to store or to process. This review will focus on the current status of the new omics technologies regarding salivaomics in ESCC, or when not evaluated yet, the achievements in related diseases.**

Keywords: Oesophageal squamous cell carcinoma, saliva, salivaomics, transcriptomics, proteomics, metabolomics

1. Introduction

Oesophageal Cancer (EC) has two main subtypes with different pathological features, Adenocarcinoma (EAC) and ESCC (*1,2*), representing between them more than 90% of the detected cases (*3*). There is also a different trend in the geographical distribution for both EC subtypes, being that of EAC in the Western world (*4*), while ESCC is especially present in Asia, for example in China (*5-7*), Iran (*8,9*), Japan (*10*), or Kazakhstan (*11*). In this last country, ESCC is the 6th major type of diagnosed cancer, with high mortality rates among women (9th place) and men (5th place) (*11*). Early detection of ESCC and subsequent treatment would be crucial in order to decrease mortality (*12*), but as indicated by several authors (*4,13-15*) lack of early stage diagnostic tools is one of the biggest problems in ESCC diagnostics, especially because ESCC is manifested as asymptomatic lesions at first stages. Some of the current techniques used for diagnosis are non-invasive imaging methods, as well as more conventional ones that include computed tomography (CT) scan, or endoscopic ultrasound (EUS). Some of the difficulties that these techniques face are in the case of EUS the limitation that tumour enlargement pose for the passage of endoscope in advanced cases, or for

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CT the lower sensitivity displayed in comparison to a combination of PET (Positron Emission Tomography) and the use of 2-deoxy-2-(^{18}F)fluoro-D-glucose (^{18}F-FDG) (*16*). As previously indicated by Takeshita (*15*) there have been advances in the use of several techniques as those mentioned above, but still a late detection happens at very advanced stages (*17,18*), when surgical interventions are not effective and lead to recurrence and low survival rates (*19*).

Besides, an additional problem for ESCC is the multifactorial nature of its occurrence (*20*), and the influence that different habits would have over the risk of developing this disease, as it has been associated with heavy smoking, drinking, or low intake of vegetables or fruits (*21-23*). The increased risk factor in this case as indicated by Cheng and Day (*22*) may come as a consequence from a direct contact of the potential carcinogen with the epithelium, some existing transport facilitating mechanism, or derived from compounds that increase the cell turnover in the epithelial cells. Albeit yet a controversial topic, there have been also studies trying to correlate the occurrence of HPV infections and ESCC, though results showed an elevated degree of variation that did not yield a clear association between the viral infection and the development of the disease (*24-26*).

A molecular feature that could be associated to the development of this disease would serve as an indicator for a more effective treatment. In this scenario, the discovery of biomarkers would become a great advantage for clinicians allowing an early diagnosis of ESCC, as it has been for many other diseases, *e.g.* level of serum creatinine as indicator of renal function. The term biomarker (biological marker) can be defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention", definition that was proposed at the 1998 National Institutes of Health Biomarkers Definitions Working Group (*27,28*). Biomarkers can aid in diagnosis, and an efficient way to discover them is the use of available omics technologies. Transcriptomics, Proteomics, or Metabolomics (TPM) are relatively new high throughput techniques that allow performing massive screenings of different molecules. Each of them refer to the complete set of transcripts/proteins/metabolites (respectively) and their quantity, for a given cell with a given genotype, under certain environmental conditions (including developmental or physiological stage, as well as being under influence of bioregulators) (*29-32*). After this definition, it comes as a logic consequence that physiological changes that accompany the development of a disease in humans lead to changes in the TPM profile, that can be observed and measured in a tissue, or indirectly measured/observed in human fluids, *e.g.* urine, sweat, saliva. Currently due to the development of omics technologies, these profile changes can be detected and analysed (*29-32*). The advantage of using TMP "expression" biomarkers is that they are closely related to the disease, as they are usually by-products of its development. Metabolites are for example end-points of all the system, being the final products of metabolic pathways (*33*) and a reflection of the phenotype (*34*). Therefore, this approach would yield biomarkers that are potentially specific for the studied disease, and have a potentially direct application (*35*). The use of a combination of this new technologies (*36*), can largely contribute in the understanding of each disease, their evolution, and molecular mechanisms.

A very important point in studies concerning cancer or other diseases, is the validation of the model through testing in an independent set of samples (*36*). This would be, for example, a set of individuals with the same features as the control (healthy), and another set of independent affected ones, which were not used in the original screening. It will mean that the found biomarkers have the power to potentially predict or be associated with the disease in any given set of samples that fulfil the disease conditions. Otherwise, this would be (the necessary) technical validation of the study. Biomarkers that are validated in this way can be promising targets for further clinical testing assays.

Considering the final aim of developing biomarkers to routinely use them for ESCC testing in clinical settings, one of the most accessible and interesting types of human samples is saliva (*35*). It has numerous advantages, being easily accessible for clinicians, and non-invasive for patients (*37-39*). Compared to imaging techniques, saliva collection is methodologically less demanding than a ^{18}F-FDG PET assay (*16*), allowing to scale up the number of patients to sample. Compared with other identification techniques as EUS, its advantage is that saliva collection avoids the anxiety that an endoscopy may cause to the patient, or the potential disturbances afterwards. Other interesting features of saliva in comparison to blood, is that saliva does not need dedicated precautions for storage or biosecurity, besides it does not clot (*14*). Thus, its handling is very convenient for any hospital facility worldwide, especially in depressed or impoverished areas.

Saliva is a human fluid that is originated mostly in three salivary glands (*parotis*, *submandibularis* and *sublingualis*), as well as a number of minor glands, and the fluid from gingival crevice (*37,40*). It is a complex mixture of exudates derived from mucosa, plasma, microflora (or what we will refer as oral metabiome/microbiome), epithelial cells, small metabolites and different transcripts, among other minor compounds (*41-44*). This complexity must be considered as a very important feature for its study. Regarding the protein content, some studies reported that approximately 20%-30% of the proteins that can be found in blood plasma are present in saliva (*45,46*). According to

Castagnola *et al.*, most of the proteins (more than 90% of representation) are not from gland secretory origin, but common to other body fluids or tissues. However, despite this abundance, common proteins account only for 15% w/w of the salivary proteome, something that has to be considered as a potential drawback for biomarker discovery studies (*41*). An aspect of saliva that cannot be forgotten, of importance because it can affect the results, is the human oral microbiome (*47,48*). It can be defined as the population of different bacterial species that inhabit the oral cavity, while they form part of the whole human microbiota. Taking in account all of the above mentioned features from saliva, there is room for discovery of different biomarkers associated to the development of ESCC.

In conditions of disease, human saliva can reflect the physiological state as occurs in blood (*49,50*). This has been demonstrated in the work of Asatsuma *et al*. (*51*) (Table 1), in which they found significant differences in a protein between the healthy patients compared with the ones affected by primary Sjögren's syndrome (pSS). Nevertheless, this disease affects salivary and lachrymal glands (*52*), and it could be thought that these type of alterations are easier to trace in saliva than other conditions, for which could not be possible the discovery of useful biomarkers. Then it is important to address here the important findings in a disease that is not directly related with the oral cavity, Breast Cancer (BC), where saliva was the sample of choice. A successful example is the study of Zhang and collaborators (*53*) (Table 1), where they discovered and validated eight mRNA biomarkers and a protein biomarker, having a 92% accuracy in the tested sample set.

It is then clear that saliva diagnostics is a powerful tool and at the same time a promising biofluid. The current status regarding ESCC salivaomics will be reviewed when available in the following sections for the three main current high throughput technologies: transcriptomics, proteomics, and metabolomics. For clarity, the most promising results in salivary biomarker discovery have been briefly resumed in Table 1.

2. Transcriptomics

Transcriptomics studies and quantifies the set of RNA molecules produced by the genome as a result of the environmental influences, or the developmental stage. A major question that has to be addressed is the stability of RNA in saliva. It is a common presumption that RNA cannot be stable in saliva (*54*) due to its labile nature and the presence of RNases in the oral cavity (*55*). A further complication for cancer studies is the reported higher activity of RNases in gastric cancer patients (*56*), leading to a higher degradation of total RNA in the oral cavity of affected subjects. If that described situation applies for other types of cancer, being ESCC of our interest, chances to find intact RNA are lower and thus the capability to discovery useful potential biomarkers will decrease vastly. There have been successful studies focusing on saliva as the sample material for oral squamous cell carcinoma (OSCC), for example the work of Li and collaborators (*55*) (Table 1), where they found more than 1,600 differentially expressed genes. However some other studies (*54*) have described that the observed signalling molecule was in fact DNA, not RNA, what complicates the analysis. Notwithstanding, Li and colleagues were the first ones to observe more than 3,000 different RNA transcripts in human saliva (*44*) (Table 1), according to what they report in a more recent study from their laboratory, in which Park and colleagues (*57*) (Table 1) further characterized the stability of RNA in saliva. This latter study tested whether informative RNA molecules exist in saliva or not through different molecular biology assays. As reported (*57*) , until 2004 most of the detected RNA had a viral or bacterial origin. In this study Park and colleagues (*57*) used a 22,283 cDNA probes microarray (Agilent Affymetrix Human Genome U133A) to test the complexity of several oral saliva samples in terms of number of distinct transcripts that could be detected, having found more than 6,000 in the whole saliva. Regarding stability, one of the possibilities that they indicate for the stability of RNA in saliva is the association with mucin, protein that would protect from degradation, as well as other type of macromolecules. Are there additional sources of cell free circulating RNA in saliva? Amidst the possible origins some authors have indicated apoptotic processes (*58*), while other studies report the presence of exosomes (*59,60*). In keeping with this last possibility, Ogawa and collaborators (*59*) (Table 1) found for the first time the presence of exosomal vesicles in whole saliva samples from humans.

For efficient biomarker discovery, the release of RNA from the apoptotic cells has to occur in a quantity that allows an early identification and efficient tracking of the disease. The appearance of RNAs in latter stages of the disease will not have such a clinical value for ESCC, as there are other available techniques that were mentioned in the previous section, and because of the current critical need for early markers. In that way, the presence of exosomes and their nature as a communication mean between distant cell types (*61-64*), can be a key point to exploit biomarker discovery. That would be of a great interest in the case of ESCC, as the chances for detecting apoptotic derived RNA may not be so big during early stages than in advanced ones, but still tumorigenic cells may be starting to derive RNA containing exosomes for communication while the lesion is not yet detectable.

More interesting developments related with saliva that focused on studying BC and their potential salivary biomarkers, were achieved by Zhang and collaborators

Table 1. Most promising advances regarding biomarker discovery in saliva

Methodology	Model of study	Discovery	Importance	*Ref.*
ELISA; sandwich EIA	pSS	Significantly increased levels of MMP-9/TIMP-1 and MMP-9 in pSS patients	Use of saliva to study differences between affected patients and healthy ones	Asatsuma *et al*. (*51*)
Affymetrix Human Genome U133A Array	Healthy subjects	3,000 different RNA transcripts in human saliva	Large scale methodology to study transcriptomics in saliva	Li *et al*. (*44*)
Affymetrix Human Genome U133A Array	OSCC	1,600 differentially expressed genes	Differentially expressed genes between affected patients and healthy ones through large scale approach	Li *et al*. (*55*)
Affymetrix microarray platform/2D-DIGE	BC	Discovery and validation of eight mRNA, and a protein biomarker (92% accuracy)	Salivary biomarkers in a disease not related with oral cavity, broadening the field	Zhang *et al*. (*53*)
Affymetrix Human Genome U133A Array	Healthy subjects	Characterization of RNA stability in saliva	Observation of the association with mucin and other macromolecules, for protection against degradation	Park *et al*. (*57*)
Peptide sequencing/MALDI-TOF-MS	Healthy subjects	Exosomes in saliva samples	First time report of exosomal vesicles in whole saliva samples from humans	Ogawa *et al*. (*59*)
miRNA microarray	EC	Different miRNA profiles in saliva derived from healthy patients and affected ones	Four validated miRNA biomarkers in EC	Xie *et al*. (*70*)
RNAseq	Healthy subjects	20-25% of sequenced reads that align to the human genome, while another 30% aligns to HOMD	First whole RNAseq in saliva samples from healthy human subjects, methodology to differentiate microbiota from human moiety	Spielmann *et al*. (*43*)
RNAseq	Healthy subjects	Human oral and gut microbiome and transcriptome differences	Establishment of patient self-collection of samples	Franzosa *et al*. (*72*)
RNAseq	PD	Oral metabiome differences between healthy microbiome and disease microbiome	Found differences in the diversity of the community between disease and healthy states	Jorth *et al*. (*73*)
RNAseq	Healthy subjects	exRNAs as micro RNA, Piwi-interacting RNA, and circular RNA	Characterization of diverse RNA species from saliva.	Bahn *et al*. (*74*)
Peptide sequencing/MS	Healthy subjects	Characterization of the salivary metaproteome	First catalogue of metaproteome, serving as a reference for future studies	Jagtap *et al*. (*76*)
Subtractive proteomics approach, combination of separation techniques: LC, LC-MS/MS, QqTOF MS	OSCC	Differential levels of proteins between healthy and affected patients	Verification of results in independent set of patients and healthy subjects, promising biomarkers	Hu *et al*. (*81*)
CE-TOF-MS	BC, OC, PC, PD	57 metabolites for accurate prediction of the probability of each disease	Shows the feasibility to obtain valuable information and biomarkers from saliva, in a variety of cancer diseases	Sugimoto *et al*. (*85*)

ELISA: enzyme-linked immunosorbent assay; EIA: enzyme immunoassay; pSS: primary Sjögren's syndrome; MMP-9: metalloproteinase-9 ; TIMP-1: tissue inhibitor of metalloproteinase-1; 2D-DIGE: two-dimensional difference gel electrophoresis; BC: Breast Cancer; OSCC: Oral Squamous Cell Carcinoma; MALDI-TOF-MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; EC: Oesophageal Cancer; PD: periodontal disease; HOMD: Human Oral Microbiome Database; exRNAs: extracellular RNAs; LC: liquid chromatography; MS: mass spectrometry; QqTOF: quadrupole-quadrupole-time-of-flight; CE: Capillary Electrophoresis; OC: oral cancer; PC: pancreatic cancer.

(*53*) (Table 1) through a microarray platform. Amidst their results, it was found that 1,402 genes had > 2 fold up-regulation, while 2,447 > 2 fold down-regulation in their saliva samples. Their findings indicate that saliva is a relatively good source of transcripts for biomarker discovery when comparing affected subjects to healthy ones. A further selection of the 27 top up-regulated candidates based on p-value and fold-change ($p < 0.01$, fold change > 10) allowed them to select potential candidates, that in the next stage of their

study were validated through RT-PCR. This last assay rendered 11 positive genes out of 27 genes. To be able to complete their study, the 11 candidates were tested in an independent sample (30 BC patients, 63 controls), having found 8 pre-validated markers (*53*).

Microarray is a platform that despite its limitations or a lower resolution compared with RNAseq, the new high throughput platform for transcriptomics, can be still very useful for studies in saliva, as it has been demonstrated in the reviewed papers using this approach in miRNA ESCC profiles. If a search over the studies that focused on ESCC and microarrays is performed directly in Gene Expression Omnibus (GEO) using the keywords "ESCC" and "array", the outcome gives back more than 300 results. However, up to date and to our knowledge, no study in the salivary ESCC transcriptome has been performed using this platform yet, according to the last bibliographic searches performed using keywords as "saliva", "microarray", and "ESCC" or "oesophageal squamous cell carcinoma". The exception to that comes from several studies that focused in miRNA characterization and identification using salivary samples (as well as tissues).

In one of those studies, Ishibashi and colleagues (*65*) used a microarray platform to analyse ESCC expression profiles between normal and cancer affected samples from 12 individuals. The 24 paired samples come either from the tumour area with more than 80% of tumour cells, or from normal tissue at least 4 cm away from the affected zone. An interesting option for microarray analysis in ESCC, has been the comparison of tumour cells with cell lines overexpressing an important gene for tumour progression (*66*), or a different splice variant (*67*), as it can aid to clear many questions about the development of the disease. Both of these studies have been carried out in the same laboratory, and show the potential of transcriptomics, as well as other type of complementary technologies, to address fundamental questions in molecular biology being an alternative for obtaining a higher resolution profile than classic approaches.

Identification of miRNAs in ESCC has been a promising research area with a number of studies published in the field. Matushima *et al*. (*68*) have studied ESCC cell lines that were moderate and well differentiated, as well as a control squamous epithelial oesophageal line, through a microarray platform for miRNA. Besides, they have performed functional studies increasing or decreasing the expression of miR-205, a miRNA overexpressed exclusively in ESCC cell lines. Additional assays included estimation of wound healing, cellular invasion and migration, or evaluation of the regulation target, that in this case was zinc finger E-box binding homeobox 2 (*ZEB2*). An important feature of the work of Guo and collaborators (*69*), is that they obtained the distinct miRNA profiles for ESCC tumour samples in frozen archival tissues, having obtained 46 differentially expressed ones. They established a minimum set of 7 that can differentiate between cancer and normal tissues. Despite the degradation, miRNA profiles in these stored samples were displaying similar values to that of fresh tissues, enlightening the use of the extensive tissue archives to gain better understanding of the disease. Some significant examples with saliva samples can be found in the recent work of Xie and collaborators (*70*), where they found different miRNA profiles in saliva derived from healthy patients and Oesophageal Cancer (EC) affected individuals, with four of them validated in a set of independent individuals, through a miRNA microarray. According to them, their work has been the first to assess the miRNA content in EC. Comparing both of the above mentioned studies, it seems that saliva renders less information, though is still valuable resource for biomarker discovery in terms of miRNAs. As it was pointed out by Xie (*70*), saliva can be considered an end point of blood circulation, having a certain degree of representation of the molecules in blood, stating that it serves for diagnostic purposes. Further broadening this point, Wang and collaborators (*71*) carried out a meta-analysis in several papers studying ESCC miRNA profiles in Asian populations, including that of Xie *et al.* (*70*). They observed that miRNA profiles in blood have a bigger diagnostic accuracy than those derived from saliva. Despite this observation, miRNA based diagnostics is a promising field in saliva (as well as blood), due to the higher stability, reproducibility, correlated concentrations to some types of cancers, or ease of detection through RT-qPCR (*71*).

Within the new RNAseq methodologies there have been promising discoveries focusing on saliva, as the work of Spielmann and colleagues (*43*) (Table 1), in which they highlight the power of salivary transcriptome as a diagnostic tool for human diseases using a massive RNA sequencing approach. With a similar methodological approach, Franzosa (*72*) (Table 1) revealed the importance of RNA studies in related locations to ESCC within human body by studying human oral and gut microbiome and transcriptome. The work of Spielmann and collaborators (*43*) is the first whole RNAseq in saliva samples from healthy human subjects, broadening to new studies in the field that will focus on the differences between healthy and affected samples. Both types of approaches have not been used in ESCC yet, and could therefore pose a great source of different biomarker tools. As it was mentioned above, the oral microbiome is an important part to consider when studying saliva. Spielmann and collaborators (*43*) report a 20-25% of sequenced reads that align to the human genome, while another 30% aligns to the Human Oral Microbiome Database (HOMD). They have detected more than 4,000 genes (coding and non-coding), while

structural integrity of the transcripts was conserved (*43*). Considering the potential of the metabiome as a diagnostic tool, there has been a close study in the oral metabiome between healthy and affected patients of periodontal disease (*73*) (Table 1), having found differences in the diversity of the community in both cases. A very interesting suggestion was that metabolic pathways are conserved while there are geographical, ethnical, and food consuming factors that may alter the species presence. If this gets demonstrated, we may expect that microbiome in ESCC affected patients displays different expression patterns to that one observed in healthy subjects, and this could serve as a prognostic marker for the development of the disease. Another study using RNAseq in salivary samples was performed afterwards by Bahn and colleagues (*74*) (Table 1). They carried out a similar massive study on the cell free component of salivary samples than that of Spielmann (*43*), but focused on extracellular RNAs (exRNAs) as micro RNA, Piwi-interacting RNA, and circular RNA.

3. Proteomics

The development of proteomics has been notable in different body fluids as urine, blood and saliva, as Amado and colleagues (*75*) have indicated. One of the features that they highlighted was the convenience of adding proteases inhibitors, due to the presence of proteases from bacteria and saliva which may affect the downstream procedure. As it has been already mentioned in the introduction, most of the proteins in saliva (90%) are shared with blood, but they represent only 15% w/w (*41*), while there have been studies reporting that 20-30% of the proteins in saliva can be found also in plasma (*45,46*). An interesting question that arises in here, and in agreement with Amado (*75*), is about how many of those proteins belong to the human moiety, or to the metabiome. An answer to that question was obtained by Jagtap and collaborators (*76*) (Table 1), in a deep study of the salivary metaproteome of healthy subjects, where they found that most of the detected proteins had a human origin, being non-human peptides present in much lower quantities. Additionally they determined over 200 different bacterial species. Their focus was the salivary supernatant, which largely remains free of bacterial component, as the pellet fraction collects the bacteria present in the oral cavity after the initial centrifugation. Even though, by using pooled samples from 6 individuals, they were able to find bacterial peptides in this fraction. Besides these significant discoveries, their study can serve as a reference for future studies that will focus on the differences between healthy subjects and those affected by a disease, especially ESCC. An extensive review on the topic was written by Uemura and collaborators (*77*), which focuses on the proteomics advances regarding EC in its two main forms, ESCC and EAC. This thorough revision of the bibliography, gives a precise idea of the status until 2014 of the usage of proteomics for different downstream applications, such as early detection, prediction of lymph node metastasis, therapy response prediction, prognostic prediction, as well as the applications in the development of novel therapeutics, or the elucidation of molecular mechanisms of action. Although all of those studies reviewed by Uemura are focused mainly on serum or biopsy samples from ESCC or EAC tissues, they give a complete perspective on what has been done until date using the available proteomic approaches. Equally as interesting, is the review written in 2012 by Qi *et al*. (*78*) which focused exclusively on ESCC proteomics, yet there were no reported studies using saliva as the source for proteins.

One of the included studies in the paper from Qi, was a proteomic profiling of cancer tissues from Chinese ESCC subjects, carried out by Du and collaborators (*79*), who found differential expression of 22 proteins (17 up- and 5 down-regulated) through MALDI-TOF (Matrix-Assisted Laser Desorption/ionization- Time of Flight) or LC-ESIT-IT MS (Liquid Chromatography-Electrospray/Ionization Ion Trap) approaches. According to Uemura (*77*), this work can be classified into the group of prognostic prediction biomarkers, considering that one of their findings was a correlation between poor prognosis and the expression levels of calreticunin, and 78-kDA glucose-regulated protein (GRP78) (*79*). The biological functions that were represented in the differentially expressed proteins are related with terms as glycolysis, regulation of transcription, cell proliferation, cell motility or cell signal transduction among others, what is related with the kind of processes that occur within a group of malignant cells, for example the Warburg effect (*80*).

But coming back to the field of salivaomics, one interesting approach was followed by Hu and colleagues, who used saliva as the source material to study the proteome of 64 healthy subjects compared with another group of 64 affected patients, although the disease in this case was OSCC (*81*) (Table 1). The methodology included a subtractive proteomics approach for their in-depth study, through a combination of separation techniques such as LC and LC-MS/MS, together with a QqTOF MS. About the methodologies, further explanation of the techniques can be consulted in the two above mentioned reviews from Uemura (*77*) and Qi (*78*). In their study Hu and colleagues concluded with a verification of their experimental results, being promising targets for biomarker discovery in OSCC. Regarding saliva and ESCC, we were not able to find studies relating both, only the reported ones using saliva in related affections as OSCC. This situation makes proteomic biomarker discovery in ESCC a promising and unexplored field of research.

4. Metabolomics

A remarkable review about the application of metabolomics for biomarker discovery can be found in the work of Armitage and Barbas (*82*). They have highlighted a variety of key pathways that are altered in cases of cancer, but considering all the potentially involved metabolites there is no current platform that can detect all of them at the same time. The choice of analytical platform can range between the two main techniques used in metabolomics analysis, one of them being MS (Mass Spectrometry) based approaches, with a deep profiling capacity; and NMR (Nuclear Magnetic Resonance). The latter has the advantage of being fast and reproducible (*83*), while there is no need to disrupt the tissues or samples, a feature that can be used for *in vivo* studies as showed by Morvan and Demidem (*84*), where they analysed the response of tumours to a chemotherapy treatment in tissues and mice. Albeit these advantages, it shows lower resolution than MS based choices, for which there have been advances in separation techniques with the coupling of MS to different complementary methodologies, for example CE-MS (Capillary Electrophoresis), GC-MS (Gas Chromatography), or LC-MS (Liquid Chromatography) (*82*).

Unfortunately, up to date there have been no published studies on metabolite analysis in saliva of ESCC patients. However some works as that of Sugimoto *et al*. (*85*) (Table 1), used the saliva of subjects affected in breast (30 subjects), oral (69 subjects), and pancreatic cancers (18 subjects), as well as periodontal disease (11 subjects), compared with a set of 87 healthy ones. In their approach they used a combination of CE-TOF-MS methodologies, having found a set of 57 metabolites for accurate prediction of the probability of each disease. Moreover, they show that it is possible to obtain valuable metabolomic information and biomarkers from saliva, in a variety of cancer diseases that affect other areas of the human body than oral cavity. One of the important metabolites discovered, choline, is relevant because choline-containing metabolites participate in phospholipid metabolism of cell membranes, and that has been associated to malignancy, as it has been reported by other authors that is a reflection of the increased proliferation state of tumorigenic cells.

A recent review has been published by Abbassi Ghadi (*86*) focused on studies using any type of sample in gastric and oesophageal cancers, being the most interesting studies for ESCC salivaomics, those performed in biofluids as serum or urine. The variety of analytical platforms from the studies included in this review range from GC-MS, High Resolution-Magic Angle Spinning-NMR, CE-MS, LC-MS, and Selected Ion Flow Tube-Mass Spectrometry. Due to the inherent differences in each platform, sensitivity, sample preparation, or type of sample source, there was a notable variability among the reviewed references, having found that glutamine is the most consistent biomarker for both cancers across many studies. An interesting idea highlighted by these authors is that potentially useful biomarkers should be further tested in other analytical platforms, and using different statistical approaches, to lower as much as possible the false positives discovery.

Wu and colleagues (*87*) focused on the screening of 20 paired samples from the same patients affected by EC (18 ESCC and 2 EAC), including both non-affected tissue and tumour samples (with at least 90% cancer cells), resulting in the identification of 20 metabolites through GC-MS. Other metabolomics studies focusing exclusively on ESCC as the disease model, include the one from Yang and collaborators (*88*), where they have studied the profile changes in ESCC tumours at different stages derived from tissue samples using a NMR based approach. They have addressed, according to their results, the possibility that some metabolic changes arises before any morphological alterations could be detected, and that is precisely what could pose an advantage in the early screening of this disease. Jin and collaborators (*89*) have pointed out that ESCC metastasis advances primarily through the lymphatic system, acting as well as a key prognostic factor. In their study they used GC-MS to elucidate the possible alterations in a set of ESCC serum samples (including metastatic and non-metastatic ones), versus healthy controls. One of the key elements of their study was the evaluation of the metabolomics differences in those subjects with lymph node metastasis. They have found a marked Warburg effect (*80*) on ESCC cells due to the enhanced glycolysis, which leads to decreased levels of glucose and glutamine in blood, as well as a notably higher content of lactic acid. This last metabolite was found in higher quantities in those patients with lymph node metastasis than non-metastatic ESCC subjects. Some other metabolites usually associated with tumour cells, are for example the observed increased levels of certain long chain fatty acids, that could be derived from a stronger *de novo* fatty acid biosynthesis, with some fatty acids being up-regulated in the metastatic samples compared with non-metastatic ones. Glutamine is another metabolite that was found to be decreased in metastatic cells, at its lowest levels from the three groups. They demonstrated that a combination of altered metabolites in cancer cases, could be used as a metabolomic signature for discrimination between patients in different stages.

Xu and collaborators (*90*) studied the metabolomics differences in ESCC, through a RR-LC-MS (Rapid Resolution) platform. They analysed different blood samples from healthy and affected patients, as well as other samples derived from ESCC patients that underwent a chemoradiotherapy treatment, being

divided in two groups, Overall Responders and non-Overall Responders. Among their results, 11 of the discovered metabolites were classified as tentatively potential biomarkers, while another set of 18 metabolites were classified in other group that potentially will serve as biomarkers for the diagnosis of ESCC. In keeping with the results from the study of Jin, there was an observation of an abnormality in the levels of several fatty acids. A similar finding was reported by Liu *et al*. (91), who used peripheral blood in order to isolate cell-free plasma through a UPLC-ESI-TOF-MS (ultraperformance liquid chromatography-electrospray ionization-accurate time-of-flight) platform, having found 6 metabolites related with the phospholipids metabolism, out of 11 potential metabolite biomarkers.

5. Conclusion

Despite the potential drawbacks that saliva may have, as lower representation of molecules that could be used as biomarkers in comparison with other body fluids, a higher degree of degradation of its components due to the exposure of the oral cavity to the open environment, or an enhanced RNase activity as reported for some cancer types, it has been demonstrated by many authors that it is possible to use saliva as a sample source, as it has been reviewed through this text.

Amidst its many advantages, it is an easily accessible biofluid, that fulfil the requirements for fast and efficient collection for many hospital settings all over the world, with minimum storage and biosecurity measurements, while it represents a non-invasive way to test patients, increasing their well-being. Once that the biomarkers have been validated and approved for their clinical use, the type of analysis that can be carried out to test for the different molecules as metabolites, proteins, or transcripts are relatively easy performed with minimum technical requirements. It is only for the discovery of those biomarkers when sophisticated and dedicated technologies must be applied.

For example, the transcription levels for given expression biomarkers can be carried out through RT-qPCR an accurate and quick method available at any diagnostic facility nowadays. In the case of proteins or metabolites, a targeted approach can be followed as well, what makes analyses more affordable. In agreement with the reviewed bibliography, we can conclude that a panel of biomarkers that cover the three main omics will have more discriminating power than focusing on measuring separately gene or miRNA expression, proteins, or metabolites.

There has not been a great development of salivaomics in ESCC patients despite the successful stories from other type of cancers, except for those efforts in miRNA analysis of saliva. Thus, it is a promising field for ESCC biomarker discovery with enough room for improvement.

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