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REVIEW ARTICLE

Novel technologies for oral squamous carcinoma biomarkers in diagnostics and prognostics

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Abstract

Background. Oral squamous cell carcinoma (OSCC) is a highly prevalent malignant pathology of the oral cavity. Despite the significant progress accomplished in the field of OSCC, the diagnosis is performed mostly in advanced stages; thus, novel biomarkers need to be developed for the diagnostic and prognostic of this malignancy. Many new technologies are used to provide indispensable information related to the pathogenesis of OSCC. The molecular profiling studies that incorporate genetic and epigenetic alterations need to be integrated in clinical practice as routine approaches to facilitate a better diagnostic and prognostic. ***Review.*** In this review, the authors present a summary of these novel technologies in the field of genomics, transcriptomics or proteomics, capable of generating data related to personalized diagnostic and treatment.

Key Words: *oral squamous cell carcinoma, biomarkers, transcriptomics*

Introduction

Squamous cell carcinoma (SCC) is an epithelial disorder implying numerous anatomical locations, with a substantial metastatic capacity [1]. Oral squamous cell carcinoma (OSCC) is a highly frequent oral cancer which causes over 500,000 new cases globally every year [2,3]. This pathology represents ~ 90% of all oral cancer cases [4], but early diagnosis assures a good survival rate (over 85%) [5].

The incidence of this malignancy increased in the last years in the Romanian population. Despite that, OSCC has not yet raised the interest of many researchers. A preliminary search on OSCC on the PubMed Central page on NCBI returns only 4943 results, while searching for 'breast cancer' returns 148,846 results and for 'lung cancer' 83,526 scientific articles. However, this is bound to change, especially because mortality rates for this type of malignancy have shown a steady increase during the past years, especially in countries that belong to the Eastern European block. For example, according to medscape.com, the situation has become severe in countries like Hungary, Germany and the Czech Republic, where mortality due to oral cancer has increased by almost 10-times during one generation in men aged between 35–44. At global level in men, in the year 2012—according to the Globocan website—OSCC ranked 11th among the most frequent types of cancer (198,975 cases, 2.7%), with a mortality of 2.1% (97,919 people) and a 5-year prevalence of 467,157 (3%, 7th most prevalent form of cancer). In women the situation is less drastic, with OSCC being the 17th most frequent form of cancer (1.5%), the 16th cause of death by cancer (101,398 cases, 1.5%), with a 5-year prevalence of 1.4% (234,992 cases). Nonetheless, with an incidence of over 300,000 new cases per year in both sexes combined, OSCC needs to be addressed more thoroughly by oncology researchers in order to learn

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more about the molecular mechanisms that lead to the development of this type of cancer, to eventually come up with biomarkers for early detection and prognosis and new targeted therapies.

OSCC pathogenesis

Based on its anatomical localization, OSCC has the advantage of an accessible biopsy for the case of clinical manifestation followed by the histopathological tests for the diagnosis confirmation [6]. OSCC is associated with modifications in histologically normal type oral mucosa due to multiple genetic and epigenetic alterations, leading from papillary hyperplasia or different stages of dysplasia (mild/moderate/severe), to the development of carcinoma *in situ* or well differentiated, invasive squamous cell carcinoma. Most oral cancers display an activation of tumor microenvironment and function of cancer-associated fibroblasts, along with the presence of classical tumor biomarkers (Ki-67, p53, homeobox genes and collagen type IV). This emphasizes a direct correlation with tumor cell proliferation and basal lamina degradation [7]. Cyclin D1 and Ki-67 were proved to be highly expressed in well-differentiated OSCC [8].

The implementation of molecular biology approaches in the diagnosis of oral pre-cancerous lesions or cancer contributes to the early detection of alterations that are not observed histologically. The same approaches are useful for the understanding of the molecular basis of oral cancer. This includes the microarray technology, deep sequencing approaches or proteomics. Until now, high-throughput methods were used for the identification of oral cancer biomarkers in certain biofluids (saliva and serum) or for prognostic markers from tissue specimens [9].

OSCC is considered to be the result of successive accumulations of genetic and epigenetic alterations, linked with the presence of the pre-cancerous lesions (such as leukoplakia, erythroplakia or combined pathologies) [10].

OSCC is a complex disease whose etiology includes interactions of various carcinogenetic and genetic risk factors that lead to malignant development and progression. The principal risk factors for developing OSCC include chronic tobacco and alcohol use. At the same time, chronic inflammation, viral infections (human papillomavirus, particularly HPV type 16 and 18) or genetic pre-disposition are additional factors that lead to this pathogenesis.

In order to ameliorate survival by enhanced prevention and therapeutic alternatives, it is mandatory to comprehend the fundamental molecular mechanisms which support oral tumorigenesis [11], mechanisms which can lead to novel therapeutic options. Oncogenic research can improve patients' prognosis in two ways: (1) early diagnosis and (2) the prediction of implications of risk factors (dietary, environmental or genetic). All this can eventually lead to the implementation of prophylactic screening programs meant to reduce incidence and mortality caused by this illness [12].

In the last years, genetic and epigenetics alteration has become a topic of interest for numerous research groups, shedding some light on the novel mechanistic understanding of OSCC. This review paper provides a comprehensive description of the presently available technologies in miRNA/mRNA profiling studies, deep sequencing or proteomics investigation related to OSCC diagnosis and prognosis.

Genetics and epigenetics alteration in OSCC

Despite the assertive management comprising surgery and radiotherapy in parallel with chemotherapy, the prognosis of this disease continues to be unfavorable in most cases. There is an urgent demand for more effective biomarkers for screening and diagnosis, but also for developing novel targeted therapies [1,2].

During recent years, a new method of analysis has become more and more present in molecular biology laboratories, especially in the study of cancer, which can aid in the progress of personalized medicine in the field of OSCC (Figure 1). Because of ever increasing output and reduced costs, high-throughput technologies are giving researchers not only new and invaluable in-depth knowledge regarding cancer genomics, but also provide data for transcriptomics, epigenomics, miRNA gene regulation and DNA–protein interactions [13]. Trying to shed light on these driving molecular mechanisms and improve the lives of patients by early diagnosis methods, prognostic biomarkers and targeted therapies with reduced side-effects, several research teams have employed these technologies in comparative studies between tumors and matching healthy tissue on other biological specimens or on SCC cell lines treated with various therapeutic compounds [7,14].

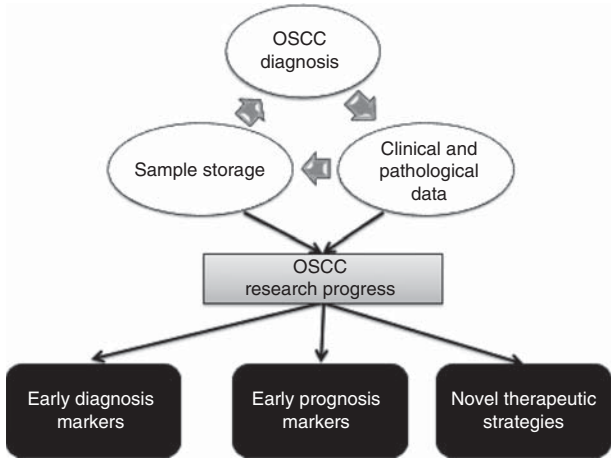

Figure 1. Application of the profiling studies in personalized medicine of OSCC.

In one study, an integrated analysis has shown a higher number of somatic events, classified in four main molecular pathways (mitogenic signaling, Notch, cell cycle and TP53) [15]. The Notch pathway was displayed as defective in 66% of patients and also repeated mutations of caspase-8 (CASP8) were observed, thus describing a novel sub-type of OSCC characterized by only a few copy number variations [10].

More recently, high-throughput platforms were used to study genome-wide quantitative methylation or for other epigenetic alterations, allowing a more comprehensive overview of DNA epigenetic modifications. Differential methylation patterns for the tumor tissue sample vs the matched normal or a specific methylation signature which correlates with the OSCC recurrence was observed. Therefore, the epigenetic alteration of *NOTCH4* signaling that was observed might represent a probable methylation signature for OSCC recurrence. Also, methylation alterations in HPV-driven patients were observed, but were proved to be less significant than other similar studies presented in the literature [16].

Transcriptomic expression profiling in OSCC

It is known that microarray data-sets are easily generated and collected in a short period of time. Gene expression studies are mainly focused on comparing relative gene expression levels in tumor tissue vs normal specimens. The main problem is the discrepancy of the data sets due to using different platforms and sample preparation protocols or other technological differences [17,18].

Kim and Cha et al. [19] combined two data-sets of public microarray data generated on the same platform (Affymetrix U133A), revealing 51 significant genes that were upregulated in OSCC, most of them being cancer-related genes. They proposed some new OSCC-related genes like STAT1, SKP2, IFI16, RHEB, IFI44, SOD2 and GREM1 [10,11] and also identified a set of genes capable to discriminate the normal tissue from OSCC, among which are MMP1, SOCS3 and ACOX1 [4].

A panel of 23 CXC-chemokine ligands and corresponding receptors were assessed using microarray and qPCR and linked with the response to therapy based on a logistic regression method. The expression of CXCL10 was significantly related with the response to radiotherapy, and the group with CXCL10 over-expression responded poorly [20]. The other microarray investigation revealed that DNA repair genes over-expression was connected with a reduced therapeutic response to radiotherapy, signifying that DNA repair genes can predict post-radiotherapy clinical outcome [21].

Another microarray data-set (using Affymetrix GeneChip/Human Genome-U133 plus 2.0 GeneChip) emphasized 167 over-expressed genes in radioresistant cells (based on X-ray irradiation). From this gene list, 40 were mapped to three highly significant genetic networks (using Ingenuity Pathway Analysis), including 25 cancer-related genes that shaped as one network and were classified by the role into genes involved in cell growth and proliferation, apoptosis and adhesion [22].

A different molecular profiling of OSCC revealed biological connections with basal-like breast or lung cancer. A panel of genes specific for breast cancer (CBX2, CNTNAP2, S100A8, SCUBE2 and STK32B) proved to have a biomarker role in OSCC, playing a significant function in cancer-related processes (hedgehog signaling, regulation of cell cycle progression, cell proliferation and metastasis). CBX2, SCUBE2 and STK32B were related to relevant clinical and pathological characteristics for OSCC (inflammatory infiltration, metastatic dissemination and tumor size). Therefore, SCUBE2 and STK32B regulate hedgehog signaling, while CNTNAP2 and S100A8 expression were connected with disease-free survival or overall survival rate [23].

microRNAs (miRNA) are a highly conserved group of small RNAs of 18–22 nucleotides that specifically modulate gene expression. miRNAs play important roles in various physiological and pathological processes, including tumorigenesis, cancer progression and invasion.

A microarray study of 1200 human miRNAs on 20 whole blood specimens lead to a specific signature for OSCC. The most relevant miRNAs were validated by qRT-PCR and displayed a 2-fold upregulation in the case of miR-494 and miR-3651 and down-regulation of miR-186 [24]. Another study described 114 miRNAs with differentially expressed levels between OSCC and normal oral tissue, of which miR-375 had the highest inhibition and miR-31 was the most over-expressed. The same study performed a molecular classification of OSCC based on 61 miRNAs with an accuracy of 93%. Human papilloma virus (HPV) infection leads to an alteration of 21 miRNAs, the most relevant being miR-127-3p and miR-363. The impact of HPV on miRNA profile may supply a mechanism for the different clinical behavior of HPV-infected tumors [25].

miR-491-5p inhibition is related to reduced survival in OSCC and leads to the inhibition of migration in *in vitro* studies and was proved to be a direct target for miR-491-5p [26].

Another investigation on OSCC revealed miR-375 down-regulation, miR-127 over-expression and miR-137 hypermethylation. Also, miR-200 and miR-205 were epigenetically activated in tumor tissue vs normal specimens, but they were inhibited in the absence of DNA hypermethylation, particularly in CD44 (high) OSCC. Observing the alteration of miR-375 and miR-200a, as well as miR-200c methylation, is a useful non-invasive biomarker, as proved by a study

performed on the saliva of OSCC patients vs healthy volunteers. Proposing miRNA signature from saliva has promising clinical utility as a biomarker for early diagnosis and prognosis in OSCC [27].

A recent paper presents an increased level of miR-483-3p in the case of final stages of OSCC, targeting CDC25A and inhibition of keratinocyte proliferation, leading to anti-tumor effects. miR-483-3p was proved to sensitize SCC cells in an experiment performed in serum deprivation condition leading to increased apoptosis rate. This pro-apoptotic effect resulted by direct targeting of API5, BIRC5 and RAN. These data were confirmed in xenografted mouse models [28].

Abnormal expression of several long non-coding RNAs (lncRNAs, with size larger than 200 nt) were connected with tumorigenesis and metastasis, including OSSC [29]. Gibb *et al.* [30]. reported the first lncRNA heat map for the case of normal oral mucosa vs pre-malignant lesion. Several recent studies present the utility of novel lncRNA as non-invasive biomarkers from saliva for diagnosis and prognostic purposes. In a recent study, HOTAIR detected from saliva was proved to have significant prognostic significance, the expression level being correlated with lymph node metastasis. Another lncRNA involved in OSSC metastasis is UCA1 [31] and FOXC1 [32]. Lnc-PPP2R4-5, lnc-SPRR2D-1, lnc-MAN1A2-1, lnc-FAM46A-1, lnc-MBL2-4:1 and lnc-MBL2-4:3 were proved to be over-expressed in OSCC [33]. HOTAIR, NEAT-1 and UCA1 were over-expressed in metastatic tumors and connected with MEG-3 down-regulation [28].

Next generation sequencing (NGS) profiling studies in OSCC

Because the debut and development of squamous cell carcinoma are due to alteration at genomic level, as in the case of other malignancies, NGS is a useful tool in discovering the mutations and variations in copy numbers that underlie these modifications [7].

Stransky et al. [34] conducted whole-exome NGS studies on matched DNA pairs—from tumors and peripheral whole blood—from 74 patients with head and neck SCC and discovered an average number of 130 coding mutations/tumor. Taking into account that 14% of all tumors were found to be HPV-positive, the sequencing studies revealed that these presented a mutation rate which represented ~ 50% of that found in HPV-negative tumors, which strengthens the belief regarding the molecular differences between the two sub-types of SCC. Because of the high sensitivity of NGS technology, the molecular characterization of these two SCCs can go even deeper, further sub-dividing HPV-negative tumors not only according to their exact localization, but also based on the frequency of substitutions of particular nucleotides. The results obtained by Stransky et al. were similar to those of other researchers, reinstating the already known role of several genes which present mutations in SCC, such as TP53, PIK3CA, HRAS, PTEN. Aside from these, a notable observation was the mutated status of NOTCH1 in ~ 11% of all tested tumors, since it was for the first time that somatic mutations in this gene were associated with squamous cell carcinoma development and progression. This study brings new insight regarding the tumor biology of this type of cancer, suggesting the importance of the alterations that occur at different stages in squamous differentiation on the various genetic mechanisms that underlie SCC development [7,13]. A significant role of TP53 mutations in the development of this disease was clearly presented in the case of young age patients (non-smoker) [35].

Another team, Pickering et al. [14] [**Error! Bookmark not defined.**], conducted a cohesive, broad genomic analysis of oral squamous cell carcinoma, aiming to identify the molecular mechanisms that drive the malignant transformation process, as well as to find biomarkers for classifying this disease and for prognosis and response to therapy. They used fresh-frozen tumor tissue and corresponding non-malignant tissue from 38 patients with oral SCC, on which Pickering's team conducted copy number alterations (CNA) studies at genome level and found strong existing correlations between gene expressions and relative copy numbers for 1721 genes, including CCND1, BIRC2, IKBKB, FADD and ORAOV1. By running DNA methylation assays, they found almost 3700 methylated probes that were differentially expressed, which were grouped in two separate clusters by applying unsupervised hierarchical clustering. The first cluster presented a more enhanced DNA methylation profile; similar to the CpG island methylator phenotype (CIMP) specific to colorectal cancer, but the samples from this group did not display a high mutational profile or MLH1 methylation. Another significant particularity found by means of next generation sequencing was the fact that, in about one tenth of the tumor samples, the NOTCH1 gene presented several missense and truncating mutations, which they validated on a panel of several HNSCC (head and neck squamous cell carcinoma) cell lines. They obtained similar results with the previously mentioned research group. The lack of protein expression, together with other tests that were performed—including *in vivo* studies on murine models—supported the idea that Notch signaling pathway is involved in tumor suppressive mechanisms in SCC [8].

This new but increasingly employed technique can offer a solution not only for studying the primary molecular devices that are involved in SCC onset and evolution, but can and have been used to decipher other potential players with active roles in the malignant transformation process for this type of

cancer. For instance, based on the known correlations between certain bacterial infections and the progression of certain cancers, such as *Helicobacter pylori* in gastric adenocarcinoma, or the possible implications of other bacteria, like *Propionibacterium acnes* in prostate cancer or *Chlamydophila pneumoniae* in certain lung tumors, Pushalkar et al. [36] wanted to study the mechanisms by which bacteria can be involved in carcinogenesis, especially in the case of SCC. Thus, they obtained saliva samples from three patients with oral SCC, as well as from two matched healthy subjects as controls and the genomic bacterial DNA was extracted, prepared and pyro-sequenced. Following data analysis, the team of researchers identified various species of bacteria belonging to eight taxonomic phyla, of which the best represented in the samples vs controls was phylum *Firmicutes*. At species' level, NGS studies revealed the fact that bacteria are differentially expressed in the oral environment of SCC patients compared to healthy subjects, which suggests the possible implication of these micro-organisms in the development of the disease. The same research team continued their study on matched malignant/non-malignant tissue pairs obtained from SCC patients and further emphasized the importance of these novel investigative tools in studying the complex and multi-faceted aspects of squamous cell carcinoma transformation [37].

Proteomic profiling studies with impact in diagnosis and prognosis

Proteomics represent an encouraging assay in the detection of novel biomarkers with prognostic and diagnostic roles [38,39]. Assessment of the cellular whole protein complements or from different biological fluids like serum, plasma or saliva may lead to the development of novel biomarkers in OSCC [4]. An important aspect is that there are several studies that propose a patient classification-based saliva proteomic profiling study [40].

Multiple investigations were focused on protein expression levels in OSCC samples and normal tissue or different *in vitro* systems for this pathology [41]. Studies revealed an alteration of multiple proteins implicated in cell metabolism and structure, cellular adhesion or cell motility, as well as signal transduction proteins and oncoproteins [42,43].

Plasma proteomic profiling showed a raised expression of haptoglobin and apolipoprotein A1 precursor that were up-regulated in the mice with OSCC. In humans, the expression of haptoglobin plasma indicated a strong correlation between the increasing levels of haptoglobin and the clinical stages of OSCC, proposed as a plasma biomarker for early detection of patients with OSCC [44].

A SELDI-TOF ProteinChip system was used to screen proteins in saliva from pre- and post-treatment OSCC samples, displaying a panel of 26 candidates with an altered pattern. In the case of pre-treatment saliva samples, the study showed a truncated cystatin SA-I of 14 kDa, having a deletion of three amino acids at N-terminus [45]. The authors proposed that ProteinChip analysis may provide a reliable screening test for early diagnosis of OSCC and that truncated cystatin SA-I might be a useful tumor biomarker for OSCC. In another study, transglutaminase 3 (TGM3) down-regulation was connected with OSCC loss of histological differentiation [46].

A comparative proteomics study led to the identification of a panel of eight differentially expressed proteins, from the total of 52 with statistical significance. The study was carried out on 10 OSCC samples, in parallel with the normal corresponding tissue [47]. Important attention was given to RACK1, a scaffold protein for several kinases, implicated in various biological pathways, including in OSCC pathogenesis [8].

An atypical expression was observed in the case of matricellular glycoprotein SPARC, a protein which has a significant role in tissue re-modeling and also in neoplastic transformation. Modified SPARC expression was identified both in stromal cells associated with cancer and in tumor cells [3]. A study using SDS-PAGE and MALDI TOF/TOF mass spectrometry showed elevated levels in Annexin A8, Peroxiredoxin-2 and Tyrosine kinase in diabetes and OSCC and these proteins were proposed as potential biomarkers for OSCC diagnosis [48].

Mass spectroscopy proteomic analysis of saliva samples of tumor vs normal specimens reveals an altered panel of 213 novel proteins. From these, Profilin, Cofilin, S100A9 and MMP9 were already identified as salivary biomarkers in head and neck cancer and Vimentin is known to be related to epithelial mesenchymal transition and metastasis [49].

Cytokines and chemokines are key proteins involved in inflammation and carcinogenesis and can be easily quantified by ELISA. This facilitates the development of novel salivary biomarkers for diagnosis and prognostic of OSCC. Still, the data concerning the expression levels remain inconclusive [50]. In 2004, Il-6 and IL-8 were proposed as potential biomarkers for oral cancer, but until now have not been validated as valid indicators [51].

Chemokines are secreted as a reaction to the presence of cytokines. CCR7 was positively correlated with lymph node metastasis, tumor size and disease stage. CCR7 may have a significant role in lymphatic spread [52]. It was observed that CXCL12 and its receptor (CXCR4) are related with OSSC metastasis [53].

Perspectives and conclusions

Regardless of the improvements in diagnostic and therapy approaches, the mortality rates for OSCC

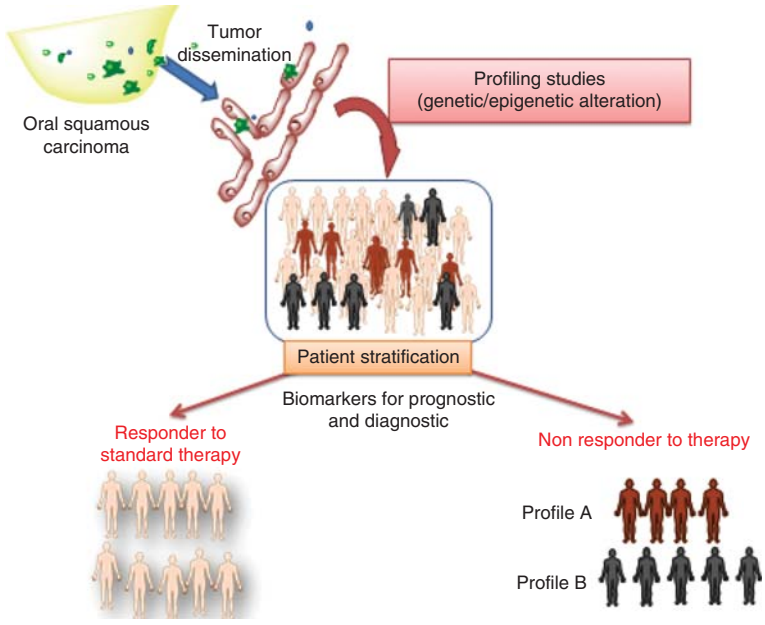

Figure 2. Profiling studies prior to therapy could identify responders vs non-responders as well as patients at risk for minimal residual disease after the therapy.

remain elevated, thus creating urgency for developing novel early diagnosis and prognostic biomarkers. In addition, efforts should be made for the improvement of screening and diagnostic techniques, in parallel with the development of new therapeutic molecules for the patients diagnosed in advanced stages. At the same time, further studies should take into account the wide range of the tumor types and the connection with the anatomical site, considering that these tumors show varying degrees of relationships to risk factors, of which HPV infection presented the highest association.

The early diagnosis of a malignant lesion or a squamous cell carcinoma is fostered by a complex diagnosis network that involves biopsies, which constitute an invasive approach. This should be replaced by non-invasive biomarkers, along with the integration of risk factors for oral cancer or for patient's response to therapy (Figure 2). The progress of these novel technologies should be the support of dental practitioners, in order to screen potential oral malignant pathologies, along with complex prevention programs, particularly aimed at the risk population.

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