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The promising impact of molecular profiling on treatment strategies in oral cancers☆

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is a major cause of cancer-associated morbidity and mortality. Although OSCC may develop from easily accessible oral preneoplastic lesions (OPLs), no intervention has been reported so far that reduces the rate of malignant transformation. A comprehensive molecular characterization of oral carcinogenesis may help refining treatment strategies both in patients with OPLs and OSCC. Herein, we review main molecular alterations occurring at different steps during oral carcinogenesis and show how molecularly-based medicine and surgery may impact the outcome of OSCC in the future.

1. Introduction

Head and neck cancers are commonly squamous cell carcinomas and are due to excessive alcohol consumption and smoking exposure. Head and neck squamous cell carcinoma (HNSCC) is the 7th most common cancer with a worldwide incidence exceeding half a million cases annually [1]. The past decade has witnessed significant advances in head and neck reconstructive surgery with the increasing use of perforator flaps [2], in radiation strategies with intensity-modulated radiotherapy (IMRT) [3] and in the management of metastatic/recurrent HNSCC with the promising impact of immunotherapies [4,5]. However, HNSCC remain a major cause of cancer-associated mortality and morbidity, particularly oral cavity SCC, the most common anatomical subsite of HNSCC. Oral squamous cell carcinoma (OSCC) is associated with the greatest risk of death [6], commonly related to locoregionally advanced disease [7]. Because OSCC may develop from oral premalignant lesions (OPL), the most common one being oral leukoplakia (OL), prevention of OPL malignant transformation as well as early detection of oral cancer may substantially improve outcome.

The development of new technologies for molecular profiling, such as next generation sequencing (NGS), has allowed providing a comprehensive molecular characterization of a wide variety of tumor types including OSCC [8,9]. Overall, this accumulated knowledge is an opportunity to make precision medicine a clinical reality [10]. Deciphering the molecular mechanisms associated with oral carcinogenesis may help move the field of OPL and OSCC management into the new era of precision medicine/surgery.

In this article, we describe the main molecular alterations occurring during oral carcinogenesis and discuss how molecular medicine is thought to change our current vision of OPL and oral cancer care.

2. Molecular characterization of oral carcinogenesis

2.1. A multistep process

Oral carcinogenesis is commonly described as a multistep process corresponding to the accumulation of genetic events driving the transformation of a normal mucosa into an invasive carcinoma. The so-called "genetic events" includes a large panel of molecular alterations including somatic mutations and copy number alterations (amplification, deletion) of tumor suppressor genes or oncogenes [11], epigenetic changes *i.e.* aberrant DNA methylation, histone modifications and miRNA deregulations [12],

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and gene/protein based pathways deregulation, that have been reported during oral carcinogenesis. Some of these alterations that accumulate during tumor development may confer to clonal population of cells selective advantages for tumor progression, and are therefore considered as drivers of tumorigenesis. They confer distinctive and complementary biological capabilities, named cancer hallmarks, that enable tumor growth, invasion and metastatic dissemination [13]. Those include genomic instability, immune escape, angiogenesis, resistance to apoptosis, or replicative immortality. The variety of molecular events may affect the expression and function of thousands of genes and proteins, and explain the remarkable diversity and complexity of neoplastic diseases.

Researchers proposed a model of tumor progression, which includes driver and sequential molecular alterations, and is commonly referred as "vogelgram", in reference to the first publication by Vogelstein et al. [14]. A "vogelgram" has also been described in HNSCC [15]. This genetic progression model that includes successive chromosomal losses at specific loci during head neck carcinogenesis (*i.e.* normal > hyperplasia > dysplasia > carcinoma in situ > SCC), has also provided some insight into the field cancerization concept, a phenomenon by which an entire field of tissue exposed to a defined carcinogen, may develop premalignant and malignant changes [16,17]. The concept of field cancerization and multistep carcinogenesis form the basis of chemoprevention. Most of the literature in this field has focused on the easily accessible oral cavity [18], with various clinical presentations (leukoplakia, erythroplakia, lichen planus...) grouped as oral premalignant lesions (OPLs) [19]. Most of the publications in the oral cancer prevention field refer to oral leukoplakia (OL), the most common OPL [19,20].

2.2. Molecular alterations at early steps of oral carcinogenesis

Because OL is easily accessible to physical examination, oral carcinogenesis is a good model for prevention. However, its clinical natural history remains poorly understood [21], the reported rate of malignant transformation is highly variable, from 0% to 36% [20,22]. Additionally, the presence and the degree of dysplasia observed in OL is inconsistently associated with the risk of oral cancer development for several reasons:

- OL without dysplasia may still progress to cancer;
- a high inter- and intra-observer discordance rate has been reported in the evaluation of dysplasia;
- and OL may be reversible even when dysplasia is identified [23].

The poor value of clinical and pathological factors to predict the risk of malignant transformation of OL has provided the rational to develop molecular-based prevention strategies.

The most robust and validated biomarker of oral cancer risk is loss of heterozygosities (LOH) at specific chromosomal loci, first reported by Mao et al. in 1996 [24], and subsequently validated by larger retrospective and prospective studies [25–27]. Other biomarkers of risk have been proposed such as deltaNp63 and podoplanin increased protein expression [28,29], as well as *AGTR1*, *FOXI2* and *PENK* increased promoter methylation and decreased global DNA methylation [30]. Gene expression profiles have been shown to be altered early during oral tumorigenesis and associated with oral cancer development [31,32]. The underlying stroma and its interaction with the epithelium may also play an important role during oral carcinogenesis. Indeed, a low CD3+ T cells infiltrate [33] as well as a declined inflammatory phenotype [34] have been associated with malignant transformation of OPL.

Overall, key-molecular events occur at early steps of oral carcinogenesis that result in an increased risk of OPL malignant transformation. A comprehensive molecular characterization of OPLs and paired OSCC is now required to allow an integrated molecular analysis of these molecular alterations. This effort will allow proposing a comprehensive model of longitudinal molecular changes occurring during oral carcinogenesis and will hopefully help refining prevention strategies.

2.3. Molecular characterization of oral squamous cell carcinomas

Compared to most other cancer types, HNSCC harbor a high level of somatic mutations [35–37]. Genomic instability, as defined by high levels of CNAs and mutations, is a dominant feature of OSCC. At the chromosomal level, more than 50% of OSCC harbor arm-level CNAs, particularly gains of 8q (63%) and 3q (58%), as well as losses of 3p (76%) and 18q (58%) [8]. At the gene level, focal deletions occur in chromosomal region 9p that contains the tumor suppressor gene *CDKN2A*, whereas focal 11q amplifications is associated with overexpression of *CCND1*, *FADD*, *ORAOV1*, *IKBKB* and *BIRC2* [8]. *EGFR* demonstrates a high-level amplification in more than 10% of samples and is overexpressed in a majority of HNSCC, leading to the development of EGFR inhibitors, including cetuximab, the only approved targeted therapy in locally advanced or advanced HNSCC. Additionally, some mutations are common in OSCC, particularly inactivating mutations of *FAT1* (30%) that plays an important role in cell differentiation and cell growth, *CASP8* (10%), and the tumor suppressor gene *TP53* (60%). Interestingly, recurrent *NOTCH1* mutations have been reported, that may be associated with loss-of-function especially in Caucasians [8,38] or with gain-of-function in the Chinese population [39], underlying the complexity of some molecular alterations occurring during oral carcinogenesis.

At the pathway level, mitogenic signaling including *EGFR*, *HRAS* and *PIK3CA*, cell cycle, and TP53 pathway were altered in most of OSCC. Additionally, epithelial-to-mesenchymal transition as well as degradation of the extra-cellular matrix are frequently altered during oral carcinogenesis particularly in oral tongue SCC [40].

In order to provide an integrated overview of these molecular alterations, HNSCC have been classified into distinct molecular subtypes [41–43]. The classification into 4 subtypes (atypical; basal; classical and mesenchymal subtypes) is the most commonly used [9,44,45]. In this classification based on gene expression profiles, the "classical", "basal" and "mesenchymal" subtypes exhibit canonical genomic alterations such as focal EGFR amplification, high frequency of *HRAS* mutations and upregulation of EMT-related genes respectively, while the atypical subtype is characterized by *PIK3CA* activating mutations and the lack of chromosome 7 amplification. Using data from The Cancer Genome Atlas (public repository), we show the distribution of those molecular subtypes in OSCC and other HNSCC (Fig. 1). In the oral cavity, the basal (42.7%) and the mesenchymal (34.8%) are the two main subtypes.

3. The impact of molecular profiling to refine treatment strategies of oral cancer

While the 20th century was marked by significant medical advances particularly in diagnosis imaging, molecular medicine may be one of the breakthroughs of the early 21th century. Indeed, since the first draft of the human genome sequence published in 2001, the dramatic decrease of sequencing cost as well as the development of new technologies such as next generation sequencing allowed generating huge amounts of data, commonly named "big data", in the field of molecular oncology [46]. The

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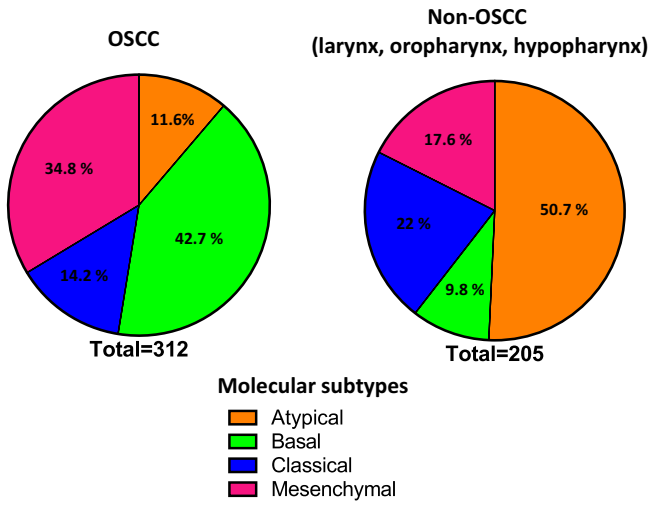

Fig. 1. Molecular subtypes of oral squamous cell carcinomas. Using clinical annotation of 517 HNSCC from The Cancer Genome Atlas (public repository), we show the percentage of "atypical", "basal", "classical" and "mesenchymal" tumors in OSCC (A) and non-OSCC including laryngeal, oropharyngeal and hypopharyngeal SCC (B).

comprehensive molecular characterization of a large variety of tumors types has provided a strong rational for "precision medicine", which consists in personalizing treatment strategies according to specific molecular alteration(s) found in a given patient's tumor. Because HNSCC are heterogeneous at the clinical and molecular levels, precision medicine is particularly relevant in this disease.

3.1. Targeted therapies and immunotherapies

Precision medicine based on specific genomic alterations is being actively evaluated [47,48] (NCT01774409). Using targeted DNA sequencing, the main goal of these trials is to identify targetable genomic alterations (mutation and/or copy number alterations) in a given patient's tumor, in order to tailor treatment to the genomic alteration. The molecular information is commonly discussed in a molecular tumor board and is thought to improve rationally-based decision making. Of note, HNSCC represented 6% of tumors included in a recent molecular profiling program in France [47]. Less than half of the patients may benefit from these molecularly-based treatment strategies, mainly because genomic alterations identified are not often targetable and patients with advanced and refractory disease where included in those trials. Immune escape is another well-established tumor mechanism that has been shown to be targetable using different immunotherapies strategies. While immunotherapies are providing unprecedented advances in the management of HNSCC [4,5,49], the microenvironment has not been assessed in early molecularly-based trials. This evaluation will need to be included in future precision medicine programs and the challenge will be to understand in which clinical setting to use these agents. In this context, we recently showed the importance of the microenvironment of HPV-negative OSCC from never-smoker never-drinker (NSND) patients compared to smoker-drinker, suggesting a higher clinical benefit of IDO1 and PD1-PDL1 blockade in NSND [50].

In addition to refine treatment strategies based on specific genomic alterations, it has been proposed to use the gene expression-based classification of HNSCC as described above to tailor patients' treatments. Interestingly, different patterns of drug sensitivity were associated with the molecular classification of HNSCC [43], suggesting its relevance for precision medicine. For example, Nutlin 3A, a pro-senescent drug acting via stabilization of p53, may be more efficient in tumors from the classical subtype compared to other subtypes.

In the prevention field, precision medicine may also play a pivotal role in the future. The recent clinical trial Erlotinib Prevention of Oral Cancer (EPOC) was the first randomized chemoprevention trial to utilize molecular risk stratification as a core selection strategy [27]. In this study, patients with OPL or who underwent surgical resection for OSCC, were stratified by their risk of oral cancer as defined by LOH status in OPL or normal mucosa. High-risk patients were randomized to the EGFR tyrosine kinase inhibitor erlotinib arm or to the placebo arm. Although the trial did not reach its primary objective, it allowed validating LOH status as a robust biomarker of oral cancer risk. Markedly, as previously described by J.E. Bauman and J. Grandis, EPOC marked "the beginning of an epoch of molecular selection" [51] and moved forward the oral cancer prevention field.

3.2. Radiation strategies

Radiotherapy plays a major role in the management of HNSCC and benefits approximately 75% of patients with HNSCC [52]. However, the complex anatomy of the upper aerodigestive tract makes it difficult to safely deliver an efficient dose to the tumor. Although different molecular mechanisms or biomarkers have been associated with radiosensitivity/radioresistance in HNSCC [53–56], precision medicine in radiation oncology has been poorly studied. HPV-positive oropharyngeal tumors are associated with a higher radiosensitivity, thought to be linked to specific molecular alterations such as p16 overexpression that decreases DNA repair capacities by inhibiting the recruitment of RAD51 to the site of DNA damage [57]. Based on this rational, de-escalation treatment protocols are currently being evaluated in clinical trials in this setting, with the goal to decrease morbidity without decreasing efficacy [58–60]. Similarly, because some specific molecular alterations such as activation of the EGFR have been associated with radioresistance in HPV-negative HNSCC [61,62], it is tempting to envision similar approaches based on biological differences across HPV-negative HNSCC.

Interestingly, a study has shown the relevance of a Genome-based model for Adjusting Radiotherapy Dose (GARD) to tumor radiosensitivity. GARD is derived from a gene expression-based radiation sensitivity index previously proposed in HNSCC [63] and a linear quadratic model. A high GARD value predicted a high therapeutic effect of radiotherapy in a large cohort of patients suffering from different types of cancer. Interestingly, median GARD was higher in patients with oropharyngeal SCC compared to non-oropharyngeal HNSCC. Thus, the GARD may serve as the basis for precision medicine in radiation of HNSCC.

3.3. Surgical strategies

Because oral cavity is a complex anatomical site critical for speech, swallowing and appearance, oral cancer surgery is associated with two main challenges:

- to perform a complete resection of the tumor disease (R0), including the primary tumor site and regional lymph nodes to cure patients;
- and to perform a functional and esthetic reconstruction to improve quality of life of patients.

3.3.1. Margin

Positive margins are recognized as a strong poor prognostic factor in OSCC [64–67]. As previously described [68], intraoperative

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guidance is primarily based on human vision and tactile information, especially for peroperative margin assessment. In order to control margin without resecting unnecessary normal tissue, 0.7 cm was described as the ideal distance in oral squamous cell carcinomas [64]. However, a millimetric accuracy remains a challenge for the surgeon, and one centimeter is the usual distance approximatively measured around the tumor to perform resection. Additionally, although histopathology is the gold standard for margin assessment, peroperative pathologic assessment of the margin status can be difficult because of tissue shrinkage, inaccurate sampling, and improper orientation [66]. Furthermore, correlation of orientation/localization between the tumor and tumor bed are distorted after excision [69]. Interestingly, the use of biomarkers such as a gene expression signature [70] or p53 and eIF4E immunohistochemical expression [71] have been proposed in histologically negative margin to predict recurrence. Whether reproducible molecular margin assessment is associated with improved prognosis needs to be further validated prospectively.

Several technologies for intraoperative tumor visualization have been recently described [72]. Optical fluorescent imaging, based on fluorescence variation between normal and tumor tissue, requires a fluorophore agent, which is autogenous (autofluorescence) or exogenous (target fluorescent probe). Compared to non-targeted fluorescent probes (e.g. indocyanine green), targeted fluorescent probes (corresponding to specific molecules) may be more specific to tumor tissue and improve accuracy. Targeted fluorescent probes are selected according to "TArget Selection Criteria" (TASC) scoring system, including percentage of target overexpression as well as its tumor/normal cell ratio [72]. Overall, the main goal is to target relevant biomarkers of cancer corresponding to the previously published cancer hallmarks [73]. A comprehensive molecular profiling of OSCC may help for the identification of the best candidates for targeted-probe fluorescence imaging using near-infrared. A recent paper showed the relevance of the Gastrin Releasing Peptide Receptor (GRPR) and its binding peptide TM1-IR680, for surgical margin prediction in a murine orthotopic model of oral cancer [74].

An alternative approach for refining margin resection strategies may be to predict the preoperative probability of positive margins, as previously described in breast cancer [75]. Molecularly-based preoperative assessment of the probability of positive margin according to the tumor biology may therefore refine surgical strategies, particularly the classical one centimeter margin.

3.3.2. Nodal involvement

Cervical lymph node dissection is a well-established treatment in clinically node-positive (cN+) OSCC. In early-stage (cT1-T2) and cN0 OSCC, an elective neck dissection should be performed when the probability of occult nodal disease is greater than 20% [76]. Interestingly, the incidence of occult cervical metastasis was 9.8% in hard palate and maxillary alveolus SCC [77] while buccal OSCC were associated with a rate of 1.8% and 10.6% for cT1 and cT2 tumors respectively. Based on these results, a "watchful waiting" strategy has been proposed for early stages SCC of the hard palate and maxillary alveolus.

A tumor gene expression signature has been proposed to be associated with lymph node metastases in HNSCC [78] and then validated in OSCC for prediction of lymph node positivity [79]. Interestingly, the negative predictive value of this signature was higher in early-stage OSCC (89%), the most relevant subset of OSCC in the context of node metastasis prediction. Thus, preoperative molecular assessment of the probability of occult disease may refine surgical strategies in early-stage OSCC from the hard palate and maxillary alveolus.

4. New technologies may bridge the gap between basic research and clinical practice

Because most of the studies described above have been performed in frozen samples and a research context, their main pitfall is their relevance in routine practice. In order to transfer those advances in molecular-driven medicine including radiation, targeted therapies as well as surgical strategies, new technologies able to address the challenges associated with molecular profiling of formalin fixed paraffin embedded (FFPE) patient samples have been developed that allow targeted DNA/RNA sequencing. They may help bridging the gap between basic research and clinical practice.

As part of this effort, we have initiated the "Cavity Oral Squamous Molecular Screening" (COSMOS) project (Fig. 2) to make clinical use of the molecular information generated by new sequencing technologies dedicated to FFPE samples. This is a collaborative project between the department of translational research at Centre Léon-Bérard (Lyon, France), the department of maxillo-facial surgery and the department of pathology (Pr I. Brochériou, Dr G. Hervé) at Hôpital Pitié-Salpêtrière (Paris, France). Using the HTG Edge Seq technology (HTG Molecular Diagnostics, Tucson, AZ, USA), we are currently generating targeted-gene expression profiles of OSCC to identify therapeutic targets, and to assess the relevance of gene expression signatures associated with radiosensitivity, margin positivity, and lymph node metastasis (Fig. 3). Using only one 5 μm tumor section, this technology allows generating targeted-gene expression profiles in FFPE samples (~2 days and less than 4 hours hand-on-time, for 96 samples). The expression levels of a panel of ~2560 genes involved in cancer hallmarks are measured in a single experiment, in a specific area of the tumor selected by a pathologist on an adjacent HES stained slide.

In conclusion, recent insights into the molecular comprehension of oral carcinogenesis as well as the development of new molecular profiling technologies available in clinical routine may

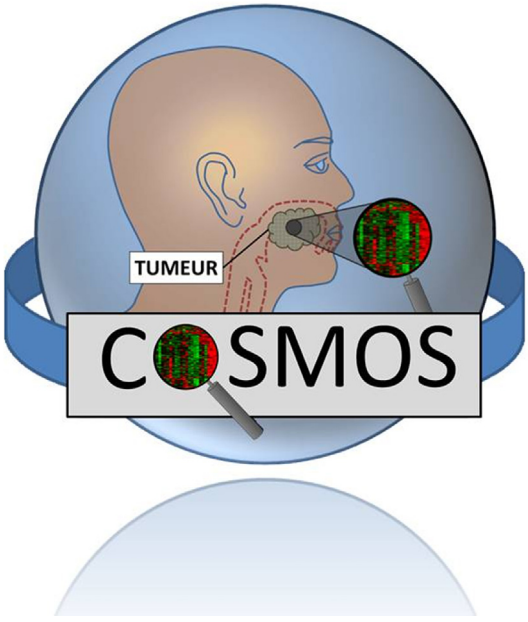

Fig. 2. Cavity Oral Squamous Molecular Screening (COSMOS). COSMOS is a collaborative project between the department of translational research at Centre Léon-Bérard (Lyon, France), the department of maxillo-facial surgery and the department of pathology (Pr I. Brochériou, Dr G. Hervé) at Hôpital Pitié-Salpêtrière (Paris, France). Our goal is to make clinical use of the molecular information generated by new sequencing technologies dedicated to FFPE samples, in order to refine treatment strategies in oral cancers.

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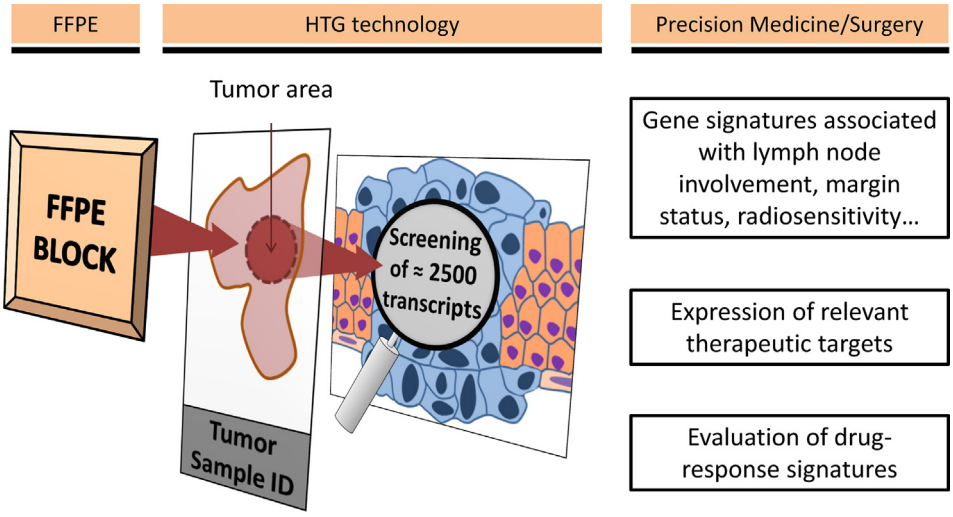

Fig. 3. Targeted-RNA sequencing using the HTG EdgeSeq technology. The HTG EdgeSeq technology requires only one 5 μm section for generating targeted-gene expression profiles in FFPE samples. The expression levels of a panel of ~2500 genes involved in cancer hallmarks are measured in a single experiment, and may be used in precision medicine and surgery.

allow refining treatment strategies in patients suffering from OPL and OSCC. Thus, precision medicine and surgery may decrease the substantial morbidity and mortality associated with oral cancer.

Disclosure of interest

The authors declare that they have no competing interest.

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