Oral Oncology 74 (2017) 83–89

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Review

Liquid biopsy: An emerging prognostic and predictive tool in Head and Neck Squamous Cell Carcinoma (HNSCC). Focus on Circulating Tumor Cells (CTCs)

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ARTICLE INFO

Keywords:
Liquid biopsy
Head and neck squamous cell carcinoma
Circulating tumor cells
circulating tumor DNA

ABSTRACT

Molecular diversity and continuing evolution of metastatic tumors are not easily captured by tissue biopsies. Development of non-invasive diagnostic tools, such as analysis of circulating tumor DNA (ctDNA), Circulating Tumor Cells (CTCs) and exosomes provides the opportunity to assess a blood sample in order to monitor tumor change and extract molecular information from cancers at a given time. "Liquid biopsy", which refers to molecular analysis of tumor's genetic features based on circulating genetic material in the peripheral blood, is already used to monitor disease response and track mechanisms of drug resistance in solid tumors. Head and Neck Squamous Cell Carcinoma (HNSCC) is a malignancy associated with advanced disease at presentation and dismal outcomes; furthermore, there is lack of biomarkers to monitor disease burden. Incorporation of liquid biopsy in the management of HNSCC might help identify patients with occult metastatic disease earlier and in a non-invasive manner. Herein, we aim to review current knowledge regarding CTCs and ctDNA in HNSCC and address open questions in this fast-evolving field of research.

Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is sixth most common malignancy worldwide, accounting for approximately 6% of all cases and is responsible for an estimated 1–2% of all cancer deaths [1]. HNSCC has been historically associated with tobacco and alcohol use; however, in the past decade, infection with high-risk human papillomaviruses (HPV) and especially type 16 has been implicated in the pathogenesis of a subset of HNSCCs, mainly those arising from the oropharynx. HPV-associated oropharyngeal cancer represents a distinct biological and clinical entity with a more favorable prognosis [2,3]. The majority of HNSCC patients present with locally advanced disease for which multimodality therapeutic approach is employed, including surgery, radiotherapy and cisplatin-based chemotherapy. These treatment modalities have resulted in improvement of local control that has not translated into a significant overall survival advantage for patients with locally advanced disease. Dismal survival rates partly reflect the lack of severe impact of multimodality treatment on the development of distant metastases [4,5].

Dissemination of tumor cells may represent a fundamental step in haematogenous metastasis. Circulating Tumor Cells (CTCs) are rare epithelial cells identifiable in the bloodstream that are considered to arise from the primary tumor and obtain genetic heterogeneity and specific properties during evolution, which allow them to colonize distant organs in order to seek improved biological conditions to enhance their survival [6,7]. Their detection in the peripheral blood of cancer patients has been shown to be of prognostic and predictive relevance [8]. More specifically, the presence of CTCs has been shown to correlate with overall survival (OS) in patients with breast [9,10], colorectal [11] and prostate cancer [12]. Detecting the presence of tumor cells outside the primary tumor could serve as important evidence for an early occult spread of tumor cells, as a relevant risk factor for metastasis and as a biomarker for monitoring treatment susceptibility [13]. However, the role and capacity CTCs in metastasis is yet to be established, and the presence of CTCs is not always correlated with disease burden or stage [14]. Therefore, despite detection of CTCs, definite evidence of metastatic disease should be required. Serial CTC measurement might also serve as a therapeutic tool; it offers the opportunity for the identification of drug resistance mutations, abrogating the necessity for repeat tumor biopsies; indeed, phenotypic and molecular characterization of CTCs may help to identify important drugable cellular molecules (i.e. EGFR, HER2, BRAF, etc.) [15–18].

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http://dx.doi.org/10.1016/j.oraloncology.2017.09.012
Received 21 April 2017; Received in revised form 7 September 2017; Accepted 14 September 2017

The term “liquid biopsy” refers to the extraction of molecular information from the tumor by analysis of circulating genetic material in the bloodstream, including CTCs, circulating tumor DNA (ctDNA), circulating miRNAs and exosomes. It is a minimally invasive and easily performed method that can serve as a diagnostic, prognostic and therapeutic tool. In HNSCC and other aggressive solid tumors, liquid biopsy can provide early and definite evidence of metastatic disease and aid in the identification of high-risk patients that could benefit from adjuvant therapy after surgical removal. In this review, we aim to capture the essence of current knowledge regarding CTCs and ctDNA as biomarkers in HNSCC and discuss future trends in this field.

CTCs

Introduction and historical background

The knowledge that tumor cells can travel through the vasculature to distant organ sites dates back over 100 years [19]. However, the presence of CTCs was first postulated in 1869 by Thomas Ashworth, who first described cells in the bloodstream that resembled those of tumors [20]. In 2005, Braun et al. first demonstrated the clinical usefulness of micrometastasis in the bone marrow of breast cancer patients, defined as the presence of disseminated tumor cells (DTCs), which was associated with poor prognosis at the time of diagnosis [21].

Development of overt metastasis is based on the notion that cancer cells that detach from the primary tumor get access to circulation either directly into blood vessels or after transit in lymphatic channels. In the head and neck region that is characterized by high vascularization, a CTC could potentially enter into circulation after physical manipulation during surgery, through lymphatics or veins that return blood to the superior vena cava to the right atrium and subsequently the ventricle of the heart [22]. CTCs are believed to be exceedingly rare among the population of primary tumor cells [23]. While in circulation, tumor cells have a short half-life and only a fraction survive to metastasize due to harsh conditions (apoptosis, elimination by the immune system, shear forces and fluid turbulences) [24–26], in keeping with the ‘seed and soil’ theory originally postulated by Stephen Paget and revisited by Langley and Fidler [27]. This theory proposes that the cancer cells (the seeds) have a preference to metastasize in a certain organ (the soil) seeking favorable conditions for their growth and development.

Epithelial-to mesenchymal-transition of CTCs

CTCs can undergo an epithelial-to-mesenchymal-transition (EMT), which results in a significant change in cell phenotype through downregulation of epithelial markers and acquisition of mesenchymal properties, such as increased capacity for migration and invasion, causing disruption to basement and extracellular matrix [28]. EMT is a fundamental process in metastatic cascade and enables disassociation of CTCs from the primary site. Furthermore, it has severe impact on CTC analysis; a majority of systems that detect CTCs, including the CellSearch system, are based on the expression of the epithelial marker EpCAM and cannot identify CTC subtypes with mesenchymal markers. Only recently EMT-related markers, such as TWIST and ALDH1, have been applied in CTC studies [29]. Mesenchymal CTCs have been associated with disease progression in breast cancer [30,31]. In HNSCC cells, it has been shown that EGFR, neurothropin receptor B and inflammatory cytokines (interleukin-1β) are involved in EMT [32,33]. The reverse process of mesenchymal–epithelial transition (MET), where cells acquire a more epithelial phenotype, plays a key role in allowing CTCs to seed in distant organs and establish metastasis [26].

CTC analysis

CTCs are extremely rare in the peripheral blood and it has been roughly estimated that patients with metastatic cancer will have one CTC to one billion of blood cells. As a consequence, they are usually co-isolated with a background of peripheral blood mononuclear cells [34]. Highly standardized protocols are needed for CTC analysis, which typically includes (a) isolation/enrichment (b) detection (c) enumeration and (d) molecular characterization [35]. Novel technologies of isolation/enrichment exploit different properties of CTCs to separate them from normal blood cells and include (a) systems based on physical properties (size, density, electric charge), which use microfluidics, and (b) systems based on biological properties of CTCs, such as expression of specific cell surface proteins, which utilize immunomagnetic beads. Immunomagnetic procedures use antibodies either against tumor-associated antigens (positive selection) or the common leukocyte antigen CD45 (negative selection) [36,37]. Positive selection is the most widely used CTC isolation/enrichment system and among various antigens that have been exploited for the positive immunomagnetic isolation, EpCAM is the most common. However, EpCAM is not expressed in cells undergoing EMT, for which cell surface marker vimentin has been used [38]. Negative selection for CTCs might be superior to positive selection, because it is not limited by an initial selection on cell surface marker(s). In a recent published report, Wu and colleagues have developed a rare cell enrichment methodology which allows further analysis for a wide range of cell markers. This methodology includes an initial negative depletion process, which consists of red blood cell (RBC) lysis and the subsequent removal of CD45 expressing cells through immunomagnetic depletion. Subsequently, an optimized, four-color immunofluorescence staining protocol is conducted on the cells retained after the CD45-based immunomagnetic depletion process. The use of these additional markers including the mesenchymal markers, vimentin and N-cadherin, the epidermal growth factor receptors, EGFR and HER2, markers related to the Hedgehog pathway, Gli1 and Smo, and a DNA damage indicator, gammaH2AX demonstrate the range and heterogeneity of these rare cells [39].

Molecular characterization of CTCs might lead to the identification of new driver mutations, providing valuable information for the development of novel drugs that target those mutations with the view to overcome drug resistance. CTC detection and molecular characterization is achieved by: (a) immunofluorescence, and (b) molecular assays, including RT-qPCR, fluorescent in situ hybridization (FISH) and next generation sequencing (NGS) and (c) detection of tumor-specific proteins released by CTCs (EpiSpot assay). The most validated procedure is detection of epithelial CTCs by immunofluorescence using anti-CK antibodies. However, detection of CTCs by classical immunofluorescence, which is typically done by pathologists through visual observation of stained CK-positive epithelial CTCs, is time-consuming and largely dependable on pathologists’ experience, whereas molecular assays provide objective and quantifiable CTC measurements [10,40,41]. PCR assays are sensitive, automated, relatively low cost and amenable to quantifiable quality control [41]. Furthermore, they require a small amount for analysis. On the other hand, multiplex RT-PCR enables simultaneous evaluation of molecular targets from the same sample, providing a multiparametric approach. However, PCR assays are based on the detection of mRNA markers that are specifically expressed in CTCs and not expressed in leukocytes. Hereby, there is an increased risk of false-positive results due to the amplification of non-specific RNA. Downregulation of gene transcription in CTCs due to EMT might be a problem in this approach as well [42].

NGS technologies contribute to improved understanding of a tumor’s biology; in contrast to single tumor-biopsies, not only they determine patient-specific genetic mutations in the primary tumor, but also provide insight into intra-tumor heterogeneity that reflects molecular changes during tumor evolution [43,44]. NGS analysis of CTCs may provide a better picture for individualized treatments, since they represent the type of cells that have disseminated from the primary tumor, but also from secondary metastases. In a recent study by Heitzer et al., molecular characterization of CTCs using NGS was performed in patients with colorectal cancer. It was found that driver genes