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Review

Liquid biopsy in head and neck squamous cell carcinoma: Prognostic significance of circulating tumor cells and circulating tumor DNA. A systematic review

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Introduction

Head and neck cancers (HNCs) are sixth most common cancer globally and they comprise malignant tumors originated from the oral cavity, nasal cavity, pharynx and larynx. The predominant histological type is squamous cell carcinoma (SCC), that represents more than 90% of HNC [1,2].

Their global incidence is 500,000 new cases annually, and they are responsible for 350,000 deaths every year: an estimated 1–2% of all cancer deaths [3,4].

The overall survival of head and neck cancer patients has remained unchanged for decades: currently 5-years survival range is between 25% and 60% and it is only slightly improved (3–5%) in the last two decades [4]. Among these, oral cancer (OSCC) is definitely the most prevalent with 145,000 deaths worldwide every year [5,6].

HNSCC are characterized by a multifactorial etiology: known risk factors include tobacco smoking, alcohol consumption, betel nut chewing and infection with Epstain Barr virus (EBV) and Human Papillomavirus (HPV), in particular HPV-16, which is responsible for 30.8% of oropharyngeal cancers [7–11].

Head and neck tumor may interfere with breathing, swallowing and eating: due to this reason and to their high mortality, they should be treated as soon as possible [12,13].

Early diagnosis is one of the most important elements for the treatment success in HNSCC: in fact, high mortality rate is largely attributable to late diagnosis (40% of patients have a stage IV disease at the time of diagnosis) [1,6].

An innovative tool that could allow these tumors to be detected early is liquid biopsy, a noninvasive diagnostic tool, based on the extraction of molecular information from the tumor by detecting circulating genetic material in the bloodstream, including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and micro-vesicles such as exosomes, containing miRNAs and protein [14–16]. This genetic material represents the exact copy of mutations present in the tumor and it has been increasingly considered as an option for detection of cancer as it can provide real time information about tumor in a minimally invasive manner [17].

Liquid biopsy can serve as a diagnostic, prognostic and treatment-monitoring tool, which allows to obtain a molecular profile for each patient, with the aim of achieving a more personalized approach to cancer management [14,18,19].

In order to evaluate the effective utility of liquid biopsy in the clinical setting and its potential role in the treatment of head and neck cancers, we have reviewed the main clinical studies conducted on the subject in the last years.

Material and methods

We examined the main clinical studies based on the detection and analysis of CTCs and ctDNA in HNSCC patients. A systematic search was conducted in the PubMed and Scopus electronic database, by using a combination of the following search items: *head and neck cancer, head and neck squamous cell carcinoma, liquid biopsy, circulating tumor cells* and *circulating tumor DNA*.

The search was run in November 2018 and a limited update literature search was performed in January 2019.

Eligibility assessment was performed by 2 independent reviewers: one of the authors of the review extracted the data from the included studies and a second author verified them. The disagreements between the reviewers were resolved with discussions between the two authors and if consent was not reached, the decision was taken by a third author.

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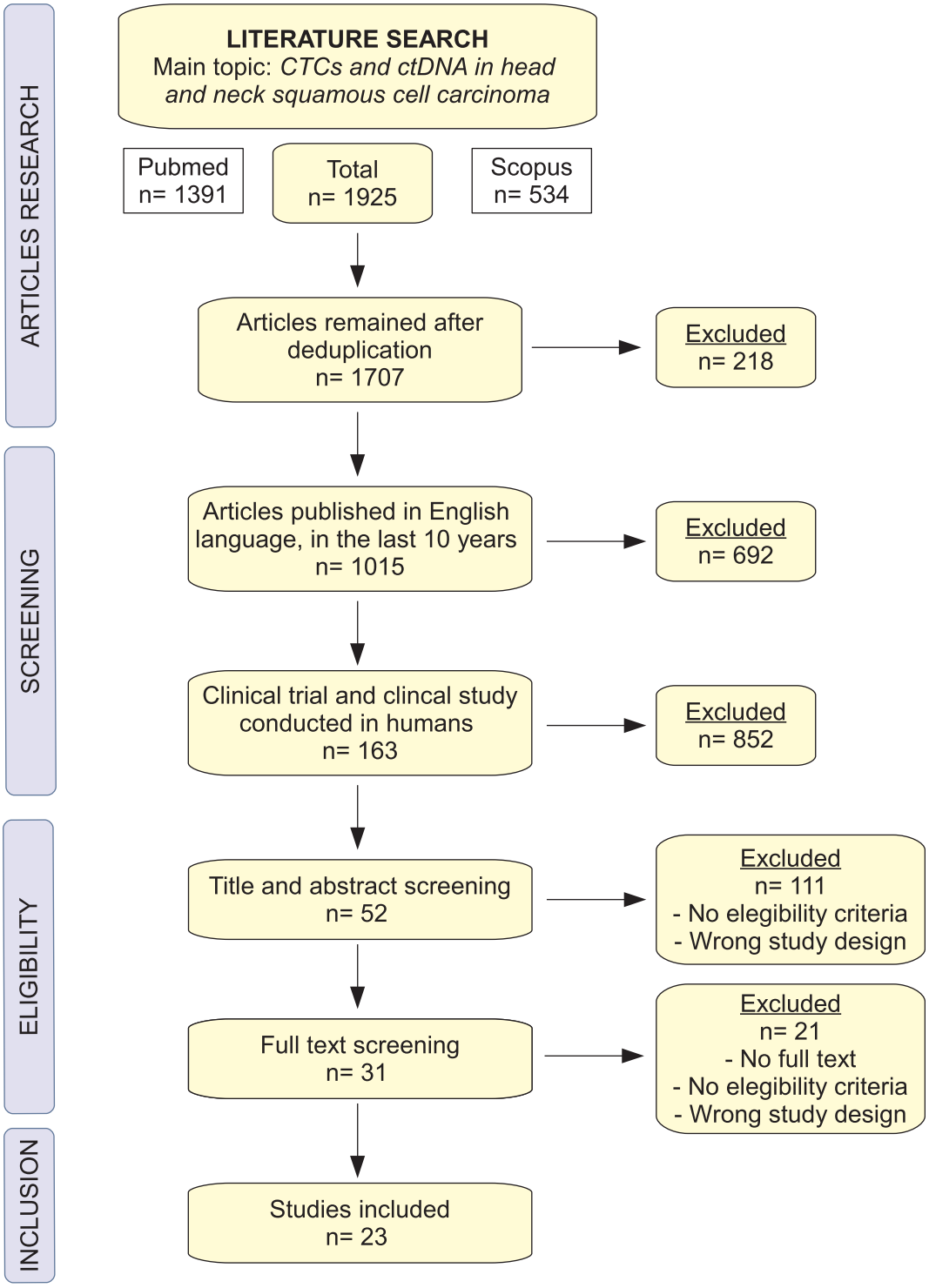

Fig. 1. Flow chart showing selection of studies for the review.

The results were automatically filtered, including only studies published in English language, in the last 10 years. Of these, all the literature reviews and the case-reports were discarded, and only clinical trials and clinical study conducted in humans were considered.

As eligibility criteria, we excluded all the studies which used a source of CTCs and ctDNA different from blood, serum, plasma or saliva, and all the studies conducted on patients with cancer different from squamous cell carcinoma.

After deduplication, the first search provided a total of 1707 studies. By excluding articles published in languages other than English, those published before 2009 and considering only clinical studies and clinical trials only 163 articles remained.

Finally, after abstract and full text screening we excluded 132 articles because they did not meet the eligibility criteria or because the full-text was not available. Furthermore, only studies that follow REMARK guidelines (REporting recommendations for tumour MARKer prognostic studies) were selected [20]. 23 studies were included in our review. The detailed procedures of the literature search and article screening are shown in Fig.1.

Results

The included studies use two different biomarkers: circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA).

CTCs STUDIES

12 studies have as objective the detection of CTC in the peripheral blood of HNSCC patients.

They analyzed samples of patients of different sizes: the largest population was 144 patients, while the smallest one was 9 patients [21,22]. A total of 718 cases were evaluated, with an average of 59,83 patients per study.

Only 6 studies specify the presence of scientific control samples: in these studies, the number of controls is significantly lower than the number of patients, except for the study conducted by Hsieh et al. in which the number of controls exceeds the number of patients [23].

All of these patients received a diagnosis of squamous cell carcinoma: 4 studies take into consideration only oral and/or oropharyngeal cancer, while others also evaluate patients with hypopharyngeal and laryngeal cancer. Only 4 studies included paranasal sinus and nasopharyngeal carcinomas.

In relation to the stage of the tumor, 3 studies reported both TNM classification and stage, 3 only TNM classification and 6 only the stage. In detail, 5 studies take into consideration only locally advanced tumor (stage III or IV).

To research CTCs, authors used different methods, all based on peripheral blood as biological source: 7 studies used Cell Search system (EpCAM + based), 2 used RT-PCR for EGFR detection, 1 employed PowerMag system (EpCAM + based) with Biomark HD system for PDPN detection, 1 used Maintrac system (EpCAM + based) and 1 employed a SERS-based system.

Blood sampling with the aim of detecting CTCs was carried out at different times: 8 of the 12 studies performed a single sample (before or after treatment), one study carried out two samples (before and after treatment), and 3 study took three sample (before, during and after treatment, or before treatment, after treatment and at follow-up).

All studies evaluated the prognostic response as the main outcome. Only one study also evaluated treatment response. The goal of most studies is to identify the prognostic significance of CTCs, in terms of overall survival and disease-free survival. Some studies have then investigated the existence of a correlation between the detection of CTCs and the presence of metastases or between the number of CTCs detected and the survival.

Characteristics of the mentioned studies are shown in Table 1.

ctDNA STUDIES

11 studies have as objective the detection of ctDNA in plasma, serum or saliva of HNSCC patients.

They analyzed samples of patients of different sizes: the largest population was 259 patients, divided into 2 group (training and testing samples), while the smallest one was 6 patients [24,25]. A total of 1126 cases were evaluated. Control populations are described only in 5 studies.

All the patients received a diagnosis of squamous cell carcinoma: 4 studies evaluated only oropharyngeal cancer, probably because the aim of the study was to detect HPV DNA, typically associated with this type of tumor, while others also evaluate patients with oral, hypopharyngeal and laryngeal cancer. Only 1 study included nasopharyngeal carcinoma [26].

In relation to the tumor stage, all the patients selected in these studies are variously distributed in the 4 cancer stages.

To research ctDNA, all the authors used PCR or quantitative-PCR; only 2 study used droplet digital PCR. Plasma was the source of ctDNA for 8 studies, while the other 3 studies used serum. Three authors also used saliva for detecting ctDNA.

The genetic target was HPV 16/18 for 4 studies, while the others target was a panel of genes, often associated with HNSCC: TP53, EGFR, KRAS, CDKN2A.

In addition, 9 studies also evaluated the positive HPV status.

The main aim of all the studies is to assess the prognostic value of ctDNA or to evaluate the usefulness of ctDNA in monitoring the response to treatment.

Characteristics of the mentioned studies are shown in Table 2.

None of the studies taken into consideration evaluated the possible influence that the administered therapy (chemotherapy, radiotherapy or surgery) may have on the prognostic response and on the concentration of biomarkers in the blood.

On the other hand, no significant differences in the marker values were found in relation to the age and sex of the patients, probably due to the fact that the majority of the patients included in the studies were over 50 years.

Discussion

Nowadays surgical biopsy is the gold standard in the diagnosis of HNSCC, but it is hampered by some limitations: biopsies are invasive, time-consuming and costly [27,28], moreover they are difficult to repeat, they depend on patient's age and comorbidity and they potentially lead to clinical complications, such as increased risk of cancer seeding to other sites [29–31].

In addition to this the main downside of surgical biopsy is the fact that it does not reflect the biological heterogeneity of a tumor, because it consists in the analysis of only a single site of the tumor in a single point in time [32].

Various area within the primary tumor can in fact harbor different genomic profiles, in particular in most advanced cancers: consequently, a single site biopsy may underestimate mutational burdens [27,33]. Anyway, multisite tissue biopsies in order to assess tumor heterogeneity is impracticable due to the high risk of complications, the cost and the sampling bias [34–36].

Moreover, compared to other type of cancer, for HNSCC there is also a paucity of disease-specific biomarkers: this problem always forces clinicians to rely on conventional diagnostic tool [13].

An additional method, which can integrate conventional procedures and overcome these limitations, is represented by the analysis of CTCs and ctDNA.

CTCs

Definition

Circulating tumor cells (CTCs) are rare epithelial cells identifiable in the bloodstream that have been shed from the primary tumor. CTCs share most of the mutational profile with the primary tumor and it mirrors tumor heterogeneity [17].

The knowledge that tumor cells are capable of traveling through the bloodstream to distant organ dates back to 1869, when Thomas Ashworth reported the presence of circulating tumor cells in peripheral blood of a cancer patient [18].

CTCs are not found in all cancer patients, but they are generally detected in 30–40% of them. In the included studies the percentage of CTC reported varies from a minimum of 12.5% in the study by Gröbe et al. to a maximum of 80% in the study conducted by Inhestern et al. The average value is 36.56% [37,38].

Once entered into the bloodstream CTCs have a short half-life and only a small part of them survive and become able to metastasize [14]. CTCs generally circulate alone, but they are also able to form clusters that have a greater metastatic potential: CTCs cluster is detected in about 25% of HNSCC patients [15].

The exact role of CTCs clusters in metastasis remains unknown, however two recent studies have tried to demonstrate the correlation

Table 1
Characteristics of studies using CTCs as biomarker.

Study	Country	N[1]	Controls	Tumor site[2]	Tumor stage	Detection mode	Assessed for
Jatana et al. 2010	USA	48	10	OSCC, OPSCC, HPSCC, LSCC	I: 4 II: 13 III: 9 IV: 22	RT-PCR (EGFR +)	Prognostic outcome
Hristozova et al. 2011	Germany	42	NR	NPSCC, OSCC, OPSCC, HPSCC, LSCC	T_0: 2 T_{1-2}: 6 T_{3-4}: 34	Cell Search system (7,5 ml of peripheral blood)	Prognostic outcome
Nichols et al. 2011	Canada	15	1	OSCC, OPSCC, HPSCC, LSCC	III: 10 IV: 5	Cell Search system (7,5 ml of peripheral blood)	Prognostic outcome
Buglione et al. 2012	Italy	73	9	PNSSCC, NPSCC, OSCC, OPSCC, HPSCC, LSCC	I: 1 II: 5 III: 11 IV: 56	Cell Search system (7,5 ml of peripheral blood)	Prognostic outcome
Bozec et al. 2013	France	49	10	OSCC, OPSCC	III: 15 IV: 34	Cell Search system (7,5 ml of peripheral blood)	Prognostic outcome
Gröbe et al. 2013	Germany	110	NR	OSCC	T_1: 29 T_2: 32 T_3: 21 T_4: 28	Cell Search system (7,5 ml of peripheral blood)	Prognostic outcome
He et al. 2013	China	9	NR	OPSCC, HPSCC, LSCC	III: 3 IV: 6	Cell Search system (7,5 ml of peripheral blood)	Prognostic outcome
Grisanti et al. 2014	Italy	53	3	PNSSCC, NPSCC, OSCC, OPSCC, HPSCC, LSCC, CESCC	I-II: 44 III: 9	Cell Search system (7,5 ml of peripheral blood) and detection of EGFR +	Prognostic outcome
Hsieh et al. 2014	China	53	61	OSCC, OPSCC, HPSCC, LSCC	II-III: 11 IV: 42	PowerMag system (peripheral blood) and detection of PDPN +	Prognostic outcome
Tinhofer et al. 2014	Germany	144	NR	OSCC, OPSCC, HPSCC, LSCC	III: NR IV: NR	RT-PCR (EGFR +)	Prognostic outcome
Inhestern et al. 2015	Germany	40	NR	OSCC, OPSCC	T_2: 15 T_3: 15 T_4: 10	Maintrac system	Prognostic outcome and Treatment monitoring
Morgan et al. 2018	USA	82	NR	PNSSCC, NPSCC, OSCC, OPSCC, HPSCC, LSCC	I: 2 II: 4 III: 3 IV: 43 Recurrence: 10 Remission: 20	SERS	Prognostic outcome

[1] Population sample.
[2] NPSCC: nasopharyngeal squamous cell carcinoma, PNSSCC: paranasal sinus squamous cell carcinoma, OSCC: oral squamous cell carcinoma, OPSCC: oropharyngeal squamous cell carcinoma, HPSCC: hypopharingeal squamous cell carcinoma, LSCC: laryngeal squamous cell carcinoma, CESCC: cervical esophageal squamous cell carcinoma.

between the presence of these clusters and the onset of metastases. Kulasinghe et al., in 2018, conducted a clinical trial with the aim of identifying CTCs clusters in patients with HNSCC, using straight microfluidic chips: they reported that CTCs clusters were found in 9 of 21 patient samples and they also demonstrated the higher metastatic capacity of these clusters, due to the presence of EGFR amplified single CTCs within the cluster volume [39].

The study conducted by Kulasinghe et al. in 2019 reported that the presence of CTCs clusters associated with the development of distant metastatic disease (p-value: 0.03) and that they represent an important prognostic marker of locally advanced disease. They also found that 20% of detected CTCs clusters contained leukocytes: the incorporation of white blood cells seems to be very important as it could provide a mechanism by which these CTCs clusters evade the immune system [40,41].

CTCs occur at an extremely low concentration in cancer patients: it is estimated that, on average, there is 1 CTC per billion of normal blood cell [34]. The studies included in the review, in fact, reported variable values: the number of CTCs found is between 1 and 3.2 CTCs per 7.5 ml of blood, with an average of 1.7 CTCs. Two of the analyzed studies reported a number of CTCs which is significantly different from the average: Inhestern et al. reported an average value of 3295 CTCs/ml, while in the study conducted by Morgan et al. the average value is 941 CTC/ml. The reason for this difference could be found in the different detection systems used in these studies compared to others [38,42].

Moreover, some studies have shown a possible correlation between the detection of CTCs and the anatomic site of the tumor: Buglione et al. found that CTCs was more prevalent in paranasal sinus, hypopharyngeal and oropharyngeal cancer (p-value: 0.05), while for Hristozova et al. the oral cancer is the tumor associated with a greater quantity of CTCs [43,44],45.

Detection methods

In order to overcome the limitations due to the reduced amount of circulating tumor cells, finding a standardized way to detect and isolate CTCs, which is also highly sensitive and specific, remains an underlying challenge [34].

Novel technologies of enrichment and detection of CTCs comprise a large panel of methods based on different properties of CTCs that separate them from the surrounding normal blood cells [46]. They include systems based on physical properties and systems based on biological properties of CTCs [14].

Methods based on physical properties include: density gradient centrifugation, filtration through special filters, differentiation based on size and deformability of cancer cells (generally larger and stiffer than blood cell, except for apoptotic CTCs), photoacoustic flow cytometer and the dielectrophoresis cell separation technique [34,46].

Biological properties are mainly based on the use of microfluidics devices (panel of magnetic nanoparticles) or on immunological procedures with antibodies against either tumor-associated antigens (positive selection) or the common leukocyte antigen CD45 (negative selection). These technologies have always been used with ex-vivo blood samples [46]. Recently a new in-vivo technology has been developed and it allows the enrichment of CTCs directly in the arm vein of the patient

Table 2
Characteristics of studies using ctDNA as biomarker.

Study	Country	N[1]	Controls	Tumor site[2]	Tumor stage	Detection mode	DNA source	HPV + patients	Genetic target	Assessed for
Cao et al. 2012	USA	40	10	OPSCC	I: 1, III: 4, IV: 35	qPCR	Plasma	14	HPV 16/18	Treatment monitoring
Ahn et al. 2014	USA	93	NR	OPSCC	NR	qPCR	Plasma, saliva	81	HPV 16	Prognostic outcome
Dahlstrom et al. 2015	USA	262	NR	OPSCC	I-II: 20, III: 26, IV: 216	PCR	Serum	212	HPV 16/18	Prognostic outcome
Wang et al. 2015	USA	93 (*saliva*) 47 (*saliva + plasma*)	NR	OSCC, OPSCC, HPSCC, LSCC	I-II: 20, III-IV: 3	PCR	Plasma, saliva	30	HPV 16/18, FBXW7, NRAS, PIK3CA, HRAS, TP53, CDKN2A	Prognostic outcome
Braig et al. 2016	Germany	20	NR	OSCC, OPSCC, HPSCC, LSCC	II: 2, III: 3, IV: 15	PCR w/targeted sequencing	Serum	2	EGFR, KRAS/NRAS, HRAS	Prognostic outcome
Mazurek et al. 2016	Poland	200	15	NPSCC, OPSCC, HPSCC, LSCC	I-III: 83, IV: 114	TERT, PCR	Plasma	28	KRAS G12C, EGFR	Prognostic outcome
Mydlarz et al. 2016	USA	100	50	OSCC, OPSCC, HPSCC, LSCC	I: 11, II: 9, III: 11, IV: 69	qMSP-PCR	Serum	NR	EDNRB, DCC, p16	Prognostic outcome
Perdomo et al. 2017	South America	36	49	OSCC, OPSCC, LSCC	I: 6, II: 8, III: 8, IV: 14	PCR	Plasma, saliva	0	TP53, NOTCH1, CDKN2A. CASP8, PTEN	Prognostic outcome
Schrock et al. 2017	Germany	137 (*training samples*)122 (*testing samples*)	122	OSCC, PSCC, LSCC	*Training samples*: T_{is}:1,T_1:22,T_2:42,T_3:30,T_4:32,NA:10 *Testing samples*: T_{is}:1,T_1:31,T_2:43,T_3:29,T_4:28,NA:9	qPCR	Plasma	NR	SHOX2, SEPT9	Prognostic outcome
Van Ginkel et al. 2017	Netherlands	6	NR	OSCC, OPSCC	II: 1, IV: 5	ddPCR	Plasma	0	TP53	Prognostic outcome
Hanna et al. 2018	USA	17	NR	OPSCC	III-IV: 17	ddPCR	Plasma	17	HPV 16/18/31/33/45	Prognostic outcome and Treatment monitoring

[1] Population sample.
[2] NPSCC: nasopharyngeal squamous cell carcinoma, OSCC: oral squamous cell carcinoma, PSCC: pharyngeal squamous cell carcinoma, OPSCC: orapharyngeal squamous cell carcinoma, HPSCC: hypopharingeal squamous cell carcinoma, LSCC: laryngeal squamous cell carcinoma.

[47].

However other enrichment and expansion strategies aim to increase the CTC concentration from blood are being developed, like the short term ex-vivo expansion of HNSCC CTCs [48].

Molecular characterization

After enrichment, the CTC fraction usually still contains a substantial number of leukocytes, and CTCs need to be identified at the single-cell level by a method that can distinguish tumor cells from normal blood cells [46].

Currently the only US FDA-approved platform for isolating CTCs is CellSearch™, a standardized semi-automated system that enables positive selection of CTCs based on the expression of the epithelial marker EpCAM [34]. It has been approved for a prognostic use in colon, prostate and breast cancer [17] but many authors used it also in HNSCC patients with promising results [49].

In relation to head and neck squamous cells carcinomas, EpCAM and EGFR are the principal molecular markers for the identification of HNSCC CTCs [50]: in fact, in the studies analyzed, EpCAM turns out to be the most used marker, followed by EGFR and by PDPN, a protein which results to be often upregulated in squamous cell carcinoma.

The selection of CTCs does not take place only through the identification of protein targets, but also through the detection of mRNA or new therapeutic targets like the immune checkpoint regulators such as PD-L1, which is frequently expressed on a variable percentage of metastatic cells circulating in the blood [51].

The enumeration and molecular characterization of CTCs are achieved by immunofluorescence, detection of specific proteins released by CTCs, and molecular assays including RT-PCR. The immunofluorescence using anti-CK antibodies represents the most validated procedure, although the use of the RT-PCR, which is more sensitive and automated, is increasingly considered [52].

Association with clinical features

Many authors have tried to explain why CTCs were present only in some cancer patients and they have hypothesized the existence of correlations between the detection of CTCs and the characteristics of the tumor.

In the study conducted by Gröbe et al. the detection of CTCs and their presence in high concentrations seem to be associated with a higher T stage (p-value: 0.04) [37]. In contrast, Hristozova et al. and He et al. did not find any correlation between CTCs and T stage [22,43].

However, several authors, including Hristozova et al., agree that there is an association between CTCs and the presence of metastases or lymph node involvement: in fact, although CTCs+ cases were also observed in the N_0 and N_1 stage patient group, their frequency was statistically significantly increased in patients with N stage 2b or higher (61%, p-value: 0.013) [43].

In relation to metastases, Gröbe et al. demonstrated that there is a statistically significant correlation between detection of CTCs and presence of metastasis (p-value: 0.004), and Morgan et al. have shown that a higher number of CTCs is associated with a low distant metastasis-free survival (p-value: 0.017) [37,42].

Even Nichols et al. have evaluated the existence of a possible correlation between the detection of CTCs and the presence of distant metastases, in particular of lung nodules: in fact, they showed that 80% of patients with lung nodules was CTCs positive at diagnosis and that the percentage of CTCs positive patients among subjects without metastasis was only 20%. For this reason, the presence of CTCs was statistically significantly associated with the presence of suspicious metastases (p-value: 0.04) [53].

Moreover, Buglione et al. noted that the percentage of survivors at follow-up was significantly lower among patients with CTCs positivity at diagnosis (p-value: 0.009) [45].

Prognostic and predictive significance of CTCs

Many studies have been carried out on the role of CTCs in HNSCC, with promising results in relation to their possible diagnostic and prognostic role [54]. However, to this day, as shown by the studies mentioned above, CTCs are more likely to be a prognostic marker rather than an early diagnostic marker in head and neck cancer [55,56].

In this regard, CTCs are considered to be an independent prognostic marker for disease-free survival and overall survival [21,57].

Jatana et al. reported that patients with no CTCs showed improved disease-free survival (p-value: 0.01) [58], and Hsieh et al. found that EpCAM + CTCs and PDPN + CTCs were a significant prognostic factor for poor progression-free survival and overall survival (p-value: 0.016 e 0.015) [23].

Besides, Grisanti et al. reported that a number of 2 or more CTCs was related to a poorer prognosis (both in term of overall survival and progression-free survival) [58]. Even Jatana et al. and Inhestern et al. claimed that patients with a higher number of CTCs were more likely to have worse clinical outcome (p-value: 0.04) [38,58].

On the contrary, Tinhofer et al. stated that, in oropharyngeal cancer, detection of CTCs had a good prognostic value (p-value: 0.059), but the reason remains unknown [21].

The presence of CTCs also seems to influence treatment response: according to Buglione et al. 90% of those who had a positive clinical response had no CTCs at diagnosis (p-value: 0.017) [45].

Finally, CTCs detection is also related to the risk of recurrences [37]: according to He et al. recurrences or progressive disease was observed in 2 of the 3 patients with CTCs and only in 1 of the 6 patients without CTCs [22]. Even Grisanti et al. reach similar conclusions: disease control was obtained in 8% of CTCs positive patients as opposed to 45% in CTCs negative ones (p-value: 0.03) [59].

Contrary to previous studies, Bozec et al. reported that, at follow-up, among 13 patients who developed recurrences, only 2 were CTCs positive at diagnosis: there is no significant correlation between CTCs detection before treatment and recurrent disease after therapy (p-value: 0.87) [60].

CtDNA

Definition

It has been shown that cells release fractions of their DNA into the bloodstream: this genetic material is known as circulating free DNA (cfDNA). When cfDNA is released by cancer cells it is called circulating tumor DNA (ctDNA) and it consists in a very small fraction of the total circulating DNA [14].

As early as 1948 Mandel and Metais found fragmented DNA and RNA in human plasma [18]. In 1977 Leon et al. reported an increased circulating free DNA (cfDNA) concentrations in circulation of cancer patients, providing concrete evidence that tumors release cfDNA into the bloodstream (36). But the last step towards confirming the ctDNA usefulness is represented by two publications in the *New England Journal of Medicine* and *Nature*, respectively, showed that circulating DNA in plasma is a specific and sensitive biomarker to monitor breast cancer [61,62].

Although the exact mechanisms behind the release and dynamics of cfDNA remain unknown, the most widely accepted theory is that tumor cells release DNA in a passive mechanism (via apoptosis or necrosis) or in an active one (cell secretion of DNA fragments in the tumor microenvironment, in order to affect susceptible cells at distant sites) [31,33].

The apoptosis, in particular represents the main way for the release of ctDNA: in fact, if we measure the length of ctDNA strands, we can note that they often assume the classic pattern in integer multiples of 180 base pairs, characteristic of the apoptotic process [63].

The reason is to be found in the rapid growth and in the increase in volume of the tumor, which induces also an increase in cellular turnover and in the number of apoptotic cells: as a result, we assist to the accumulation of cellular debris and its inevitable release into

circulation [18].

The key point of ctDNA is that its genetic alterations reflect those of the primary tumor: mutations in oncogenes, microsatellite instability, chromosomal translocations, epigenetic alterations or even viral DNA [64].

Detection methods

Multiple methods have shown that the percentage of ctDNA within total cfDNA can vary greatly between patients, from 0,01% to 90%, or, as suggested more recently, can even be detected at fractions of 0.01% [33,65,66].

CtDNA detection includes different quantification methods, that are based on the identification of different mutations: spectrophotometric methods, fluorescent dyes, qRT-PCR or droplet digital PCR [18].

For cfDNA analysis, plasma has been shown to be a better source than serum, although the amount of cfDNA in serum can be 2–24 times higher than in plasma: for this reason, many laboratories recommend the use of plasma for liquid biopsy [67]. Regarding our review, most studies used plasma as a source of ctDNA.

Two potential processes have been described for performing ctDNA analysis: the first is a targeted method, which consist in the analysis of known genetic changes from the primary tumor, in a small set of frequently occurring driver mutations (see Table 3).

A recent study has identified the main mutations found in head and neck tumors: in 239 HNSCC tumor samples, TP53 was found mutated in more than 83% of all tumors, while the mutation rates of CDKN2A, PIK3CA, HRAS, CDK4, FBXW7 and RB1 was between 0% and 21%. In particular mutational co-occurrence predominantly existed between TP53 and PIK3CA, HRAS and PIK3CA, and TP53 and CDKN2A [68]. There is also a correlation between PIK3CA and HPV-KRT subtype head and neck cancer, characterized by elevated expression of genes in keratinocyte differentiation and oxidation-reduction process, which show the gain of PIK3CA mutation [69].

The alternative approach is an untargeted method, which involves scanning DNA, extracted from plasma, for mutations of interest, without any prior knowledge of a particular change in a tumor (because the tumor tissue was not initially assessed). This last one represents an extremely expensive approach [18,36,70].

Specific DNA mutations

In our review, the study conducted by Wang et al. confirmed that TP53 was the most frequently found mutation in HNSCC (86% of the sample analyzed) [71].

Even Van Ginkel et al., by examining ctDNA in pretreatment plasma samples by ddPCR, found that all the samples were positive for targeted TP53 mutations in varying degrees, showing that detection of tumor

Table 3
Population characteristics of the included studies.

Study	N[1]	Sex	Age	Therapy[2]	Exclusion criteria
Jatana et al. 2010	48	M: 67% F: 33%	NR	SURG (only 1 CT + RT)	Systemic diseases, unsuccessful CTCs enrichment
Hristozova et al. 2011	42	M: 83% F: 17%	NR	CT, RT	Inoperable locally advanced HNSCC
Nichols et al. 2011	15	M: 73% F: 27%	> 60y: 53% < 60y: 47%	SURG, CT, RT	Previous cancer
Buglione et al. 2012	73	M: 76,7% F: 23,3%	NR	SURG, CT, RT	Treatment discontinuance, technical problems with CTCs samples
Bozec et al. 2013	49	M: 89% F: 31%	> 60y: 51% < 60y: 49%	SURG, CT, RT	Previous cancer, severe systemic disfunctions
Gröbe et al. 2013	110	M: 67,3% F: 32,7%	> 60y: 42% < 60y: 58%	SURG, CT, RT	Neoadjuvant therapy
He et al. 2013	9	M: 89% F: 11%	> 60y: 44% < 60y: 56%	SURG, CT, RT	NR
Grisanti et al. 2014	53	M: 79% F: 21%	> 70y: 21% < 70y: 79%	SURG, CT, RT	Other concomitant neoplasm, poor health status
Hsieh et al. 2014	53	M: 94% F: 6%	NR	SURG, CT, RT	Previous cancer, age < 20 years, renal or liver severe disfunction
Tinhofer et al. 2014	144	M: 87,5% F: 12,5%	NR	SURG and then CT + RT	Cancer stage I or II
Inhestern et al. 2015	40	M: 83% F: 17%	NR	SURG, CT, RT	Distant metastatic disease, life expectancy < 3 months, pregnancy, previous cancer, serious medical conditions, other concurrent treatment, previous treatment for HNSCC
Morgan et al. 2018	82	M: 73,2% F: 26,8%	NR	SURG, CT, RT	NR
Cao et al. 2012	40	M: 92,5% F: 7,5%	> 58y: 50% < 58y: 50%	CT, RT	NR
Ahn et al. 2014	93	M: 87,1% F: 12,9%	> 60y: 13% < 60y: 87%	SURG, CT, RT	Missing pre- or post-treatment saliva or plasma samples
Dahlstrom et al. 2015	262	M: 85,5% F: 14,5%	NR	NR	Distant metastases, unknown primary tumor, laboratory data not available
Wang et al. 2015	140	M: 83% F: 17%	NR	SURG, CT, RT	NR
Braig et al. 2016	20	NR	NR	CT	NR
Mazurek et al. 2016	200	M: 80% F: 20%	> 63y: 35% < 63y: 65%	CT, RT	Metastases, relapse
Mydlarz et al. 2016	100	M: 77% F: 23%	NR	NR	Previous cancer, new cancer during follow-up, premalignant oral cavity lesions
Perdomo et al. 2017	36	NR	NR	NR	NR
Schrock et al. 2017	259	NR	NR	SURG, CT, RT	Cancer in the previous 3 years
Van Ginkel et al. 2017	6	M: 67% F: 33%	NR	NR	NR
Hanna et al. 2018	17	M: 95% F: 5%	NR	CT, RT	NR

[1] Population sample.
[2] SURG: surgery; CT: chemotherapy; RT: radiotherapy.

specific TP53 mutations in low level ctDNA from HNSCC patients could be a valuable prognostic tool [25].

Also, RAS mutations, in particular KRAS e HRAS, were found to be quite common: Braig et al. studied a population of patients treated with cetuximab therapy and chemotherapy regimen, and 30% of these patients acquired activating RAS mutation during therapy. They also reported that 0% of treatment responsive patients had mutations, confirming that RAS mutations were related to disease progression (p-value: 0.032) [72].

Several studies published in recent years show that EGFR is also often mutated in HNSCC patients. However, in the studies we examined, two authors reported that no patients with mutated EGFR were found [26,72].

Instead, other mutations have been reported: Mydlarz et al. found that 10% of subjects had amplification of EDNRB (20% and 10% of these patients had also DCC and p16 amplification, respectively), showing that EDNRB could be a highly specific, but not sensitive, biomarker for HNSCC (p-value: 0.03) [73]. The study by Schröck et al., which analyzed a training and a testing population of HNSCC patients, reported that 59% of subjects were methylation-positive for SHOX2 or SEPT9 genes [24].

Also, PIK3CA oncogenic pathways are frequently mutated in head and neck squamous cell carcinoma, including virally driven HNCs: gene copy number alterations of PIK3CA have been found to be a prognostic and predictive biomarker for therapy response. A high polysomy of PIK3CA was found in 20% of HNSCC cases: PIK3CA copy number gain was significantly associated with shorter disease-specific survival and larger tumor volume [74,75].

Finally, as reported by the study of Perdomo et al., included in the systematic review, it is possible to state that, on average, typical HNSCC mutations is detected in about 42% of subjects with head and neck cancer [76].

Prognostic and predictive significance of ctDNA

CtDNA levels are characterized by high variability in cancer patients: this finding has led several authors to evaluate the existence of a correlation between ctDNA levels and tumor characteristics [27,77]. Recent studies, in fact, states that circulating cfDNA is so sensitive in drawing an overall picture of the malignant disease that it could serve as a definite source of diagnostic and prognostic DNA [36,78].

Currently ctDNA has been reported to be a highly sensitive genetic biomarker of disease in pancreatic, colorectal, prostate, lung, melanoma and breast cancer, but its applications in head and neck cancer are still largely limited [35]. Nevertheless, the use of ctDNA in HNSCC is increasingly considered.

Potentially, ctDNA can be used to monitor tumor burden, to predict treatment response, and to detect minimal residual disease. It would also be possible to use it for the early diagnosis of cancer, but actually several studies have demonstrated its superiority in prognosis rather than in diagnosis of cancer [78].

The measurement of ctDNA as a marker of tumor burden is certainly more advantageous than the use of conventional protein biomarkers or even imaging studies: due to the short half-life of ctDNA, that is approximately of 2 h, it is possible to evaluate tumor changes in hours rather than weeks to months [30].

A recent preclinical study using mice models of head and neck cancer found a correlation between ctDNA levels and tumor burden. Moreover, they detected significantly ctDNA changes before a tumor could be spotted by CT scan [79].

Association with clinical features

The study conducted by Schröck et al. and included in our review, found that high methylation levels of SEPT9 and SHOX2 in ctDNA correlated with tumor and nodal category (p-value: 0.001) [24].

Even Mazurek et al. demonstrated that cfDNA levels in patients with clinical N2 or N3 disease was higher than in patients with a clinical N0 or N1 disease (p-value: 0.015). It was also higher in patients with cancer stage IV compared with stages I-III (p-value: 0.011) [26].

On the contrary, Shukla et al. analyzed ctDNA in the plasma of 390 patients with oral cancer and 150 healthy controls, but they did not observe significant differences between the groups [80].

Furthermore, the analysis of ctDNA may predict for treatment response both in the early stages of therapy, when it could be possible to modify treatment regimen, and during the course of treatment (after weeks or months), when it is possible to monitor response to therapy [18].

Recent studies have also shown that liquid biopsies can be used effectively to monitor the emergence of multiple resistance clones during the course of treatment [81].

CtDNA is also useful for determining overall survival: one of the studies analyzed reported that increased methylation levels of SEPT9 and SHOX2 were associated with a higher risk of death (p-value: 0.001 and 0.024, respectively) [24], while another study, conducted by Perdomo et al., reported that no association was found between ctDNA TP53 mutation and overall survival [76].

Salivary ctDNA

CtDNA can be found not only in the blood, but also in other body fluids, such as urine, saliva, seminal plasma, pleural effusions, cerebrospinal fluid, sputum samples and stool samples, that can be used for a liquid biopsy [15,16].

In particular, detection of ctDNA in saliva has gained increased attention, as it would seem to be a sensitive and completely non-invasive method for HNSCC diagnosis.

Both the study conducted by Spafford et al. and the one conducted by Okami et al. have demonstrated that the genetic mutations found in salivary ctDNA reflect those found in plasma and that these mutations are not present in the healthy subjects of the control group [82,83].

In the study conducted by Wang et al. and included in the review, ctDNA was collected from plasma and saliva of a sample of HNSCC patients: they demonstrated that plasma ctDNA was more sensitive biomarker than salivary ctDNA for oropharynx, hypopharynx and larynx cancer, while salivary ctDNA showed better sensitivity than plasma ctDNA (100% vs 80%) in oral cancer. Furthermore, by combining findings from plasma with DNA detected in saliva, diagnostic sensitivity increased to 96% [71].

We can conclude that the combination of ctDNA detection both in plasma and saliva may be a valuable tool to increase sensitivity in HNSCC screening.

Detection of HPV DNA

In addition to searching for tumoral DNA, liquid biopsy can also be used to detect viral DNA, particularly HPV DNA. In fact, HPV-16 is often identified as one of the main causes of the occurrence of OPSCC, while it is etiologically linked only to a limited subset of oral cavity cancer [84].

The 2017 edition of the WHO Classification of Head and Neck Tumours classified HPV-positive OPSCC as a specific tumor entity with a distinct epidemiological profile, specific genetic features, clinical presentations and outcomes [85].

As reported by Tang et al. HPV-positive OPC is linked with a more favorable prognosis, but with high recurrences within two years of diagnosis when compared to HPV-negative disease [86].

Six of the studies included in our review also looked for circulating HPV DNA.

Mazurek et al. analyzed cfDNA levels of 200 HNSCC patients by examining HPV-16/18 and they found a higher level of viral DNA in 14% of subjects, the majority of whom had the type HPV16 (96.4%). They also demonstrated that HPV DNA detection is significantly greater in patients with oropharyngeal cancer in comparison with other HNSCC [26]. These results prove the diagnostic potential of plasma-based HPV cfDNA tests for the early detection of HPV-positive HNSCC.

Three studies included in the review also looked for possible correlations between viral DNA, tumor burden and survival, with the aim of using liquid biopsy as a tool for monitoring HPV-induced tumors: Hanna et al. found that total tumor burden strongly correlated with HPV cfDNA levels (p-value < 0.001) and that median plasma HPV cfDNA levels negatively correlated with overall survival (p-value: 0.05) [87].

In this sense, high values of viral DNA and an elevated viral load (> 10 copies/50 ng) was significantly associated with the advanced stages of OPSCC, and consequently they represent a negative prognostic index which should direct the clinician towards a specific and more aggressive treatment plan.

Another recent study has highlighted the need to implement, as recommended by the revised WHO/IARC standards, the analysis of oropharyngeal tumors, introducing, in addition to the immunohistochemical examination of the p16 expression, also HPV DNA tests based on PCR.

This study also showed that the overall survival was significantly lower in patients with

p16-positive and HPV-negative OPSCC when compared with the group in which both p16 and HPV DNA were positive, further reinforcing the importance of performing additional HPV DNA testing in OPSCC patients [88].

Cao et al. reported that pretreatment plasma HPV DNA copy number significantly correlated with metabolic tumor volume and nodal classification. They also found a rapid decline of HPV DNA after RT completion, and an increase in its level when metastasis appeared [89].

The study by Dahlstrom et al. showed that patients with high N category and stage IV disease had higher rates of detectable pretreatment serum HPV DNA. They also reported that subjects with HPV-positive tumors had better progression-free survival than patients with HPV-negative tumors, and that, among HPV-positive patients (81%), those who were negative for serum HPV DNA (43.5%) had better progression-free survival than those who were positive, but this result was not statistically significant [90].

In conclusion, the detection of HPV DNA in patients with head and neck tumors should become a routine procedure, as it allows to develop a specific therapeutic plan and to improve patient follow-up, and also, through the evaluation of the viral load, it allows to monitor the progress of the disease and the results obtained by the treatment. This is what also emerges from the study by Rutkowski et al., which have reported that the presence of HPV-DNA in patients controlled by radiotherapy or chemotherapy suggests sub-symptomatic relapse and/or dissemination, what opens a chance for successful salvage [91].

An innovative and advantageous method is the detection of viral DNA in the saliva. In recent years, several studies have evaluated the sensitivity and specificity of these methods, reporting only slightly lower values than those obtained from plasma.

Snietura et al. reported that the novel method of HPV status assessment using RT-PCR and superficial scraps appeared to be highly sensitive, specific and useful in predicting the clinical outcome, which is generally associated with an excellent prognosis and low risk of recurrences and disease-related death [92].

Differences between plasma and saliva are evident in the study conducted by Ahn et al. on the detection of HPV DNA. They reported that sensitivity of combined pretreatment saliva and plasma for detecting tumor HPV-16 DNA were 76.1%, while the sensitivities of pretreatment saliva or plasma alone were 52.8% and 67.3%, respectively. Moreover, positive post-treatment saliva HPV status was associated with higher risk of recurrence and reduced overall survival (p-value: 0.002), and this association does not apply to patients with HPV-positive status in plasma alone [93].

Comparison between CTCs and ctDNA

Recent studies indicated that ctDNA appeared to be a better prognostic marker than CTC count when combined analysis of tumor-specific mutations in ctDNA and CTCs was performed [18].

Even the studies analyzed show that CTCs are highly specific but not very sensitive biomarkers.

Both markers can be valid diagnostic and prognostic tools: the analysis of CTCs require more blood and their isolation is really difficult due to the rarity of these cells, but, on the other hand, the isolation of ctDNA is still not completely standardized.

Moreover, some cancer cases examined had detectable ctDNA levels but no detectable levels of CTCs [56]: while in most patients CTC number and ctDNA levels are mutually correlated, such cases illustrate that exceptions exist and that the underlying biology of both CTC and ctDNA release is still poorly understood [33].

Conclusion

Liquid biopsy represents a great promise for its potential to detect cancer before clinical signs occur. It represents a very advantageous tool, as it is rapid and minimally invasive in monitoring cancer: in particular its main applications should concern the early diagnosis of cancer, the prognostic evaluation, the assessment of treatment response and the evaluation of recurrences.

In relation to HNSCC compared to other cancer anatomic locations, the impact of liquid biopsy in the clinical setting is still limited: the study of CTCs and ctDNA has been increased in order to identify precise correlations between the tumor burden and biomarkers levels and to assess how these levels vary during and after treatments.

However, in order to do this, it is necessary to overcome the problem of tumor heterogeneity, by identifying the main mutations underlying head and neck cancers: in this regard an appropriate addition to the TNM staging system would be the inclusion of the molecular typing of the tumor, which would help to personalize therapies, decreasing the risk of overtreatment or undertreatment [94,95].

Despite the numerous analyzed biomarkers in these studies, we can consider as reliable only a limited number of these: for that concern CTCs the only standardized and reliable method that could be applied to HNSCC is the detection of EPCAM, while in relation to ctDNA, the main genetic alterations that can be correlated to head and neck cancers are those related to EGFR, RAS and TP53 genes, or viral DNA detection (HPV).

Finally, despite the several methods and technologies for CTCs and ctDNA detection, there is still a lack of standardized methods. In addition, there are no studies evaluating the influence that different therapies and different types of cancer may have on the detection of CTCs and ctDNA: the heterogeneity of the sample populations, the different therapeutic protocols and the different stages of the tumors included in the clinical trials still represent important limitations.

Anyway, results are promising and provide ground for more large-scale studies with standardized serial assessment of patient samples in the future.

Declaration of Competing Interest

None declared.

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