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Review Article

Liquid biopsy as a diagnostic and prognostic tool — A systematic review

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ABSTRACT

Liquid biopsy refers to a non-invasive technique of obtaining body fluid in order to analyse circulating biomarkers indicative of carcinomatous diseases. The characterization of these liquid biomarkers help in diagnosis and to choose the therapeutic strategy for each different case. Oral and oropharyngeal cancers are the largest group of those cancers which fall into the head and neck cancer category. Common names for it include mouth cancer, tongue cancer, tonsil cancer, and throat cancer. This systematic review summarizes 11 years (2009 -2020) studies done on liquid biopsy in oral and head and neck carcinoma from electronic search engines such as PUBMED, MEDLINE, GOOGLE J-GATE to get the overview of clinical significance of circulating biomarkers (CTCs, ctDNA, Exosomes, miRNA, lncRNAs, Cytokeratin 20mRNA) as diagnostic and prognostic tool. The impact of liquid biopsy in clinical settings is still limited thus allowing further studies in a bigger perspective to discover the best scenario for its application.

1. Introduction

Oral cancer is the sixth most common type of cancer with India contributing to almost one-third of the total burden and the second country having the highest number of oral cancer cases globally. In India, around 77,000 new cases and 52,000 deaths are reported annually due to oral cancer, which is approximately one-fourth of global incidences.[1] The cases of oral cancer are increasing widely in India and it has become matter of concern as about 70% of the cases are reported in the advanced stages (American Joint Committee on Cancer, Stage III-IV).[1] Oral cancer is preventable and curable if detected in early stages but as majority of cases are often diagnosed at advanced stages, chances of cure are very low, leaving five-year survival rates around 20% only.

Carcinogenesis is a complex process in which heterogeneity plays an important role in the development and progression. The development of oral cancer is related to numerous factors, including environmental and lifestyle risk factors.[2] Among these risk factors, the most frequently associated with oral cancer are alcohol intake and smoking.[3] Other causative agents of oral cancer are Epstein Barr virus (EBV), human papillomaviruses (HPVs) or Candida albicans infection responsible for the activation of prooncogenic stimuli.[4,5] All these risk factors are responsible for the development of both genetic and epigenetic alterations that can promote tumor development and progression by altering key cellular mechanisms, such as apoptosis or cell proliferation.[6] Thus molecular profiling plays role in early detection and better prognosis. In this sense, liquid biopsy has emerged as a revolution in the field of oncology.

The National Cancer Institute (NCI) defines liquid biopsy as “a test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for pieces of DNA from tumor cells that are in the blood”.

Liquid biopsy uses body fluids, such as blood, plasma, serum, urine, and gastric juice, to reflect the disease state.

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Saliva being non-invasive tool is easily accessible and is plentiful in resources of biomarkers for diseases as well as pre-symptomatic and health status. As oral cancer and potentially malignant disorders are exposed to oral cavity, salivary biomarkers may be useful for their early detection.[7]

The aim of this systematic review is to get the overview of clinical significance of circulating biomarkers as diagnostic and prognostic tool in oral and head and neck carcinoma.

1.1. History of liquid biopsy assays

1. As early as 1869, Thomas Ashworth first observed CTCs in a patient with metastasis.[8]
2. In 1948, Mandel and Metais first detected and quantified cfDNA in both healthy and diseased patients.[8]
3. In 1965, Bendich and collegues hypothesized that cf DNA could be involved in metastasis.[8]
4. In 1966, Tan and colleagues observed high levels of cfDNA in SLE patients.
5. In 1977, Leon et al. revealed cfDNA in oncology patient.[8]
6. In 1994, specific mutations in cfDNA were detected by scientists.[8]
7. In 2000, Veridex introduced first commercially available liquid biopsy assay, the CELLSEARCH CTC test.[8]
8. In June 2016, FDA approved first liquid biopsy test, the cobas EGFR Mutation Test.[8]
9. In August 2020, FDA approved two new tests which use next-generation sequencing (NGS) to target genes in advanced cancer patients, including mutations. These are Guardant Health's Guardant 360 CDx assay, and FoundationOne Liquid CDx, marketed by Foundation Medicine.[9] "These tests allow oncologists to focus care on the molecule of origin rather than the cell type," said William G. Cance, MD, chief medical and scientific officer of the American Cancer Society. "Approval of these tests is another step in the pathway to precision medicine and targeted therapeutics."

2. Material and Methods

1. *Search stratergy:* This systematic review comprises an analysis of 11 years study articles done on potential biomarkers of oral carcinoma in liquid biopsy. A search limited to English language articles published between 2009 and 2020 was performed via PubMed, Medline, Google J-Gate. Keywords that were used are liquid biopsy, oral cancer, head and neck carcinoma, circulating biomarkers, precision medicine.
2. *Review methodology:* Review of full texts, abstracts, review articles, case studies of relevant topic were selected and evaluated to match our inclusion criteria of clinical significance of circulating biomarkers in oral and head and neck carcinoma as diagnostic and prognostic tool.
3. *Data collection and extraction:* The articles were collected and evaluated independently by the reviewer.
4. *Evaluation of articles:* The articles selected as per inclusion criteria were evaluated for the type of study and newer technologies to extract those biomarkers were also ruled out along with future perspectives.

3. Results

The systematic review was done to rule out the clinical significance of circulating tumour markers (biomarkers) from body fluids in oral squamous cell carcinoma (OSCC) or oral carcinoma and Head and Neck carcinoma (HNC) as diagnostic and prognostic tool.

A total of 87 articles were analysed. Among these 11 articles were similar,18 articles were related to other cancers of the body (breast cancer, lung cancer, prostate cancer, gastroesophageal cancer, pancreatic cancer, liver cancer, etc.) therefore a total of 58 articles were included for this review study given in the flow diagram (Figure 1).

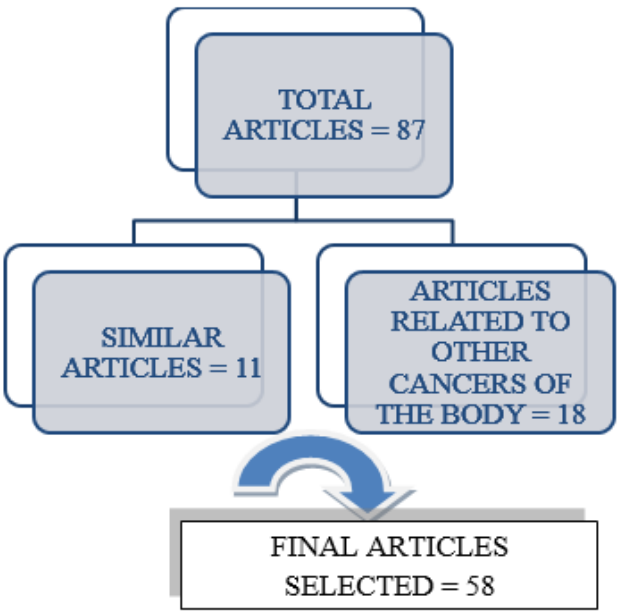

Fig. 1: This figure shows the overall process from the literature search to data retrieval in the form of a flow diagram.

4. Discussion

The evidence from the research collected from these selected studies reveals the potential of liquid biopsy as an important prognostic and diagnostic tool in carcinomatous cases. It has emerged as a potential alternative to tissue biopsy. Tumors shed cells and genomic material into the

Table 1: The overall version of this review study is quoted in Table 1.

Sl No.	Study design	Reference articles
1	Prospective study	3 [10–14] [15–17] [18] [19–24] [1,6–61] [1,4,25]
2	Retrospective study	26–29 [31,33,34] [12,35,36,38] 39,40 [1,3–17,25–44] 46,47 [51,53] 22,56 62,63 [64] 49 [65]
3	Case report	

blood, due to cell necrosis or apoptosis. These circulating biomarkers are listed below with their clinical significance. Schematic representation is shown in Figure 2.

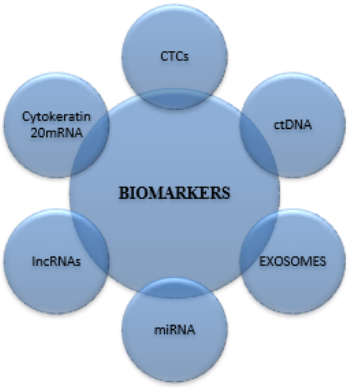

Fig. 2: Schematic diagram of circulating biomarkers.

4.1. *Circulating tumour cells (CTCs)*

CTCs are intact tumor cells which are shed from both primary tumor and metastatic sites into the blood stream. The number of CTCs present is very low counting One CTC per 10^6-10 leukocytes /ml of blood, with even lower in early stage of cancer. Studies showed that an average metastatic carcinoma patient shows that 5-50 CTCs for approximately 7.5ml of blood.[53] These CTCs represent a highly dynamic cell population as they are characterized by high heterogeneity at the genetic, transcriptomic, proteomic and metabolomic levels.[66] The phenotypic and genotypic characteristics of CTCs which can change during the course of the cancer by microenvironmental and therapeutic selective pressure. As CTCs counts run in parallel with the tumor burden of the disease, they serve to be a more accurate method for the real time monitoring of cancers than many other commonly used soluble biomarkers[53] shown in Figure 3.

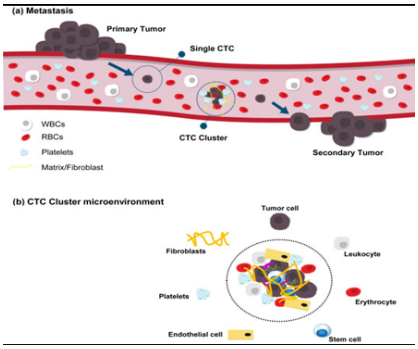

Fig. 3: (a) Circulating tumor cells (CTCs) detach from primary tumor as single cells and clusters, shed into the bloodstream, and migrate to colonize in distant organs, known as metastasis. It is assumed 1 ml of blood can comprise 1–10 single CTCs and roughly one CTC cluster, millions of WBCs and billions of RBCs. (b) The microenvironment of CTC cluster comprises immune cells, platelets, dendritic cells, cancer-associated fibroblasts, and tumor stroma. Such microenvironment can protect CTC clusters from blood shear damage and immune attacks that provides CTC cluster metastatic advantages.[67]

4.2. *Detection & isolation*

1. EpCAM — affinity based: CellSearch system, CTC-Chip
2. Physical properties-based: ISET, ScreenCell, Apostream, density gradient centrifugation.
3. Other methods: FAST, EPISPOT, PRO Onc Assay.

Isolation is followed by PCR amplification and next generation sequencing (NGS). These help in determining Hypermethylation, hypomethylation, deletions, amplifications, chromosomal rearrangement and mutations.[53]

4.3. *Clinical significance*

1. It can be used in prognosis.
2. CTCs are useful in ruling out diagnosis and could be a substitute for tissue biopsy in cases of inaccessible neoplastic sites.
3. Research is going on to use CTC as a mode to perform cancer screening.
4. Data shows that CTC count has a potential role in real-time monitoring of response to therapies.[30]
5. CTCs can also provide information on the epigenetic changes in the cancer cells.[66]

4.4. *Role in oral cancer*

CTCs have been mainly seen as prognostic and recurrence predictor in oral cancer studies.[66]

According to a study done by Partridge et al. in 2003, levels of disseminated tumour cells (DTCs) preoperatively and intraoperatively in both blood and bone marrow from

40 OSCC patients were evaluated. Presence of DTCs are associsted with high risk of loco-regional reccurence ad distant metastasis. Besides, their presence was also correlated with lower distant metastasis-free-survival and disease-free survival rates.

Another study was performed by Gröbe et al. to determine the level of CTC in various OSCC stages as prognostic marker. It was analysed that DTCs present in 20% of patients, while CTC around 12.5% with a range of 1–14 CTCs/7.5 mL. CTC detection was significantly correlated with tumour size, whereas disseminated DTCs were significantly correlated with the nodal status.

4.5. Limitations

1. Heterogeneity of CTC populations.
2. Low abundance and fragility.
3. CTC detection requires sensitive and specific analytic methods.
4. Multiplicity of technologies used for CTC isolation.[30]

4.5.1. Circulating tumour DNA (ctDNA)

ctDNA is tumour DNA which got degraded from cell free DNA fragments of tumour origin into the bloodstream. Its production depends on tumour burden, cancer stage, cell turnover and response to therapy.[53] ctDNA concentration is significantly increased in malignancy compared to that of healthy individuals.[30] The diagrammatic representation of ctDNA formation is shown in Figure 4.

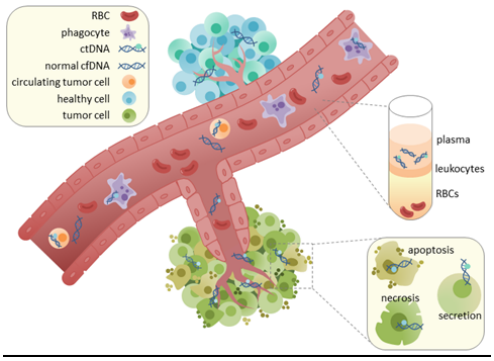

Fig. 4: Circulating tumor DNA from tumor to blood.[68]

4.5.2. Detection & isolation

1. Classical methods include — quantitative real-time PCR, fluorescent assays and spectrophotometric strategies.
2. Digital PCR-based technologies.
3. Next-generation sequencing technology.[66]
4. Targeted plasma re-sequencing (TAm-Seq), Massively paralleled sequencing (MPS), Whole-genome sequencing (WGS), Whole exome sequencing (WES).[58]
5. Sanger sequencing and Pyrosequencing.[51]

4.6. Limitations

1. Assay Sensitivity and Specificity for analysis of ctDNA[58]
2. To Discriminate ctDNA from cfDNA[66]
3. Assay standardization and Quantification[30]

4.6.1. Clinical significance

1. Cancer screening
2. ctDNA helps to detect the minimal residual disease as the amount of ctDNA formed is proportional to the residual tumor burden.
3. Response and follow up to monitor prognosis
4. To detect molecular alteration associated with resistant therapy[64]

4.6.2. Role in oral cancer

1. Shukla et al. analysed quantity of ctDNA in OSCC by spectrophometry method and found no significant difference between OSCC patients and healthy individuals.
2. Perdomo et al. reported detection of ctDNA mutations in HNC including oral cavity cancer
3. Mazurek et al. analyzed HPV detection in OSCC patients using of DNA[66]

4.6.3. I. Exosomes

Exosomes are small membrane-bound vesicles released by different cells of the body including tumour cells. It was first reported by Pan and Johnstone in 1983.[53] Exosomes consist of a lipid bilayer which contains both transmembrane and nonmembrane proteins, noncoding RNAs, mRNAs, miRNAs, DNA.[30]

The process of exosomes formation (Figure 5) begins with invagination of the plasma membrane of the cell to form small rounded structures called endosomes. As endosomes mature, inward budding of the endosome membrane results in the formation of numerous intraluminal vesicles(ILVs) which later gets released into the extracellular space, now called exosomes. Exosomes play an integral role in intercellular communication by transmitting signals and transferring contents. Exosomes are released from most cell types and can be found in all bodily fluids including urine, plasma, saliva, cerebrospinal fluid, amniotic fluid, and breast milk.[69]

4.6.4. Detection & solation

1. Conventioinal methods- Western blotting & ELISA
2. New methods
3. Nano-plasmonic sensor
4. BEAMing
5. DdPCR
6. Microfluidic exosome analysis[53]

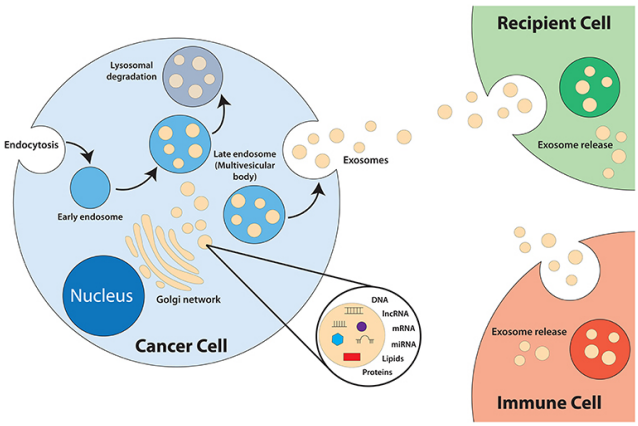

Fig. 5: Formation of exosomes.[69]

4.7. *Limitations*

- Difficult extraction and Standardization.[30]

4.7.1. *Clinical significance*

1. Tumour derived Exosomes increase TGF-β signaling pathway which contributes to progression & drug resistance of OSCC.[66]
2. Correlation found between exosomal miR-21 levels and metastasis in lymph nodes by Li et al.
3. Oral fluid-derived exosomes have been characterized morphologically in oral cancer by 3 markers CD63 (high), CD9 & CD81[Zlotgorski- Hurvitz et al.]
4. To discriminate between active-disease cancer patients & no evident disease after oncologic therapies [Ludwig et al.]
5. In cancer, exosomes are secreted by the neoplastic cells to the TME and promote tumor growth, invasion and metastasis (Zhang and Grizzle 2014).

4.7.2. *MiRNAs*

Micro-RNAs (miRNAs) are one of the important components of the cell-free nucleic acids available in different body fluids.[35] They circulate in bloodstream and are highly stable not even degraded by RNase enzyme. Thus miRNAs can be a reliable cancer biomarker.[10–12] Exosome-miRNAs are reported to represent a subset of about 3% of the entire amount of cellfree miRNAs.[13,14]

4.7.3. *Clinical significance*

According to various studies (Figure 6),

1. mir-371, mir-150, mir-21 and mir-7d were found to be potential prognostic markers.
2. mir-134, mir-146a, mir-338 and mir-371 were associated with metastases.
3. mir-21 and mir-7d were correlated with resistance to chemotherapy, while mir-375, mir-196 and Mir-125b with sensitivity to radiotherapy.[35]

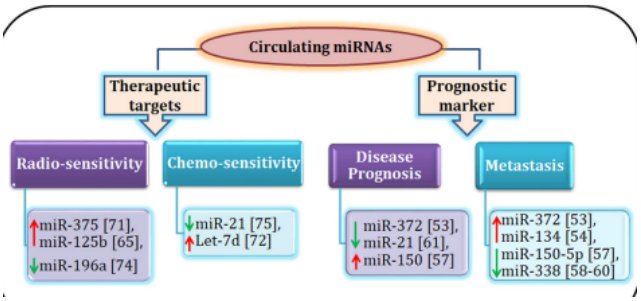

Fig. 6: Summarized representation of circulating miRNAs reported as therapeutic and prognostic markers in OSCC. The red up and green down arrows represent the high and low levels of miRNAs in the body fluids of OSCC patients with respect to disease prognosis or response to therapy.[35]

4.7.4. *III. lncRNAs*

LncRNAs are defined as RNA transcripts which are greater than 200 nucleotides in length with no or limited protein-coding potential. These are important for various processes, like gene imprinting cell differentiation and organogenesis and play an important role in tumorigenesis and metastasis. Some lncRNAs can be detected in exosomes, which are widely present in body fluids.[39]

4.7.5. *Detection & isolation*

They are isolated from exosomes by RNA extraction techniques such as phenol based techniques, combined phenol and column based approaches and pure column based methods.[40] The methods used to detect lncRNAs are described in Table 2.

4.7.6. *Clinical significance*

1. It can be used as diagnostic and prognostic marker in various types of cancers.
2. It can be used in cancer-targeted therapy as they are associated with drug resistance of tumour cells.[39]

4.7.7. *Role in oral cancer*

Tang et al. found relative abundance of lncRNAs in tissue or saliva samples of OSCC patients was investigation. Subsets of lncRNAs are expressed across non‑tumor, tumor and metastatic tissue samples. Besides, lncRNAs present in whole saliva can be used as potential marker for OSCC.[16]

4.7.8. *Cytokeratin 20mRNA*

According to a study done on postoperative peripheral blood samples of 40 OSCC patients to detect cytokeratin expression by real-time quantitative RT qPCR, it was found that

Table 2: Methods used for detecting lncRNAs.[40]

Method	Principle	Advantages	Disadvantages
Northern blot	Electrophoresis and detection with specific probe	-Fast, low-tech, cheap	-High risk of sample degradation
		-Alternative splicing products can be detected	-Low sensitivity
		-Both quantitative and qualitative method	-Only known sequences detected
		- High specific	
RT-qPCR	Transcript amplification and fluorescence signal detection after specific probe hybridization	-Cost-effective	-Splicing products no detected
		-Time-efficient	-Nonspecific binding
		- High sensitivity and specificity, l	- Maximum 4 different mRNAs can be detected simultaneously
		- Low amount of starting material	- Only known sequences detected
		- Results easy and fast to obtain	
Microarrays	Molecular hybridization to detect the expression levels	Multiple mRNAs can be analyzed in the same experiment, well defined and standardized protocols, relatively low cost	-Detection of known sequences
			-Non-specific hybridization
			-No identification of mRNA variants
			-High variability of low expressed mRNAs
RNA-seq	Next generation sequence based	-Independency from previous sequence information	-High cost
		-High dynamic range	-Complex analysis of data
		-Several isoforms of mRNA can be detected	
		-Low amount of starting material is required	

1. CK 17 and CK 19 was not detected in any sample.
2. CK 18 and CK 20 were detectable in 1 (2.5%) and in 14 (35.0%), samples respectively.
3. CK 20 was not associated with lymph node status, clinical stage, or differentiation grade, but was significantly higher in patients with T3 and T4 OSCC ($p = 0.04$).
4. Disseminated Tumour Cells in peripheral blood mononuclear cells of OSCC patients could only be detected by determination of CK 20 mRNA. Thus, detection of CK 20 mRNA in peripheral blood seems to be of relevance for prognosis in OSCC.[17]

4.7.9. Pros and cons

1. Liquid biopsy is quicker than tissue biopsy[37]
2. It can be used for real-time monitoring to see if therapy is working
3. It can be repeated easily to see the status of tumor cells or cfDNA[37]
4. Liquid biopsy tests can be used to monitor patients for mutations[37]
5. In 2019, according to National Comprehensive Cancer Network (NCCN) guidelines older liquid biopsy tests should not be replaced by tissue biopsy.[37]
6. Cance added that tumors are heterogenous, so it is impossible to know whether a liquid biopsy test analyzes DNA that represents a fraction of the tumor mutations or all mutations.[70]
7. FDA approvals call for use of Guardant 360Dx and FoundationOne Liquid CDx in conjunction with tissue biopsy. The NCCN guidelines do suggest that liquid biopsy alone can be considered in patients who are not healthy enough for biopsy or who cannot provide sufficient tissue samples, Diehn pointed out.[37]
8. New liquid biopsy tests are "not as accurate as looking at tissue. However, the technology is getting more sensitive," added Papadopoulos.[37]

5. Conclusion

In spite of many advantages, the impact of Liquid Biopsy in clinical setting in oral cancer is still hindered by many hurdles. Broadly, liquid biopsy is a quick and comparatively straightforward process that usually includes a simple blood draw, extraction of nucleic acids from the blood plasma, and amplification of the molecular targets to enable analysis of the defined bio markers.

Therefore, further studies should be conducted to overcome the challenges and assess its wider clinical application.

6. Abbreviations

1. CTC — Circulating tumour cell
2. CfDNA — Cell free DNA
3. CtDNA — Circulating tumour DNA
4. EGFR — Epidermal growth factor receptor
5. EpCAM — Epithelial cell adhesion molecule
6. ISET — Isolation by size of tumour cells
7. FAST — Fibre-optic array scanning technology
8. EPISPOT — Epithelial immunospot
9. PCR — Polymerase chain reaction
10. MRNA — Messenger RNA
11. ELISA — Enzyme linked immunosorbent assay
12. BEAMing — Beads emulsion amplification magnetics
13. DdPCR — Droplet digital PCR
14. TGF-β — Transforming growth factor beta
15. TME – Tumour microenvironment
16. CDx – Companion diagnostic

7. Source of Funding

None.

8. Conflict of Interest

None.

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