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LIQUID BIOPSY IN ORAL SQUAMOUS CELL CARCINOMA – A REVIEW

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ABSTRACT
Squamous cell carcinoma affects multiple sites of the body and exhibits high mortality rate. Early detection of the disease is the key to successful treatment of the condition. Routine tissue biopsies are often delayed because of difficult in accessibility, associated medical conditions and invasive nature of the procedure. Hence a diagnostic procedure is needed that will be easy to perform and will detect the tumour at very early stage. Tumours during their course of development release cellular components, DNA fragments and exosomes into the body fluid. These biomarkers can be analyzed from body fluids through a non-invasive method which is called as "Liquid Biopsy". In near future it will occupy a prominent role and pivotal locus in the subject of diagnostic pathology. In this review, basic concept and clinical application of liquid biopsy will be discussed.

KEYWORDS: Circulating tumour cells, Circulating tumour DNA, Exosomes, Liquid biopsy, Squamous cell carcinoma.

INTRODUCTION
Head and neck squamous cell carcinoma (HNSCC) is heterogeneous disease at clinical and molecular level. Invasion of tumour cells occurs through lymphatic and haematological route, thus causing metastasis.[1,2,3] There are two main techniques used for diagnosis of any malignancy: pathology and imaging. Pathological diagnostic technology, still a golden standard, mainly includes tissue biopsies, serological indicators test and molecular pathology test.[4] Tissue biopsies seldom reflect tumour heterogeneity and behaviour. Biopsies from multiple sites and repeated biopsies to determine progression or prognosis of any tumour, both give trauma to patients. Sometimes one single biopsy is also difficult to perform. On the other hand, imaging methods for diagnosis of malignancy include ultrasound, conventional radiographs, CT, MRI, PET-CT, endoscopy etc.[1,4] In serological indicators test, it lacks effective biomarkers for early diagnosis.[4] No such specific biomarker is available for head and neck cancer till now.[5] For this reason, highly specific and sensitive biomarkers are needed for tumour diagnosis and to monitor tumour progression dynamically. Effective tools to monitor the biological behaviour of potentially malignant disorders, minimum residual diseases, appearances of recurrences and susceptibility of the tumor to the various modes of treatment are an urgent necessity. To overcome all these problems, "Liquid biopsy" can be used as an important diagnostic tool. It is a less invasive method to monitor the real-time dynamics of cancer that can be repeated at ease, in a cost effective manner.[1,4]

Tumor cells, DNA and exosomes are released by tumour cells into the body fluid (eg. blood, urine and saliva) [6]. Liquid biopsy helps to diagnose and monitor tumor initiation and progression by detecting those biomarkers. The capacity to measure tumour cells circulating through the vasculature provides definitive evidence of the aggressiveness of a tumour prior to detection of identifiable metastases.[2] This concept offers a test approach called "liquid biopsy". We can also suggest the term "Tumour targeted blood test" instead of the term "Liquid Biopsy". Liquid biopsy can be defined as diagnosing and monitoring tumour progression by detecting and measuring specific biomarkers in the body fluid. It is defined by the U.S. National Cancer Institute (NCI) as "a test done on a sample of blood to look for cancer cells from a tumour that are circulating in the blood or for pieces of DNA from tumour cells that are in the blood". As multiple liquid biopsies can be taken at different time points to detect tumour progression or treatment outcome, so we can called as "dynamic biopsy", which is not practically possible in "static

biopsy". Less side effects, ease of operation, rapid testing and less diagnostic bias from tumour heterogeneity are also other advantages of liquid biopsy.[2,4,5,7-10] So, there will be a wonderful future of liquid biopsy in the field of early diagnosis, tumour drug design, elucidating drug-resistant mechanism, estimation of tumour's grade and stage, judging prognosis, guiding treatment plan, predict response and identify the failure of treatment early, thereby allowing a timely shift to therapeutic strategy.

CIRCULATING TUMOR BIOMARKERS

Epithelial-mesenchymal transition (EMT) and its reverse process, mesenchymal-epithelial transition (MET) play an important role in metastasis. Epithelial cells with increased migratory capacity are endowed by these processes. These processes also cause increased resistance to apoptosis. After entering the EMT/MET stage, tumour cells can detach from the primary site, enter into the circulation and invade through the surrounding tissue which are called circulating tumor cells (CTCs).[1,9-13] Circulating tumor DNA (ctDNA) which was first identified in 1948, is extracellular cell free DNA (cfDNA) which enter into the circulatory system after shedding from tumour cells, carrying somatic mutations through the mechanisms, like apoptosis, cellular necrosis, phagocytosis or exocytosis and are rapidly degraded by blood nucleases because of short half life. The degradation products are eliminated by the liver, spleen and kidney. Liver or renal disease may give false positive results of ctDNA level, thus cause problem in interpretation of tumour progression. They may present in many forms; free DNA, bound to protein complexes, cell surface bound or in vesicles (apoptotic bodies, microvesicles and exosomes).[1,5,10,14]

In 1977, Leon et al indentified that ctDNA level increase in blood in case of malignancy. They concluded that tumours release DNA into bloodstream. DNA from normal cells and ctDNA are differentiated by the presence of cancer-specific genomic alterations, such as, point mutations and the difference in DNA fragment base pair length.[5,15] ctDNA can be detected in urine, cerebrospinal fluid, saliva and other body fluids apart from blood.[1,13,16]

We have to know the role of CTCs in cancer metastasis, so that we can understand the detection method.

Tight vascular wall barriers may hamper the CTCs formation. The mechanism of CTC formation is described in Fig 1.[11,17,18]

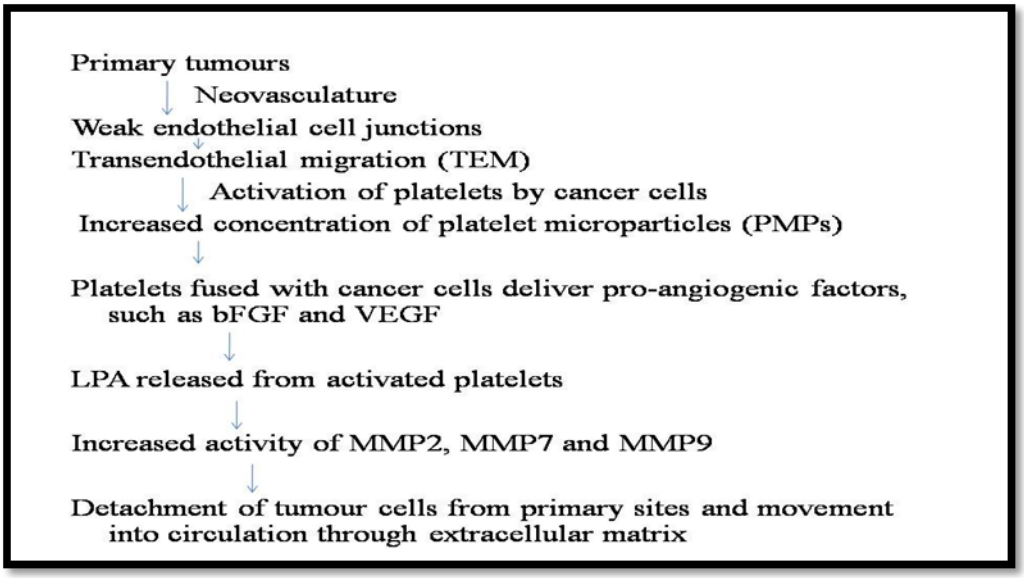

Fig 1: CTC formation (bFGF: Basic fibroblast growth factor; VEGF: Vascular endothelial growth factor; LPA: Lysophosphatidic acid).

After entering into circulation the fate of CTC is discussed here.

1. Survival of CTC in the circulation.

When CTCs are leaving their supportive microenvironment and entering into circulation, they face many survival challenges, such as immunological attack, shear forces and apoptosis. Majority of CTCs are destroyed due to these challenges and only less than 0.1% of CTCs survive. Survived CTCs help to release some agonistic mediators, such as ADP, thrombin, TXA2 and tumour-associated proteinases. Tumour cell-induced platelet aggregation (TCIPA) is increased by survived CTCs through direct contact or by those released agonistic mediators. Platelets are then adhere with the surface of CTCs through GPIIb-IIIa-fibrinogen bridge and up-regulated P-selectin.[11,17,19,20] Platelet-coating helps CTCs to survive in the circulation from immune attack by NK cells through transferring the major histocompatibilty complex (MHC) to CTCs mimicking them as host cells. Tumour educated platelets (TEPs) play very crucial role and carry information on the location of the tumour in their mRNA. Actual status

of metastatic lesions can be assessed through TEP mRNA profile.[8,11,14,17,21]

2. CTCs at distant site.
With the help of platelet coating, CTCs reach in distant organs through circulation. CTCs anchor to the luminal side of vascular endothelial cells and then break through the subepithelial extracellular matrix (ECM). Epithelial-mesenchymal transition (EMT) is induced by platelet-derived TGF-β and PDGF through triggering several specific signalling pathways. EMT helps CTCs to avoid apoptosis and vascular permeability.[7,11,17]

CTC DETECTION

CTCs can be detected by real time polymerase chain reaction (RT-PCR) through the identification of tumour-specific antigens, but one major disadvantage of this technique is lack of visual confirmation of the tumour cells. Though RT-PCR is an efficient and reproducible technique, but non-viable cellular material or amplification of non-specific genomic material can give false positive result in this method.[2,5,12,22,23]

Epithelial surface antigens (Epithelial cell adhesion molecule, i.e EpCAM) are targeted on the surface of CTCs. EpCAM can be "pulled out" of the blood sample for CTCs detection, but this method is applicable if metastatic cells are EpCAM positive because sometimes metastatic cells display significant plasticity and then they lose EpCAM expression.[2,7,23,24]

Another method for CTCs identification is cytokeratin (CK). CK 8, 18, 19 and 20 are detected in CTCs of head and neck malignancy. Weller et al conducted a study where they noted that N-cadherin (mesenchymal origin) and CD133 (stem-cell origin) were present with CTCs instead of CK in head and neck carcinoma.[2,22]

There is no such optimum CTCs detection technique among the positive selection methods as there is no normative value of circulatory CTCs as well as nonexistent surface markers. In negative depletion method, normal blood cells are removed first as many as possible so that number of CTCs can be detected. At first, RBCs are removed by lysis through immunomagnetic separation and the remaining cells undergo nuclear staining with DAPI (40,6-diamidino-2-phenylindole) followed by immunocytochemical staining.[2,16,22]

The field of microfluidics is also important in CTCs detection. Microfluidic "chip assay" method is very sensitive and specific method. Here fluid is applied to a "chip" which acts as a micro screen for detecting CTCs through DNA or RNA aptamers. It is found in many studies that this approach is highly sensitive and specific tool.[2,22,23]

There are some technological difficulties in isolation and identification of CTCs from millions of normal hematogenous cells. For this reason, ctDNA analysis can be an alternative to CTCs analysis. ctDNA fragments are also diluted with huge amounts of cfDNA from normal cells, which can be a limitation for further molecular analysis.[1,15]

Hypermethylation almost always occurs in neoplastic cells. The status of DNA methylation is very stable. The examination of abnormal methylation patterns in ctDNA, released from these cells, may help in the early detection of cancers with high sensitivity. This can be used to monitor tumour related processes and as screening and prognostic tool.[12]

Additionally, exosomes and microvesicles have also been found in blood and saliva, especially in head and neck cancer. Cancer-cell-derived exosomes seem to be able to modify tumor cell movement and metastasis.[1]

Saliva is the most popular body fluid under investigation to detect oral cancer. Advantages include accessibility in a non-invasive way, low contamination of normal material (cells, DNA, RNA, and proteins) and inhibitory substances and also less complex in comparison to blood. For head and neck tumour, the use of saliva fluid can be more useful than the exfoliated cells because swab can't be easily taken from some tumour due to its location. Especially in oral cancer, saliva sample is considered very important to search early biomarkers due to direct contact with lesions. The sensitivity of detection of tumour derived DNA from saliva is site dependent. Saliva plays important role in tumours of the oral cavity and plasma plays important role for detection of tumour derived DNA in other sites of head and neck region.

CLINICAL APPLICATION

The concept of liquid biopsy can be used to identify the disease, disease recurrence as well as disease progression.

1. Diagnostic biomarker

There are significant differences in the amounts of plasma DNA isolated from healthy individuals, patients with benign disease and cancer patients. Significantly higher concentrations of cfDNA are present in cancer patients and the quantification of cfDNA can confirm the presence of cancer or disease-free status, but only the amount of cfDNA is not a useful diagnostic tool. There are some clinical situations in which cfDNA concentrations are below optimal amounts for the detection of mutations. Recently reported diagnostic approaches involve the use of plasma EBV DNA analysis for detecting early nasopharyngeal carcinoma in individuals without a clinical suspicion of nasopharyngeal carcinoma.[6]

2. Prognostic biomarker

Some 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.[6] The ability to use ctDNA as a biomarker of disease recurrence in the post treatment surveillance phase is more valuable than its diagnostic merits.[5,13]

Oliveira-Costa et al performed whole genome sequencing of oral cancer patients using microarray analysis to identify additional biomarkers. They found that PDL-1 (programmed death ligand) expression was upregulated in the primary tumour when tumour size increased. They then examined the association of circulating tumour cells with PDL-1 and disease specific survival in a separate cohort of patients. They found an improved short-term survival benefit with the presence of cytoplasmic PDL-1 on CTCs. EGFR has also been suggested as a possible CTC biomarker for disease aggression in head-neck carcinoma. In a study, only 25% of head and neck tumours expressed EGFR. In another study, Tinhofer and colleagues also measured EGFR and its phosphorylated form, pEGFR in patients with head-neck carcinoma. In that study, Cetuximab targets EGFR, and highlights the possibility of measuring clinical response to targeted therapy with serial CTC measurement.[2,13,23]

3. Predictive biomarker

Podoplanin (PDPN), a trans-membrane protein, is involved in lymphatic formation. In some studies, it was found that PDPN can be used as a biomarker in head-neck CTCs and a possible predictor of poor outcomes in squamous cell carcinoma of head-neck region. In some studies, it was found that PDPN can be lost during de-differentiation in squamous cell carcinoma, so lack of PDPN may not an indicator of improved prognosis.[2,6,,23]

Microsatellite instability (MSI) and loss of heterozygosity (LOH) are included in microsatellite alterations. LOH is a more useful prognostic predictive marker than MSI in head and neck carcinoma. The LOH is associated with advanced high-grade disease and is a negative prognostic indicator of survival, with suggested evidence of a correlation with chemotherapy resistance.[5]

Because the dynamics of the release of ctDNA have not been fully elucidated and the timing of ctDNA analysis in relation to therapy may be important, multiple samples at different time points should be obtained from the same patients. For instance, monitoring ctDNA shortly after and during drug administration might reveal various allelic fractions of ctDNA and may provide valuable information on kinetics of ctDNA release.

FUTURE SCOPE

It is not clear yet whether ctDNA is representative of all relevant metastatic cell clones located at different sites or whether ctDNA represents DNA from distinct subclones that can promote clinical progression and/or therapeutic resistance. More studies should be done on comparative sequence analyses of plasma DNA and biopsies in combination with imaging studies. Detailed functional studies are also needed to assess clinical progression and therapeutic resistance. As intratumoral heterogeneity is not solely based on genetic changes, epigenetic changes should also be considered. Epigenetic changes are considered as an early event in carcinogenesis and may be a suitable marker for early detection.[6]

The identification of tumor‑specific genetic variations and epigenetic alterations has implications in diagnosis and assessing recurrences and therapeutic resistances. More studies should be done on these types of variations in ctDNA.[12,25]

CONCLUSION

Liquid biopsy can be a minimal or non-invasive tool with great potential to help in early diagnosis, prognosis, surveillance and treatment monitoring of cancer. An unbiased, multi-marker approach detection method is helpful in order to identify the heterogeneous population of cells. Liquid biopsies applications in head-neck carcinoma have emerged a great development, still more studies are needed to validate its application in clinical practice for a significant impact in the patient's life. Standardization of detection protocols is also needed in unifying the clinical recommendations. A better understanding of application and limitations of this technology is also required to implement it on a large scale.

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