REVIEW

Circulating Tumor Cells – A Review

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Abstract

Context: Circulating tumor cells (CTCs) are those cells that separate from the primary tumor to enter circulation. This is facilitated by Epithelial -Mesenchymal Transition (EMT) or through Non EMT based modalities by passive entry into circulation. CTCs are responsible for causing distant metastasis.

Objectives: This review article briefly describes few of the mechanisms of CTC production and survival and few methods that are used to detect CTCs

Materials and Methods: Data was collected and analyzed from published literature and electronic database searches of PubMed and Google Scholar.

Result: CTCs acquire genetic alteration that differentiates them from the primary tumor. Majority of CTCs do not survive in the circulation but the few that do, do so by adapting various survival mechanisms.

Conclusion: Detection of CTCs help in the early diagnosis of cancer, providing patient tailored therapy and for monitoring of cancer.

Key words: Circulating Tumor Cells, cancer, metastasis, epithelial-mesenchymal transition

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Introduction

Prof. Ashworth first described Circulating tumor cells (CTCs) in the year 1869[1]. These are the cells that detach from the primary tumor, following which they undergo epithelial to mesenchymal transition to enter the blood and lymph circulation[2]. These cells can act as seeds to develop secondary tumors and cancer metastasis which is responsible for cancer related death.[3,4] To date most research concerning CTCs are based on the CTCs found in circulation[5]. CTCs are seen in the peripheral blood of cancer patients. These can be appreciated even in the early stages of cancer. Previous studies have reported that CTCs are present fewer than 10 cells per milliliter in blood[6]. Circulating tumor cells have been isolated and studied in various cancers like gastric & colorectal cancer, breast cancer, thoracic cancer[7,8,9]. It has also been in studied in Oral Squamous Cell Carcinoma (OSCC)which accounts for 90% of oral cancer[10]. OSCC is considered to be the sixth most common malignancy in the world and positions itself at the top three in India[11,12]. Although there have been advances in diagnostic and therapeutic modalities, prognostic rate remains low with patients developing distant organ metastases. The 5 year survival rate continues to remain around 50%[13]. It can act as seeds for metastasis for cancer and it is reported that 90% of cancer related death is caused due to metastasis[14]. Among the oral malignancies, oral squamous cell carcinoma is considered to be the most common with high morbidity and mortality rate[15]. Morbidity rates and mortality rates associated with OSCC would decrease if detected early[16].

Circulating tumor cells could be used for early diagnosis of cancers, its recurrence and in determining therapeutic efficiency[4]. Molecular understanding of CTCs provides a diverse range of tumor information. This ranges from understanding the mutational burden and consequential genotypic changes in the cancer cell type, tumor progression, prognosis etc. An integrated under-

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standing of these parameters can therefore be used for personalized therapy.[4,2,17,18]

EPITHELIAL TO MESENCHYMAL TRANSITION: Role in generation of CTCs.

The generation and release of CTC into the blood are primarily described by two mechanisms namely epithelial mesenchymal transition (EMT) and non EMT mediated invasion[6]. Epithelial Mesenchymal Transition (EMT) contributes to majority of the CTC generation. Through the process of EMT the cells lose their properties of adhesion and polarity and achieve migratory and invasive properties[19]. In EMT many epithelial proteins such as E-Cadherin, cytokeratins, and epithelial cell adhesion molecule (EpCAM) are down regulated and many mesenchymal proteins such as fibronectin, vimentin, and N-cadherin are upregulated. Certain EMT- Transcription factors like Snail1, ZEB1, Twist etc play a regulatory role during EMT. Hypoxia and extracellular molecules present in the tumor microenvironment also induce EMT[6,19].

Figure 1: Generation of CTCs from a primary tumour and subsequent metastasis. Cells are shed off from the tumor. Most of them gets degraded in 5min and the few that do survive undergo epithelial mesenchymal transition and enters circulation. It travels to distant locations to cause secondary tumors and metastasis.

In EMT mediated invasion, there is expression Matrix Metalloproteinases[20,21] active destruction of the zonaoccludens, desmosomes and hemidesmosomes and the basement membrane and enter into circulation. CTC generation promoted by EMT occurs by encouraging intravasation and by aiding survival in the peripheral blood. EMT regulatory network promotes angiogenesis and enable intravasation[21]. Phenotypic changes to a spindle shape during EMT is also reported[20,21].

Xiao in 2017[21] have classified EMT markers as epithelial makers egEpCAM, Cytokeratins, E-Cadherin, mesenchymal markers (N-Cadherin, Vimentin, Fibronectin) and regulators (TWIST, Snail, ZEB1/ZEB2, Akt, P13

Studies have shown that metastasis can occur even on suppression of factors that regulate EMT. There was a study by Godinho, which reported that cell-cell adhesion could be disrupted by increasing Arp2/3-dependent actin polymerization.

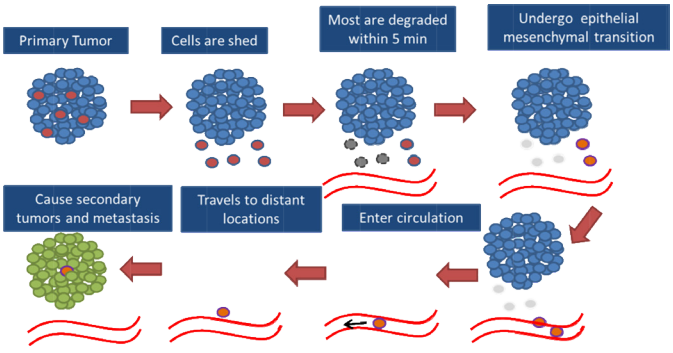

Fig.1: Generation of CTCs from a primary tumour and subsequent metastasis. Cells are shed off from the tumor. Most of them gets degraded in 5min and the few that do survive undergo epithelial mesenchymal transition and enters circulation. It travels to distant locations to cause secondary tumors and metastasis.

In Non-EMT mediated invasion, there is a passive entry of CTCs into circulation. This is owing to external factors such as a growing tumor and mechanical forces such as surgery. Once CTCs enter into the circulation irrespective of how it was generated - it presents itself as a single cell or a cluster of cells with different EMT phenotype which can further be recruited to platelets and macrophages that assist in migration.[6,22] Most CTCs undergo apoptosis due to oxidative stress, harmful elements in the blood or due to lack of cellular attachment but the few that survive lead to secondary tumors[23].

CTC BIOLOGY

CTCs acquire genetic alterations that distinguish them from primary tumor cells. Genes that are affected are primarily related to, dormancy, invasion/metastasis and anti-apoptotic genes that confers survival to the CTCs[24]. Most notably, genetic alterations in genes related to apoptosis/cell survival are of paramount importance. Apart from observed genetic alterations in CTCs, molecular factors such as surviving[25], Epidermal Growth Factor Receptor (EGFR), immunosuppressive molecules such as Programmed Death Ligand 1(PD L1)[26]. Human Epidermal Growth Factor Receptor 2(HER2)[27] estrogen receptor, prostate-specific membrane antigen, folate receptor[5], also play an essential role in survival of CTCs. Survivin, an apoptotic inhibitor, assists the escape of tumor cells by limiting the expression of apoptotic proteases and therefore increasing the resistance of malignant cells to chemotherapeutics[25]. Upregulation of HER2 has been reported in several cancers, especially in the context of CTCs, which play an essential role in impeding apoptosis, promoting tumor growth and angiogenesis[27]. For melanoma the detection is based upon markers such as HMW-MAA, CD146, and MAGE A3,[5]

SURVIVAL MECHANISMS

A majority of CTCs do not endure in the circulation. This could be due to diverse factors such as stress- physical and oxidative, dearth in growth factors and cytokines and anoikis[28]. However, the few that do survive do so by adapting various mechanisms. CTCs resist death by altering their integrin expression profile and activating certain cellular signaling pathways such as the Akt signaling pathway[29]. CTCs upregulate certain surface proteins, for e.g CD47, PD-L1 and vascular cell adhesions molecule 1(VCAM-1) to dodge immune surveillance, which bind to macrophages to evade phagocytosis.[6] Cancer cells undergo high dependence on nicotinamide adenine dinucleotide phosphate (NADPH)-generating enzyme so as to elude oxidative stress.[30] Studies have shown that cancer cells attract platelets by expressing tissue factor proteins on their surfaces, which stimulates production of antioxidants that enable them to withstand oxidative stress. Immunosuppressive molecules provide an additional layer of survival by facilitating the evasion of immune-surveillance, achieved by upregulation of various surface proteins that mask CTCs from detection. These have been reviewed in detail by Wang et al 2018.

In 2019, Szczerba et al reported that CTCs were bound to neutrophil facilitated by the cell–cell junction. CTCs clusters with neutrophils anchor to the vascular endothelium for extravasation while resisting shear stress, and the process is mediated by a series of cell adhesion proteins, such as cadherin, integrin, and surface glycoprotein. Neutrophils is said to facilitate metastasis in an indirect manner. Studies have reported that Neutrophil extracellular traps (NETs) were able to capture CTCs in the circulation and, in doing so, pro-

Table 1: Few of the methods used for CTC identification and detection

METHOD USED	DESCRPITION	Remarks
CELL SEARCH(FDA APPROVED) Veridex, New Jersey[37]	Magnetic separation of CTCs from other cells. Nucleus is highlighted using DAB and the cartridge is scanned and viewed by the operator.	1.Increased cost of equipment and per sample 2. Low detection power and low sensitivity and specificity
CELL COLLECTOR (GILUPI GmbH, Potsdam, Germany)[38]	Invivo method of CTC isolation. Has a wire with anti-EpCAM antibodies attached on its surface which is then exposed to patients blood through a cannula	1.Time required for detection and cost is more. 2. Sensitivity and selectivity is better when compared to Cell Search
CONFOCAL MICROSCOPE	Done by direct detection without enrichment method. Cells are marked with antibodies and conjugated with fluorescent agents. CTCs are counted.	1.Detection process is simple 2. Automated counting 3.More sensitive than that of cell search system. 4.Increased detection time 5. Cost of detection is high 6.No: of false negative is high
IMMUNOMAGNETIC (ANTIBODY BASED) METHOD[33]	Target CTCs using their surface antigens or by eliminating the unrequired cells.	1. Centrifugation is necessary could lead to lysis of CTCs. 2. No one antigen can be used because of the heterogeneity of CTCs
MICROFLUIDICS CTC CHIP[39] & HERRINGBONE CHIP[40]	Sorting of cells happen in a microfluidics chamber due to differential sorting of the cell sizes Uses microfluidics Samples are passed through a chamber lined with a herringbone pattern of grooves.	 1. Increased sensitivity 2. Preserve viability of the cells 3. Can process larger blood volumes 4. Able to capture cluster CTCs
DENSITY BASED FICOLL-HYPAQUE METHOD[41] ONCO QUICK	Based on the morphology of the cell. The based on differential migration of cells during centrifugation according to their buoyant density. which results in the separation of different cell types into distinct layers.RBC, platelets, and polymorph nuclear cells are collected separately, while mononuclear cells (MNCs) CTCs are collected separately. Also based on density gradient centrifugation.	 higher relative tumor cell enrichment
SCREENCELL SYSTEM[42]	Separate CTCs by cell size screen. This microfiltration process allows the nucleated cells to pass through while preventing CTC	1.can examine the neoplastic cells extracted onto the filter. 2. Can be used for tissue culture to be used in future experiments.
COMBINED METHODS CTC I-CHIP[43]	Uses both negative and positive enrichment technologies	
SIZE BASED SEPERATION	Even one tumor cell can be identified	
EPISOT ASSAY EPithelialImmunoSPOT (adaptation of ELISPOT)	Uses negative enrichment method of CTCs. It is a qualitative and quantitative method. Where Immunospot are counted and proteins are studied.	1.Proteins are captured on the membrane before being diluted in the supernatant. 2. Cell culture facility is required.
DI-ELECTROPHORESIS(DEP)[44]		1.Isolated CTCs are viable. 2.These can be sustained in culture.
FLOURESCENT FLOW CYTOMETERY[45]	It is an optical system. It identifies moving cells moving cells in a non-invasive manner.	1.Blood drawing is avoided. 2.Cell lysis, centrifugation, and enrichment, are eluded.

moted metastatic dissemination.[5] Tumor-associated macrophages (TAMs) furthers the metastatic progression within the primary tumor and promote metastasis including dissemination and extravasation of CTCs[5] CTCs can bind to platelets to form aggregates. CTCs release prothrombotic and procoagulant factors for increased aggregation. Certain mediators released by platelets, such as TGF-β encourages invasion and metastasis[5].

Methods of CTC Detection

Detection of CTCs are primarily via Liquid biopsy also called fluid phase biopsy[31]. When compared against the traditional biopsy methods liquid biopsies are non-invasive and provides a real-time snapshot of cancer progression.[7] Although peripheral blood is mainly used for detecting CTCs, it can also be detected in saliva, CSF, urine and other bodily fluids as well.[32] Real time information and molecular profiling of the tumor can be derived from liquid biopsy. Owing to its less invasive application, liquid biopsies can be repeated and disease progression and treatment can be closely monitored[33]. To isolate CTCs a number of different methods have been employed. Methods like By-line confocal microscopy and Surface-enhanced Raman scattering (SERS) allow for detection of CTCs without the need for enrichment. However, as CTCs are extremely rare events, an enrichment step allows for more efficient recovery and detection of these cells. This can be either positive enrichment where selection of required cells is done or negative enrichment through deletion of unwanted cells and this dependent on biological property.[6] This could also be based on physical properties like enrichment methodologies consisting of size/deformability-based filtration, density-gradient centrifugation, and di-electrophoresis (DEP) separation[6]. Enrichment of CTC is necessary in early cancer etection to achieve reliable detection as the concentration of is extremely low.[34,4]

Currently CTC recognition systems include, FISH (fluorescence in situ hybridization), SE-iFISH (immunostaining-FISH combined with subtraction enrichment), FACS (fluorescence assisted cell sorting), RT-PCR (reverse transcriptase polymerase chain reaction) And systems for CTC separation and isolation include size based enrichment (ISET, Cell Sieve) and microfluidic devices like Cluster-Chip, CTC-vortex chip[35]. The only FDA approved and commercially available device is Cell Search system developed by Janssen Diagnostics which is used in patients with breast, prostate and colorectal cancer[36]. (Table 1)

Conclusion

The properties of CTC vary from that of peripheral blood cells and this could be used at an advantage for development of newer technologies for identification and isolation of CTCs[7]. from cancers of different origin. Upon further research, data regarding the phenotype, genome, transcriptome, proteome and metabolome of the primary tumor from CTCs, can be obtained[42]. Development of CTC technology is still in the research phase. After its extensive advancement, research regarding this will help the clinicians for detection, therapy and monitoring of cancer. Diagnosis of circulating tumor cells are diagnosed at an early stage, metastatic prevention would be possible. This would in turn make prognosis more favourable and quality of life could increase.

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