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Recent Advances and Prospects in the Isolation by Size of Epithelial Tumor Cells (ISET) Methodology

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Current technologies to identify and characterize circulating tumor cells (CTCs) and circulating tumor microemboli (CTMs) among hundreds of millions of leukocytes in the bloodstream can be classified into tumor-marker-dependent and -independent technology. Isolation by size of epithelial tumor cells (ISET) is a tumor-marker-independent technology, in which CTCs are isolated by filtration without use of tumor-associated markers, as a result of their large size relative to circulating blood leukocytes. ISET allows cytomorphological, immunocytological, and genetic characterization of CTCs and CTMs. It offers a number of advantages, including retention of cell morphology; non-antigen dependence; amenability of cells to further interrogation by immunolabeling, fluorescence *in situ* hybridization, and RNA/DNA analysis; ability to isolate CTMs; reliability. Therefore, morphological-analysis-based and antigen-independent ISET methodology can yield more accurate and objective characterization of epithelial–mesenchymal transition. We can evaluate efficacy of chemotherapy and radiotherapy and other cancer-targeting therapies by using xenografts that are suitable models for mechanistic studies of ISET-isolated CTC/CTM biology. In addition, a new ISET-based device could be designed to increase sensitivity to CTCs/CTMs greatly and reduce the number of CTCs/CTMs directly during the blood flow, thus decreasing the possibility of tumor recurrence and metastasis while retaining normal blood cells. This article reviews recent advances and prospects in ISET methodology and provides new insights into ISET methodology, with important implications for the clinical management of cancer patients.

Key words: Circulating tumor cells; Circulating tumor microemboli; Epithelial–mesenchymal transition; Isolation by size of epithelial tumor cells.

Introduction

According to WHO 2008 statistics, cancer is the main cause of death in developed countries and the second main cause of death in developing countries (1). Although cancer is increasingly diagnosed and treated in the early phase,

Abbreviations: ISET: Isolation by Size of Epithelial Tumor Cells; CTCs: Circulating Tumor Cells; CTM: Circulating Tumor Microemboli; FISH: Fluorescence *In Situ* Hybridization; EMT: Epithelial–Mesenchymal Transition; MGG: May-Grunwald Giemsa; H&E: Hematoxylin & Eosin; CECs: Circulating Endothelial Cells; CNHCs: Circulating Nonhematologic Cells; VE-cadherin: Vascular Endothelium Cadherin Antigen; EpCAM: Epithelial Cell Adhesion Molecule; CK: Cytokeratins; PCR: Polymerase Chain Reaction; RT-PCR: Reverse Transcription PCR; EPISPOT, EPithelial ImmunoSPOT; CAM: Collagen Adhesion Matrix; CS System: CellSearch™ System; DAPI: 4′,6-diamidino-2-phenylindole; MET: Mesenchymal-to-epithelial Transition; EGFR: Epidermal Growth Factor Receptor; BC: Breast Cancer; CM: Cutaneous Melanoma; DFS: Disease-free Survival; HC: Hepatocellular Cancer; MT, Malignant Tumors (*e.g.*, NSCLC: Metastatic Carcinoma; Malignant Pleural Mesothelioma; Melanoma; Sarcoma; Miscellaneous Carcinoma); NA: Not Available; ND, Not Determined; NS: Not Significant; NSCLC: Non-small-cell Lung Cancer; OS: Overall Survival; PC: Pancreatic Cancer; PRC: Prostate Cancer; UM: Uveal Melanoma.

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most cases still appear at the point of occult or overt distant metastasis (2). Metastatic dissemination of malignant tumors cells is the most important cause of cancer fatality. Distant metastasis occurs through three pathways: (i) dissemination via hematogenous vessels; (ii) dissemination via lymphatic vessels; and (iii) direct extension (3). Although lymphatic spread of malignant tumors cells is routinely observed in human tumors and represents an important prognostic marker for disease progression, dissemination via the hematogenous vessels appears to represent the major pathway by which metastatic carcinoma cells disperse (4). Substantial challenges exist in isolating and identifying circulating tumor cells (CTCs) and circulating tumor microemboli (CTMs) among hundreds of millions of leukocytes in the bloodstream. CTCs are extremely rare in the bloodstream – approximately one tumor cell per 10^6-10^7 leukocytes (5, 6). Current technologies to identify and characterize CTCs/CTMs can be classified into tumor-marker-dependent and -independent technology. Isolation by size of epithelial tumor cells (ISET) is a tumor-marker-independent technology, first described by Vona *et al.* (7) in 2000. Use of ISET methodology for detection and identification of CTCs/CTMs is more reliable than other technologies, and requires no expensive or special laboratory equipment. Research on ISET methodology, while relatively new, has shown significant potential to unleash the promise of cancer diagnosis and treatment. However, ISET methodology has not attracted sufficient attention from researchers, who have tended to underestimate its value. As a result, research of ISET has made little evident progress since the last decade. The following review introduces recent advances in ISET methodology and discusses its future development.

ISET Methodology

Basic Principle of ISET

ISET methodology is a direct method for CTC/CTM identification, in which CTCs are isolated by filtration without use of tumor-associated markers, as a result of their large size relative to circulating blood leukocytes, and necessitates a module of filtration and a polycarbonate track-etch-type membrane with calibrated 8-μm-diameter cylindrical pores. ISET uses the following steps: (i) peripheral blood collection and processing (Figure 1A); (ii) optional enrichment of large cells by blood filtration (Figure 1B); and (iii) characterization of large cells on the membrane (Figure 1C).

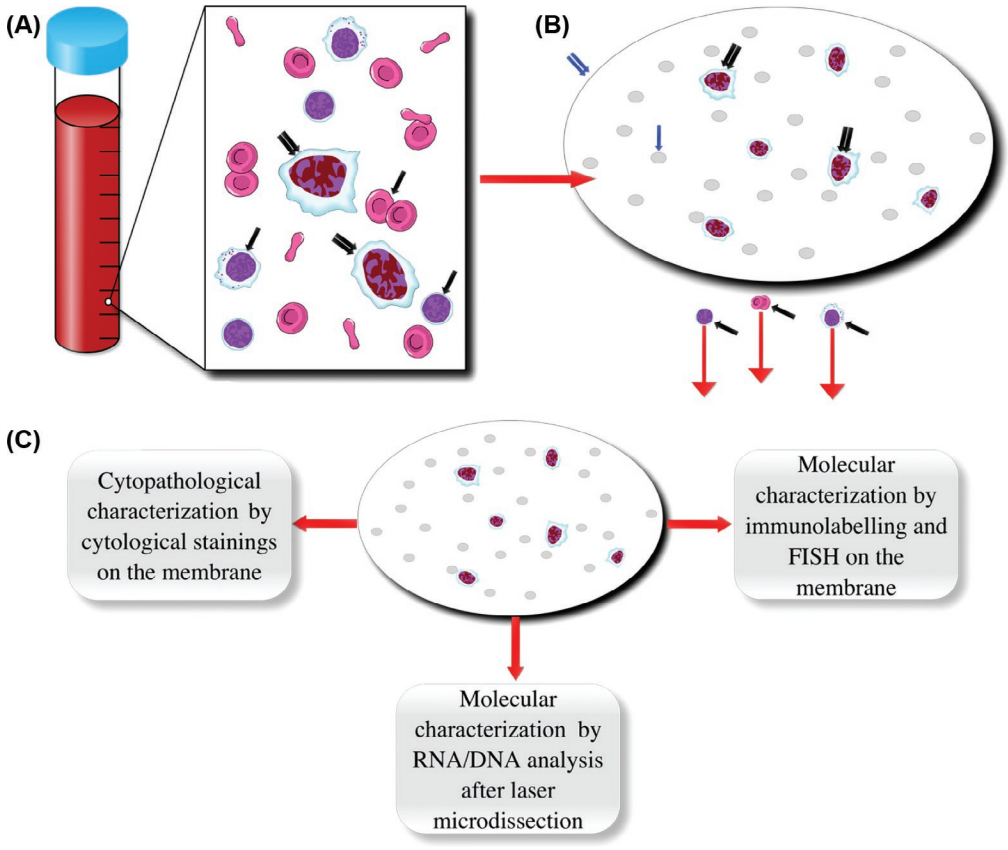

Figure 1: ISET methodology. (**A**) Peripheral blood collection from patients. (**B**) Optional enrichment of large cells by blood filtration. (**C**) Characterization of large cells on the membrane. **A, B:** Black arrows: normal blood cells; double black arrows: CTCs; blue arrows: pores of membrane; double blue arrows: polycarbonate track-etch-type membrane.

Routine First and Second Step of ISET

Peripheral blood from patients is collected in buffered ethylene diamine tetraacetic acid (EDTA) and passed through a series of processes (*i.e.*, dilution, fixation) before blood filtration. Blood samples are filtered by attaching the module to the ISET device (Metagenex, Paris, France) and applying gentle aspiration under vacuum (created by a vacuum pump). After filtration, membranes are washed once by aspiration with phosphate-buffered saline, disassembled from the filtration module, allowed to air dry, and stored at −20°C until staining (7). A key to the first and second step is maintaining peripheral blood anticoagulation to avoid clogging the membrane. Collection of patients' blood samples should avoid contamination with normal epidermal cells when the skin is pierced by the needle. To avoid this contamination, the first several milliliters of peripheral blood drawn are usually discarded. The ISET technique is based on the fact that most peripheral blood cells, including leukocytes and erythrocytes, are smaller than CTCs, with a mean diameter of 8-11 µm, while the mean diameter of CTCs ranges from 29.8 to 33.9 µm (8), and therefore can be filtered through a polycarbonate membrane with 8-µm calibrated pores. Zabaglo *et al.*, testing recovery of breast cancer cells on polycarbonate membrane with different calibrated pores ranging from 8 to 14 µm, have found that membranes with 8 µm pores are best, retaining 85-100% of tumor cells but <0.1% of common blood cells (9).

Critical Third Step of ISET

In the final step, the large CTCs/CTMs on the membrane can easily be stained cytologically [*e.g.* May–Grunwald Giemsa (MGG), hematoxylin and eosin (H&E), and Papanicolaou stain], counted, and characterized. The cytopathological analysis of CTCs/CTMs is performed rapidly and easily because the morphology of CTCs isolated on the membranes is not damaged (7, 10). A small portion of common blood cells, the rare circulating endothelial cells (CECs; reported size >10 µm (11)) and normal epidermal cells are also retained on the membrane. Cytopathological analyses of CTCs need unified criteria to distinguish between malignant and benign cells. Although currently reported criteria vary, they all identify CTCs on the membrane using the classical cytopathological criteria of malignancy already used in exfoliative and in fine-needle aspiration cytology, including: (i) large amount of cytoplasm; (ii) irregularity of the nuclear membrane; (iii) size of the nucleus (12-24 µm); (iv) anisonucleosis; (v) high nuclear/cytoplasmic ratio; (vi) presence of 3D sheets of cells; and (vii) presence of groups of adherent tumor cells (7, 12, 13). The CECs are regular-shaped, with round or oval nuclei, low nuclear/cytoplasmic ratio, and paler-stained nuclei compared with CTCs/CTMs, while normal epidermal cells are polygonal with abundant cytoplasm and small round pyknotic nuclei (14). Because of specific features of the former two types of cells, it is easy to distinguish CECs and normal epidermal cells from epithelial tumor cells by cytopathological analysis on the membrane. The few leukocytes retained in the pore lumen of the membrane are easy to identify because of their smaller size and nuclear morphology. Cytopathological analysis of cells can be performed according to the different features of cells on the membrane. Hofman *et al.* examined 808 blood samples from patients with benign and malignant diseases, and healthy volunteers to differentiate CTCs/CTMs by the ISET method (13). They defined cells on the membrane having nonhematological features as circulating nonhematological cells (CNHCs), which were stained using a modified MGG method, and photographed. The 1025 photographs were digitized and reviewed independently by 10 cytopathologists without knowledge of the patients' data. These cytopathologists classified CNHCs into CNHCs-MF (with malignant features, representing "true" CTCs/CTMs), CNHCs-UMF (with uncertain malignant features), and CNHCs-BF (with benign features) based on the above criteria of malignant tumor cells. It must be admitted that Hofman's identification and classification criteria are more accurate and reliable than other current schemes, because they examined 808 blood samples from patients – a larger cohort than in other studies – and 10 cytopathologists, blinded to patients' data, performed objective analyses. However, their results showed a false-positive rate of 5.3%, and CNHC-UMF rate of 4.9% of patients with malignant tumors, 2.6% of those with nonmalignant tumors (13). Moreover, the presence and number of CNHCs were not related to long-term prognosis and patient outcome in Hofman's research.

Furthermore, molecular analysis including immunolabeling, RNA/DNA analysis, and fluorescence *in situ* hybridization (FISH) can easily be performed to characterize their malignant nature and invasive potential. Immunological characterization should be used to improve CTC/CTM detection on the membrane. Leukocytes were identified by the presence of the leukocyte common antigen, CD45 (15, 16), using immunohistochemistry and immunofluorescence. CECs can also be identified on the membrane by immunocytology using vascular endothelium cadherin antigen (VE-cadherin) (14, 17, 18). It is difficult to differentiate epithelial tumor cells and normal epidermal cells by immunocytological analysis because of a lack of specific markers. The multiple markers can also be used to characterize CTCs/CTMs on the membrane, such as E-cadherin, epithelial cell adhesion molecule (EpCAM), cytokeratins (CKs), vimentin, and neural cadherin. However epithelial and mesenchymal markers are heterogeneously expressed in CTCs/CTMs (19, 20), due to the epithelial–mesenchymal transition (EMT) process in some CTC subpopulations with stem-cell-like properties. Therefore, ISET-isolated CTCs/CTMs were identified for immunological characterization only as a supplement. Morphological analysis can be used as a reference diagnostic method and gold

standard to recognize CTCs/CTMs on membranes. However, the biggest problem of morphological analysis is the lack of unified criteria for malignant tumor cells on the membrane.

One of the properties of ISET is its ability to use laser microdissection on the membrane, which allows for the recovery of nucleic acids from single CTCs for further molecular analysis and characterization. Vona *et al.* in 2000 collected single Hep3B cells on the membrane by ISET and captured these cells by laser microdissection, then obtained their DNA to amplify and screen for genetic abnormalities in target sequences (7). Their results demonstrate that PCR after laser microdissection for individually isolated cells on the membrane is feasible. Vona *et al.* in 2004 looked for β-catenin mutations in 60 single microdissected CTCs from 10 liver cancer patients (21). They found β-catenin mutations in only three of 60 CTCs, indicating that β-catenin mutation does not play an important initial role in tumor cell invasion. Pinzani *et al.* also extracted DNA from CK broad-spectrum monoclonal-antibody-immunostained cells recovered by laser microdissection from patients with breast cancer; they then measured HER-2 amplification by real-time PCR (22). They detected HER-2 amplification in CTCs of breast cancer patients rather than in tumor cell lines, and found good correspondence between results obtained from microdissected CTCs and those obtained using DNA extracted from the primary tumor.

Reportedly, genome analysis techniques, such as FISH, also can be used in chromosomal analyses on CTCs isolated by ISET (7), but there have been only a few studies using FISH on CTCs isolated by ISET, because FISH probes generally do not label all the target cells (10). Cytogenetic studies of CTCs that use FISH are feasible in spite of these limitations.

Comparison of RT-PCR, CellSearch and ISET

Current technologies to identify and characterize CTCs/CTMs can be classified into tumor-marker-dependent and -independent technology. There are two tumor-marker-dependent methods: immunocytology/cytometry- and PCR-based. The former methods utilize monoclonal or polyclonal antibodies against either tumor-associated antigens (positive selection) or the common blood cells antigen CD45 (negative selection) for the isolation of CTCs, and include the CellSearch™ system (23-25), CTC-chip (26-30), EPithelial ImmunoSPOT (EPISPOT) (31-35), MAINTRAC™ (36, 37), and Collagen Adhesion Matrix (CAM) assay (38). For the CellSearch™ system, CTCs are defined as positive for EpCAM epithelial-lineage marker and pan-CK and negative for CD45 pan-leukocyte antigen ($EpCAM^+/CK^+/CD45^-$ events). The principles of the other immunocytology/cytometry-based methods are similar. Immunocytology/cytometry-based methods need to maintain the integrity of the cell membrane; otherwise it cannot get a positive result. The PCR-based assays rely on the detection of known gene mutations, amplifications or methylation patterns in the CTCs. They target tumor-associated DNA (cell-free DNA) or cDNA (mRNA) markers (*i.e.* cytokeratin 19 fragment antigen 21-1, carbohydrate antigen 125, CK20, or prostate-specific antigen mRNA) for molecular CTC detection (39-41). Cell integrity is destroyed during total RNA extraction**,** therefore, the CTCs cannot be morphologically identified and isolated for further analyses. In EMT states, CTC detection rate is underestimated by both immunocytology/cytometry- and PCR-based methods. The markers used are tissue- or cell-specific rather than tumor-specific, therefore, epithelium-specific markers are often used by these two methods to detect CTCs.

Although the PCR-based molecular method is the most widely used technology for CTC/CTM detection in the bloodstream, it misses some EMT-induced CTCs, leading to false-negative results, as with the aforementioned tumor-marker-dependent technology methods. Vona *et al.* have compared sensitivity between ISET and RT-PCR (7). Cultured tumor cells mixed with 1 ml of peripheral blood were analyzed by ISET or RT-PCR. ISET was able to detect one single tumor cell in 1 ml of peripheral blood, and similar results have been found in several previous studies (7, 9, 22, 42, 43). In contrast, RT-PCR could not detect a single cell when diluted in 1 ml of peripheral blood (7, 22); several studies have reported that RT-PCR has similar sensitivity to ISET (22, 42). Choosing different epithelium- or organ-specific mRNA markers might cause different sensitivity in RT-PCR analysis. Moreover, as there is no specific mRNA marker for some cancers (*e.g.* human ovarian cancer), the sensitivity threshold of RT-PCR appears to be limited. Pinzani *et al.* have performed sensitivity tests of RT-PCR and found that samples containing one tumor cell per milliliter of blood corresponded to 0.1 tumor cell equivalents per microgram of total RNA (22); each blood sample needed enough total RNA extracted from enough CTCs to avoid false-negative results. Tissue- or cell-type specific mRNAs are used by the RT-PCR method to differentiate between nontumorous and tumorous cells in blood samples (44, 45), because none of the mRNAs are tumor specific. In most studies, RT-PCR specificity is based on the hypothesis that normal tissue cells, unlike tumor cells, either do not enter circulation, or apoptose quickly in circulation. As cell integrity is destroyed during total RNA extraction, so circulating benign cells can lead to false-positive results, because cell morphology and phenotype cannot be analyzed. Reportedly, benign nevus cells may circulate for long periods, which could result in false-positive RT-PCR results (46). RT-PCR analysis cannot reliably distinguish between benign nevus cells and melanoma cells, because it detects tyrosinase mRNA or other melanocytic markers as specific molecular markers for circulating melanoma cells (47, 48). ISET does not face this problem; in fact, circulating benign nevus cells have been

identified in peripheral blood by ISET for the first time (46). Normal epidermal cells also can contribute to a high false-positive rate in the RT-PCR method (49, 50), because they can be picked up by needles and flushed into syringes by the blood. RT-PCR analysis also cannot differentiate between normal epidermal cells and epithelial tumor cells, because it usually detects epithelium-specific mRNA for CTCs (51, 52). ISET can distinguish normal epidermal cells from epithelial tumor cells by their specific morphology. In addition, one of the major drawbacks of the RT-PCR method is low RNA stability. Therefore, blood sample preparation and storage, including RNA extraction, must be performed under circumstances that avoid RNA degradation (53). In contrast, ISET can allow storage of membrane at −20°C for later analysis after a long period.

The CellSearch™ system (Veridex, LLC, Raritan, NJ, USA) is a semiautomated enrichment and immunostaining device that has been approved by the US Food and Drug Administration for prognostication in metastatic colorectal, prostate and breast carcinomas (54-56). CTCs were defined by the CellSearch system as those coexpressing EpCAM and CKs (including 8, 18, and 19) without expressing leukocyte common antigen CD45, and positive for 4′,6-diamidino-2-phenylindole (DAPI) with a nucleus inside the cytoplasm and cell size ≥4 μm (56). It must be emphasized that CTC detection by the CellSearch system does not rely on any true morphological criteria, but rather on the magnitude of antibody fluorescent signal for CK, DAPI, and CD45. The CellSearch system is an epithelium-associated marker-dependent method, therefore, it faces technical problems similar to the PCR-based molecular method; its inability to identify EMT-induced CTCs can give false-negative results. In several studies, the ISET method and CellSearch system have been compared in CTC detection in parallel, using blood samples. Krebs *et al.* (14) in 2012 reported that CTCs were detected using an ISET method in 80% (32/40) of patients compared with only 23% (9/40) using the CellSearch system. Farace *et al.* (57) in 2011 showed that CTCs were detected using the ISET method in 95% (57/60) of patients but in only 70% (42/60) using the CellSearch system. Hofman *et al.* (43) in 2011 demonstrated that CTCs were detected in 50% (104/210) and 39% (82/210) of patients using ISET and CellSearch systems, respectively. Khoja *et al.* (20) in 2012 found that ISET detected CTCs in more patients than the CellSearch system did (93 vs. 40%). Overall, more CTCs were detected by ISET than by the CellSearch system, for two reasons: (i) the CellSearch system may miss cells if they have undergone EMT (*i.e.*, lack expression of CK and/or EpCAM), whereas ISET can be much more efficient in isolating all rare cells of interest undergoing EMT including epithelial-marker-negative tumor cells (*i.e.*, those expressing only vimentin); and (ii) the CellSearch system cannot isolate CTMs from metastatic cancer patients, whereas ISET can (14, 19). Magnetic beads, coated with EpCAM-specific antibodies, may have insufficient capacity to enrich for larger or heavier CTMs, and/or because CTMs insufficiently express EpCAM for immunomagnetic isolation and/or CKs for identification (14, 19). Therefore, the detection of blood samples that only have CTMs will be underestimated by the CellSearch systems that use epithelial-marker-positive selection. However, the CellSearch system may overestimate CTCs in peripheral blood samples if they are contaminated with normal epidermal cells and a subpopulation of circulating monocytes can be transdifferentiated into keratinocyte-like cells and express CK (58). In addition, the CTC detection efficiency varies in all relevant studies, whether by ISET or by CellSearch system. These discrepancies depend on primary tumor type. One of the main advantages of the CellSearch system is that it has the capacity to detect smaller CTCs than does ISET, because the size range of cells detected by the CellSearch system is 4-18 μm and by ISET it is 8-30 μm (14), so the CellSearch system does not miss rare cells <8 μm in size.

CTC/CTM detection technology should combine high sensitivity with high specificity. RT-PCR and the CellSearch systems have low sensitivity and specificity compared to ISET, primarily because rare tumor cells of interest cannot be morphologically isolated and identified for further analyses by the former two systems. Use of ISET for detection and identification of CTCs is more reliable than RT-PCR and the CellSearch systems, and requires no expensive or special laboratory equipment. However, ISET is not sufficiently standardized in its current form to be routinely applicable in clinical practice.

Applications of ISET

Applications in Scientific Research

It has being increasingly reported that EMT plays a predominant role in the process of tumor cell invasion and metastasis (59-61). Reportedly, some subpopulations of epithelial tumor cells undergo EMT – for example, downregulating and/or negative expression of EpCAM and/or CK, and new expression of mesenchymal vimentin (62-64) – and re-express their epithelial properties through a mesenchymal-to-epithelial transition on arrival at their target organs (65). These specific CTCs may have stem-cell-like properties (65). EMT is a complex process especially involved in carcinogenesis and is considered a prerequisite to tumor infiltration and metastasis (66). During EMT, some subpopulations of epithelial tumor cells lose their epithelial properties and acquire mesenchymal phenotypes, such as loss of cell–cell contact; increased motility and invasiveness; disinclination to apoptose; and changes in cellular morphology (67). However, although EMT is an important mechanism in increasing tumor invasive potential, there is little direct data on EMT in

CTCs/CTMs, because CTC/CTM traditional detection technology using epithelial markers for tumor cells misses these rare EMT-induced CTCs with stem-cell characteristics. Thus, morphological-analysis-based and antigen-independent ISET methodology can obtain more accurate and objective EMT characterization.

Some studies have assessed ISET-isolated CTCs/CTMs from cancer patients by immunostaining the membrane for EMT-associated markers [*e.g.*, CK, EpCAM, EGFR, E-cadherin, vimentin and N-cadherin] (14, 19, 20, 43, 68). They have found that CTCs/CTMs are heterogeneous for these EMT-associated markers in the circulation, supporting the concept that only some subpopulations of epithelial tumor cells are induced by EMT. Interestingly, Krebs *et al.* have shown that cells within CTMs tend to be positive for EGFR and CK (14), but Hou *et al.* have shown that vimentin is expressed in the majority of CTM cells (19); both using blood samples from NSCLC patients. It can be inferred from this that CTM cells may express a dual epithelial (EGFR/CK) and mesenchymal (vimentin) phenotype in the peripheral blood of patients with NSCLC.

A recent similar study used samples from six NSCLC patients and dual-color immunostaining for CK and vimentin in dual-stained CTCs/CTMs. Lecharpentier *et al.* have demonstrated for the first time that most CTCs/CTMs harbor dual epithelial–mesenchymal phenotypes (68), supporting the hypothesis that EMT increases tumor invasive and metastasizing potential; however, these results have come from only from a small number of patients and did not correlate the presence of EMT-induced CTCs/CTMs with long-term prognosis and patient outcome.

Matthew *et al.* have examined proliferation status in CTCs/CTMs by testing immunohistochemically for Ki-67 on the membrane (14). Ki-67 is present at all active phases of the cell cycle, but is absent from resting cells, and is an excellent marker for proliferating cells (69). They found that all cells within CTMs had no expression of Ki-67, whereas most single CTCs did express Ki-67, and that CTM cells might stay in G_0-G_1 phases and remain dormant without proliferating, to resist chemotherapy and immunological assault, while retaining the ability to metastasize upon reactivation (34), which suggests that CTM cells can reverse quiescence – a feature of tumor stem cells. It is highly probable that tumor stem cells share many properties with dormant tumor CTM cells (70, 71).

Hou *et al.* have identified apoptotic cells by characteristic fragmented and condensed DAPI-stained nuclear morphology, and found that apoptotic nuclei were absent from the CTMs but were present within a subpopulation of CTCs, suggesting a survival advantage (19), in which cells within CTMs might evade anoikis in aggregates via collective migration, and are protected from immunological assault by lymphocytes and natural killer cells. CTMs obviously carry a higher metastatic potential than single CTCs. They also carry more markers and can be reliably counted without damage, using ISET. ISET, unlike other CTC/CTM detection technologies, allows multiparameter immunostaining. This is a huge and unique advantage of ISET.

Applications in Clinical Practice in Cancer Patients

Significant correlations have been reported between presence and number of CTCs/CTMs detected by size and clinical relevance in various tumor types, suggesting that ISET-based CTC/CTM detection would be of use in cancer prognosis. EMT-associated markers are reported to be heterogeneously expressed in ISET-isolated CTCs/CTMs in cancer patients. Here, we briefly review current published studies on common solid tumors.

Patient cohort size and detection rates have varied between these studies (Table I). The most commonly used method for CTC/CTM detection in these studies was cytomorphological diagnosis using modified MGG staining (12, 13, 43, 72). Nucleus and cytoplasm are more clearly analyzed by MGG staining than with H&E staining (42, 48). Another method for CTC/CTM detection uses antibodies against CK (*i.e.*, pan-CK or KL1) on the ISET membrane (9, 22); however, immunocytochemical analysis tends to miss some EMT-induced CTCs, leading to false-negative results, which suggests that cytomorphological diagnosis via MGG staining is the better method. However, despite its diagnostic superiority, it would be even better to confirm these morphological criteria by immunological characterization, including immunohistochemistry and immunofluorescence. In these studies, the range of prevalence of CTCs/CTMs in malignant tumors is 26-100% (Table I). In chronic disease controls the detection rate is close to 0%, except among patients with thyroid or parathyroid hyperplasia (4.2%) (13). Reportedly, CTCs/CTMs are not detectable using ISET in blood samples from healthy controls. These results indicate that: (i) ISET may give false-positive results in patients with thyroid or parathyroid adenomas; and (ii) ISET has high specificity for CTC/CTM detection. Most published investigations have studied morphology and clinical relevance of ISET-isolated CTCs/CTMs in the bloodstream of lung cancer patients (12, 19, 43, 57, 68, 72). CTC/CTM detection in patients with NSCLC or liver cancer using ISET could be a useful aid to prognosis (12, 21, 43). Only one study has shown poor correlation between ISET-isolated CTCs/CTMs and overall or progression-free survival in a small sample set (20). In research that has used ISET methodology, the study of Hofman *et al.* had the largest patient sample, which included those with miscellaneous non-neoplastic diseases (152 cases), miscellaneous

Table I

ISET-isolated CTCs/CTMs in various tumor types.

No. of patients	Stage	Blood aspirate	Cytological analysis	EMT markers (antibody)	Characterization EMT	Range of positive cells	Detection rate cases %	Detection rate controls %	Correlation to clinical/pathol. variables	Mean follow-up (months)	Prognostic/ predictive value
210 NSCLC (43)	T1-T4	10 ml	MGG staining	Pan-CK (2, 5, 6, 8, 10, 11, 14/15, 18, and 19), vimentin	Pan-CK alone in 27/210 (13%), vimentin alone in 23/210 (11%), pan-CK in association with vimentin in 55/210	1 to 150, mean 34	50%	0/40 (0%)	NS	Median 15	DFS ($P < 0.0001$)
250 NSCLC (72)	T1-T4	10 ml	MGG staining	NA	NA	NA	41%	0/59 (0%)	NS	NA	ND
208 NSCLC (13)	T1-T4	10 ml	MGG staining	NA	NA	NA	37%	0/39 (0%)	NS	Mean 24	DFS ($P < 0.0001$), OS ($P < 0.0001$)
6 NSCLC (68)	NA	10 ml	NA	Pan-CK, vimentin	Pan-CK alone in 0/6 (0%), vimentin alone in 3/6 (50%), co-expressing pan-CK and vimentin in 6/6 (100%)	3 to 52	100%	0/6 (0%)	NA	NA	NA
3 NSCLC, 3 NSCLC (19)	T2, T4	10 ml	NA	E-cadherin, CK 4, 5, 6, 8, 10, 13, and 18, vimentin, neural cadherin	E-cadherin, CKs, vimentin, neural cadherin heterogeneously expressed in 6/6 (100%)	NA	100%	NA	NA	NA	NA
44 BC (22)	I, II, III	10 ml	NA	KL1 (cytokeratin gp)	KL1 in 12/44 (27%)	1 to 30/ml, mean ± SE: 8.5 ± 2.4	27%	0/40 (0%)	NA	NA	NA
20 BC (9)	T4	9 ml	H&E staining	Pan-CK	Pan-CK in 20/20 (100%)	NA	100%	NA	NA	NA	NA

(Continued)

Table I (*Continued*)

No. of patients	Stage	Blood aspirate	Cytological analysis	EMT markers (antibody)	Characterization EMT	Range of positive cells	Detection rate cases %	Detection rate controls %	Correlation to clinical/pathol. variables	Mean follow-up (months)	Prognostic/ predictive value
7 HC (7)	NA	6 ml	H&E or MGG staining	NA	NA	NA	100%	0/16 (0%)	NA	NA	NA
44 HC (21)	I, II, III	6 ml	NA	NA	NA	NA	52%	0/107 (0%)	NA	Mean 12	OS ($P < 0.05$)
87 CM (42)	I, II, III, IV	5-10 ml	H&E staining	NA	NA	Mean ± SE= 0.32 ± 0.09/ml (invasive melanoma), mean ± SE = 0.73 ± 0.33/ml (metastatic melanoma)	26%	0/53 (0%)	NS	22-28 (invasive melanoma), 18-16.8 (metastatic melanom)	NA
16 UM (48)	Small, Medium, Large	10 ml	H&E staining	NA	NA	NA	31%	NA	NA	NA	NA
35 PC (20)	NA	10 ml	NA	EpCAM, pan-CK, CK 7, E-cadherin, vimentin.	Heterogeneously expressed	0 to 240, median 9	93%	NA	NA	NA	NS
20 BC, 20 PRC, 20 NSCLC (57)	T1-T4	10 ml	NA	NA	NA	0 to 20, median 2 (BC), 1 to 248, median 17 (PRC), 1 to 100 median 5 (NSCLC)	85% (BC) 100% (PRC) 100% (NACLC)	NA	NA	NA	NA
569 MT (13)	T1-T4	10 ml	MGG staining	NA	NA	NA	48.90%	10/239 (1.2%)	NA	NA	NA

Abbreviations: BC: Breast Cancer; CM: Cutaneous Melanoma; DFS: Disease-Free Survival; HC: Hepatocellular Cancer; MT: Malignant Tumors (*e.g.*, NSCLC: Metastatic Carcinoma, Malignant Pleural Mesothelioma, Melanoma, Sarcoma, Miscellaneous Carcinoma); NA: Not Available; ND: Not Determined; NS: Not Significant; NSCLC: Non-small-cell Lung Cancer; OS: Overall Survival; PC: Pancreatic Cancer; PRC: Prostate Cancer; UM: Uveal Melanoma.

benign (38 cases) and malignant neoplasia (569 cases), and healthy volunteers (49 cases) (13). Interestingly, they found that ISET-isolated CTCs/CTMs were not diagnostic for a histological subtype of the corresponding primary NSCLC (72); possibly because only poorly differentiated cells without any primary tumor features were able to cross the endothelial barrier into the bloodstream (23, 73). The presence of these ISET-isolated CTCs/CTMs is not correlated with clinical or pathological variables (12, 42, 43, 72), including pTNM staging and primary tumor histological subtype. Lelievre *et al.* (74) have used ISET methodology to assess the effect of laparoscopy versus laparotomy on CTC detection in an animal model of ovarian carcinogenesis, thus assessing the feasibility of ISET methodology to test animal blood, for the first time.

Applications in Clinical Practice for Prenatal Diagnosis

Invasive prenatal diagnosis may increase the risk of miscarriage (75), therefore, development of noninvasive or minimally invasive prenatal diagnosis tests is an important goal in obstetric practice (76). Earlier work has focused on routine ultrasound screening for fetal anomalies (77) or identification of cell-free fetal DNA in the maternal circulation (78). More recently, ISET has been shown to be a promising approach to prenatal diagnosis (79-81). ISET methodology was applied to prenatal diagnosis by Vona *et al.* (79) in 2002. ISET isolates cells from maternal blood in accordance with the size of trophoblastic cells, which are larger than peripheral blood leukocytes. Cytopathological analysis with H&E staining shows that two types of fetal cell morphological features are: (i) mononucleated, cytotrophoblast-like cells with large nuclei, condensed chromatin and small cytoplasm, sometimes with few microvilli at the membrane surface; and (ii) polynucleated, syncytiotrophoblastic cells with larger diameters (79). Molecular identification of fetal cells and their genetic characterization can easily be performed by immunolabeling, FISH, and RNA/DNA analysis. Fetal mononuclear cells and syncytiotrophoblastic cells have also been identified according to the presence of KL1 (pan-CK) or antiplacental alkaline phosphatase antigen, using immunohistochemical characterization (79). In contrast, maternal cells are positive for anti-CD45 antibody (82). ISET-isolated fetal cells can be individually microdissected using single cell microdissection; gene mutations in a fetal and pure genome can be reliably detected. Vona *et al.* (79) have found that enrichment of fetal cells obtained by ISET methodology is greater than that obtained by flow cytometry and immunomagnetic cell selection; and, for the first time, that ISET is a reliable approach for minimally invasive prenatal diagnosis. Beroud *et al.* used genetic analysis of ISET-isolated fetal cells from 12 pregnant women to identify fetuses at risk of spinal muscular atrophy (80). Saker *et al.* (81) tested ISET-isolated fetal cells from blood samples of 12 pregnant women to characterize both parental alleles for mutations in the cystic fibrosis transmembrane conductance regulator gene. Three of their studies showed that maternal blood analysis using ISET as a minimally invasive means for prenatal diagnosis of genetic diseases is feasible and warrants further study with larger patient cohorts.

Perspectives

ISET allows cytomorphological, immunocytological, and genetic characterization of CTCs/CTMs. It offers a number of advantages, including retention of cell morphology; nonantigen dependence; amenability of cells to further interrogation by immunolabeling, FISH, and RNA/DNA analysis; ability to isolate CTMs; reliability.

A particular advantage is that ISET can allow immunostaining analyses with different EMT-associated markers, to explore more precisely EMT phenotypes in infiltration and metastasis of tumor cells. More detailed analysis of EMT phenotypes will allow better molecular characterization of CTCs/CTMs and may provide new insights in molecular mechanisms of resistance to anticancer treatments.

Several studies mentioned above indicate that CTC/CTM detection by ISET is a strong, independent prognostic indicator in certain tumors. Although pTNM staging is currently the only validated prognostic factor used in certain tumors, it is not comprehensive, because it does not include assessment of tumor cell invasion and dissemination via bloodstream metastasis, which suggests that a combination of pTNM staging and ISET could offer a more sensitive prognostic and predictive indicator. Quantification of CTCs/CTMs using ISET may be used as an early index of relapse in patients with early or advanced-stage tumors. Furthermore, identification of pharmacodynamic biomarkers underlying disease response in cancer patients undergoing standard chemotherapy or radiotherapy could be monitored, allowing subsequent dosages to be adjusted according to the growth or decline in the number of CTCs/CTMs in patients' peripheral circulation. Additionally, postoperative detection of CTCs/CTMs by ISET methodology could be used to identify the best follow-up and adjuvant therapy strategies after radical cancer surgery, as a minimally invasive test. ISET methodology might also be used to detect free tumor cells in hydrothorax or ascites of patients with lung or digestive system carcinoma, or in urine of patients with urological carcinoma.

Although Vona *et al.* have described development of ISET methodology over the past decade, relatively few studies have been done in the field of oncology, mainly due to: (i) lack of unified criteria in cytopathological analysis of CTCs/CTMs; (ii) ISET methodology cannot detect small-cell ($<8\,\mu m$) carcinomas, for example, oat cell carcinoma; and (iii) timing

patterns of CTC/CTM metastasis and dissemination are still unclear and require further study for practical use of ISET methodology. The morphological and molecular characterization of CTCs/CTMs correlates directly with that of primary tumor cells and characterization of secondary tumor cells has not been reported previously, because CTCs/CTMs are extremely rare and influenced by EMT in the bloodstream, which complicates differentiation between primary and secondary tumor cells, morphologically and biologically. An experiment involving ISET had specific gene-labeled tumor cells implanted into immunocompromised animals to form xenografts. The animals' peripheral blood was collected periodically, and filtered and analyzed by ISET. This method identified CTCs/CTMs with specific gene markers [*e.g.*, green fluorescence protein (GFP)] and avoided the influence of EMT, common blood cells, CECs and normal epidermal cells on the membrane, which are without specific gene markers. It also allowed easy establishment of a unified criterion of CTCs/CTMs from observations, as well as exploration of patterns of metastasis and dissemination for circulating malignant tumor cells. These xenografts are also suitable models for mechanistic studies of ISET-isolated CTC/CTM biology to evaluate efficacy of chemotherapy and radiotherapy and other cancer-targeting therapies (Figure 2). The authors' research group has carried out experiments in xenograft models and the partial results are presented in Figure 2.

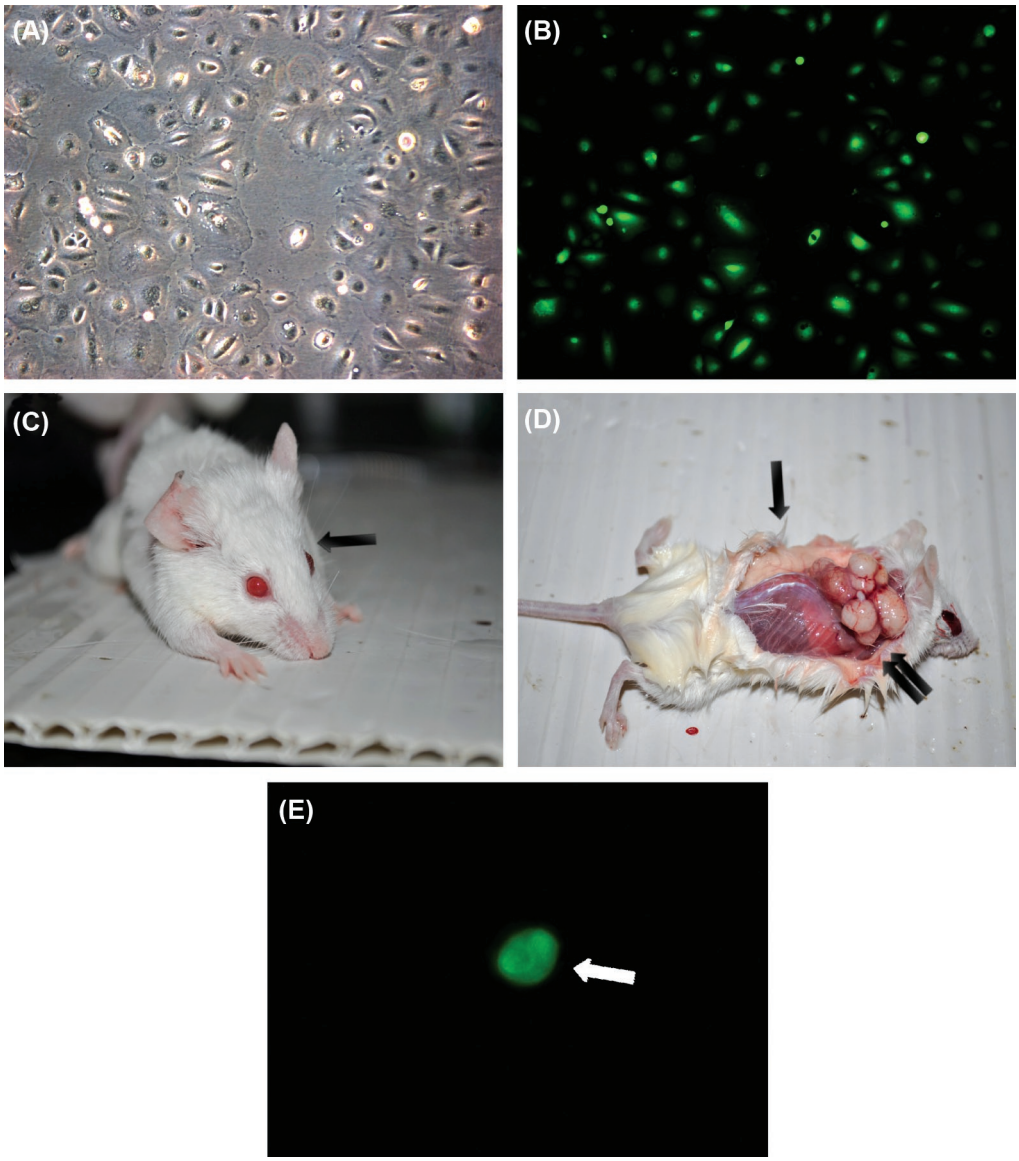

Figure 2: Establishment of a tumor cell xenograft model and CTCs filtered by ISET methodology. (**A**) Tumor cell lines. (**B**) GFP-labeled tumor cells. (**C**) Immunocompromised mouse (black arrows). (**D**) Formation of tumor cell xenografts (double black arrows). (**E**) ISET-isolated CTCs with GFP (white arrow).

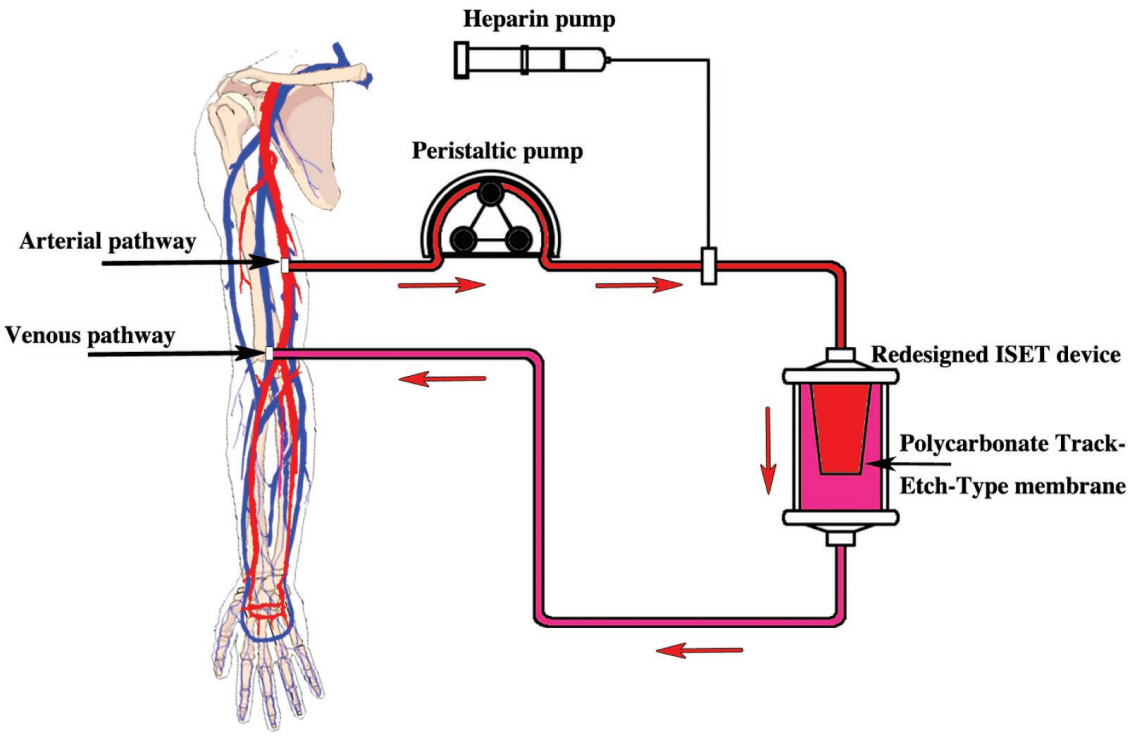

Figure 3: Under the impetus provided by the peristaltic pump, arterial blood comes through the ISET device, and is then returned to the circulatory system via a vein.

Patterns of exfoliation and dissemination of CTCs/CTMs from primary tumors are unclear, because the half-life of circulating CTCs seems to be only 1.0-2.4 h (8), few tumor cells are released into the blood, and many CTCs rapidly undergo apoptosis (83), especially in patients with early-stage cancer. For these patients, especially, we must improve ISET sensitivity.

Possibly, an ISET-based device could be designed using arteriovenous access in patients, to divert as much peripheral blood as possible from the artery, through the ISET device, and then back into the circulatory system via a vein, to offer a continuous blood analysis, which would both greatly increase sensitivity to CTCs/CTMs and directly reduce the number of CTCs/CTMs during the blood flow through the ISET device, thus decreasing the possibility of tumor recurrence and metastasis while retaining normal blood cells (Figure 3). The authors' research group is conducting feasibility studies in this area.

In conclusion, although many challenges remain, the most significant of which is more attention and intensive research in this area. The current status and future directions of ISET methodology are elaborated in this review. Finally, ISET methodology will provide new insights into the complex biology of CTCs/CTMs, with important implications for the clinical management of cancer patients.

Conflict of Interest

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with submission to TCRT. The authors have no conflicts of interest to declare.

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