The Journal of Molecular Diagnostics, Vol. 17, No. 3, May 2015

ELSEVIER

the Journal of Molecular Diagnostics

jmd.amjpathol.org

SPECIAL ARTICLE

Do Circulating Tumor Cells, Exosomes, and Circulating Tumor Nucleic Acids Have Clinical Utility?

A Report of the Association for Molecular Pathology

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Accepted for publication February 2, 2015.

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Diagnosing and screening for tumors through noninvasive means represent an important paradigm shift in precision medicine. In contrast to tissue biopsy, detection of circulating tumor cells (CTCs) and circulating tumor nucleic acids provides a minimally invasive method for predictive and prognostic marker detection. This allows early and serial assessment of metastatic disease, including follow-up during remission, characterization of treatment effects, and clonal evolution. Isolation and characterization of CTCs and circulating tumor DNA (ctDNA) are likely to improve cancer diagnosis, treatment, and minimal residual disease monitoring. However, more trials are required to validate the clinical utility of precise molecular markers for a variety of tumor types. This review focuses on the clinical utility of CTCs and ctDNA testing in patients with solid tumors, including somatic and epigenetic alterations that can be detected. A comparison of methods used to isolate and detect CTCs and some of the intricacies of the characterization of the ctDNA are also provided. *(J Mol Diagn 2015, 17: 209—224; http://dx.doi.org/10.1016/j.jmoldx.2015.02.001)*

Circulating Tumor Cells

A 1-cm carcinoma that has been growing for >10 years contains approximately one billion cells. Such a tumor doubles once every 150 days and weighs just more than half a gram.[1—4] This paradigmatic carcinoma likely manifests the hallmarks of cancer, including harboring a subpopulation of stem or tumor-initiating cells, each of which is characterized by four to seven gene mutations in a small subset of approximately 125 driver genes.[5—7] The genetic changes arise stochastically and radiate under selection pressure for increased proliferation and adaptation to the tumor microenvironment.[8—10] In some cases, tumor cells of epithelial origin will undergo a phenotypic conversion consisting of a transition to more mesenchymal characteristics.[11—16] This epithelial-mesenchymal transition will permit the tumor-initiating cells to invade the local tissue of origin.[13,17—20] Local invasion of a basement membrane, for most carcinomas, precedes extravasation,[21—24] in which the

Supported in part by the Association for Molecular Pathology and the Intramural Research Program of the National Cancer Institute, NIH.

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The Circulating Tumor Cell Working Group is a working group of the Association for Molecular Pathology Clinical Practice Committee. The 2013 to 2014 Clinical Practice Committee consisted of Matthew Bankowski, Milena Cankovic, Jennifer Dunlap, Larissa V. Furtado, Jane S. Gibson, Jerald Z. Gong, Thomas Huard, Linda Jeng, Loren Joseph (Chair 2013 to 2014), Annette Kim, Marilyn M. Li, Melissa Miller, Mary Lowery Nordberg, Carolyn Sue Richards, Paul Rothberg, M. Fernanda Sabato, and Patrik Vitazka.

http://dx.doi.org/10.1016/j.jmoldx.2015.02.001

cells slough off the edges of the tumor entering the circulation (or lymphatics). They can remain unitary in the vasculature, cluster together as they disseminate, or lodge themselves in new tissues to form metastases. Whatever the path of circulating tumor cells (CTCs), they potentially hold valuable information about tumor composition, invasiveness, drug susceptibility, and resistance to therapy. Each of these tumor characteristics is potentially amenable to molecular and cellular characterization through its isolation.

An average metastatic carcinoma patient has between 5 and 50 CTCs for approximately every 7.5 mL of blood (<1 to >5 CTCs/mL).[25–28] This small cell number places a technical limitation on the ability to resolve a relatively small subpopulation of tumor stem cells that carry the set of mutations defining the tumor and bearing self-renewal capability.[29–31] Visualization and separation of CTCs from leukocytes are, therefore, dependent on reliable cell-surface markers. Such markers have become available in the past decade. In that time frame, new technologies have, for the first time, allowed the isolation of CTCs from patient blood samples.[28,32,33] Initial methods for CTC isolation relied on physical properties of the cells.[34] Because CTCs sediment with the leukocyte fraction during low-speed centrifugation, it is possible to enrich for the population through separation on the basis of buoyancy.[35] In addition, CTCs are generally larger than average leukocytes; thus, a size-based filter further enriches for CTCs and permits separation from white cells.[36]

In more recent devices, CTC isolation techniques have depended on antibodies against epithelial cell adhesion molecule (EpCAM), a protein that protrudes from the outer surface of CTCs, but not healthy blood cells (Table 1).[33,37,38]

Because this technology proved sufficient to provide prognostic information,[25,55] the US Food and Drug Administration cleared the Veridex CellSearch platform, now owned by Johnson & Johnson (Raritan, NJ), to isolate CTCs. That platform uses EpCAM antibodies attached to magnetic beads to isolate tumor cells in conjunction with proprietary CellSave venipuncture tubes that preserve CTC structure. By using the Veridex device, CTCs can be pulled out of suspension using a magnet.[56] Other cell-capture devices using antibodies are also available. In particular, at the Massachusetts General Hospital (Boston, MA), a microfluidic silicon chip has been designed with tens of thousands of EpCAM antibody-coated microposts that bind CTCs as blood samples flow through.[57] Recent efforts to engineer this silicon chip microfluidic system have shown that herringbone channels coated with antibody maximize the efficiency of CTC-antibody interactions.[58–61] Unfortunately, this method has thus far failed to permit subsequent CTC separation from the microfluidic device and dovetailing with molecular characterization. This technical hurdle is being actively addressed in several research laboratories.

Clinical Utility of CTCs

CTCs have been identified in peripheral blood from patients with metastatic and recurrent disease. As methods for isolating CTCs have matured, several investigators have studied correlations between cell number and patient disease severity. That breast cancer patients with fewer CTCs in their blood lived longer than those with more CTCs was first demonstrated in 2004.[55] Similar observations were made in other cancer types, including prostate and colorectal cancers.[62–64] More recently, the number of CTCs was shown to be a prognostic predictor of overall survival for malignant melanoma and to predict survival in breast and prostate cancers (Table 2).[65–68]

Newer studies have made efforts to analyze the genetic mutations that CTCs carry, comparing the mutations to those in the primary tumor or correlating the findings to a patient's disease stage, grade, or metastasis. In one study of non-small cell lung cancer (NSCLC) patients, CTCs carried the well-known epidermal growth factor receptor (*EGFR*) c.2369C>T (T790M) gatekeeper mutation that mediates gefitinib and erlotinib resistance.[72] The patients carrying this lesion had faster disease progression than those with other *EGFR* variants detected in their CTCs. In another study, changes to certain signaling pathways within CTCs during treatment could predict how well prostate cancer patients responded to a drug.[73] A recent report identified *KIT* and *BRAF* mutations in CTCs of melanoma patients.[74] Tumor heterogeneity was demonstrated in one case with discordant *BRAF* mutation status between CTC and the primary tumor.[74] CTC analysis may aid the assessment of tumor heterogeneity, which can be associated with therapy resistance and relapse, and help guide targeted therapies.

In contrast to tissue biopsies, CTC detection from peripheral blood represents a minimally invasive method for early and serial assessment of several predictive factors of metastatic disease at different stages of disease, including follow-up during remission. This enables real-time assessment of a variety of tumor-related properties, including characterization of treatment effects and clonal evolution. Recently, Heitzer et al[75,76] assessed mutational status of primary tumor, metastases, and CTCs in patients with stage IV colorectal cancer using next-generation sequencing and array-comparative genome hybridization. They found mutations in CTCs that had not been identified during initial diagnosis, but were found to be present at a subclonal level in the primary tumor. These findings suggest that CTC analysis can unravel relevant changes in the tumor genome that had not been either present or observed at the time of initial diagnosis.

Yet, at this juncture, molecular characterization of CTCs has raised numerous questions. In 2012, researchers analyzed the expression levels of 95 cancer-related genes in CTCs from 50 breast cancer patients using quantitative PCR and found that these cells had large variations in gene-expression patterns.[77] That study concluded that CTC collection and characterization depends on both the technical method and biological properties of the tumor cells being studied. For instance, basal-like breast cancer CTCs expressing low levels of EpCAM are unlikely to be captured using the

Table 1 Selected CTC Characterization Methods

Method name	Developer (location)	Target cancer	Antibody against	Reference	Evidence level
AdnaTest	AdnaGen (Langenhagen, Germany)	Breast, prostate, and colon	EpCAM and MUC1	39	
autoMACS/MACS	MitenyiBiotec (BergischGladbach, Germany)		EpCAM, pan-CK, HER2/neu, or CD4	40	
Biofluidica	(Chapel Hill, NC)	Pancreatic, prostate, lung, breast, and colorectal	EpCAM	41	
CellSearch	Veridex, Johnson & Johnson	Metastatic breast, colon, prostate, lung, melanoma, and urothelial	EpCAM	42	FDA cleared, many clinical trials
ClearCell System	ClearbridgeBiomedics (Singapore)	Breast		Presented at ASCO 2014	
CTC iChip	Daniel Haber and Mehmet Toner, Dana-Farber Cancer Institute and Massachusetts General Hospital (Boston, MA)	Breast, colon, lung, prostate, and pancreas	EpCAM and CD45/ cytokeratin subtraction	43	FDA IDE
Dynabeads methods	Invitrogen (Carlsbad, CA, and Heidelberg, Germany)	Colorectal cancer	EpCAM and CD45 subtraction	44	
ISET	Metagenex (Paris, France)	Melanoma, mesothelioma, and NSCLC	Size (no antibody)	45	NCT01776385 for mesothelioma and NCT00818558 for NSCLC
IsoFlux Rare Cell Access System	Fluxion Biosciences (San Francisco, CA)	NSCLC and melanoma		46	
Lymphoprep (Ficoll-Isopaque)	Axis-Shield PoC (Oslo, Norway)	Prostate	EpCAM, PSA, and cytokeratin 7/8	47	
MagSweeper	Stephanie Jeffrey and Ronald W. Davis (Stanford University, Stanford, CA)	Breast and prostate	EpCAM	48	
Nanodetector	Gilupi (Potsdam, Germany)	Breast, lung, and prostate	EpCAM	49	
Negative enrichment QMS	Jeffrey Chalmers (Cleveland Clinic, Cleveland, OH)	Head and neck and breast	CD45 subtraction	50	
OncoQuick	Greiner Bio-One (Germany and Monroe, NC)	Breast, colorectal, melanoma, and pancreatic	Density (no antibody)	51	
RoboSep/EasySep	Stem Cell Technologies (Vancouver, BC, Canada)	Myeloma	CD33, CD66, and CD138	52	
ScreenCellCyto	ScreenCell Company (Sarcelles, France)	Lung and cell lines	Size (no antibody)	53	

This table is an updated version of Table 2 first published by Parkinson et al.[54]

ASCO, American Society of Clinical Oncology; CK, cytokeratin; CTC, circulating tumor cell; EpCAM, epithelial cell adhesion molecule; FDA, Food and Drug Administration; iChip, microfluidic silicon chip; IDE, investigational device exemption; ISET, isolation by size of epithelial tumor cell; MACS, magnetic activated cell sorting; NSCLC, non-small cell lung cancer; PSA, prostate-specific antigen; QMS, quadruple magnetic sorter.

EpCAM-dependent CellSearch.[78] More study is needed to clarify the relationship between a patient's tumor burden and the number of CTCs in the circulation and to determine whether CTC burden determination possesses clinical utility, such as providing salutary outcomes for more aggressive chemotherapeutic intervention. If this relationship is elucidated, CTCs may become useful as surrogate biomarkers for tumor progression for some purposes. Therefore, for the field to move forward, a focus on characterizing the biology of CTCs, including refining and improving cell isolation methods, will be required. In addition, investigators will have to conduct rigorous studies aimed at examining whether and to what extent these promising tumor surrogates can make the transition to use as clinical biomarkers. The value of CTCs as predictive markers is still unclear.

Circulating Cell-Free and Tumor Cell DNA

The existence of extracellular or cell-free nucleic acids was first documented by Mandel and Metais in the 1940s,[79] and the rheumatologic literature routinely discussed the presence of DNA outside the confines of intact cells through the 1980s.[80] Indeed, patients with cancer were known to have

Table 2 Review of Evidence-Based Clinical Utility of Analytes

Analyte	Type of DNA/RNA	Clinical utility	Evidence level*
CTCs		Burden is prognostic	Level I for metastatic breast[68] and level II-1 for prostate cancer[67]
Exosomes and circulating microvesicles		Burden is prognostic	Level III[69]
Circulating nucleic acids	cfDNA, ctDNA	Burden is prognostic	Level II-2[70]
	cfRNA	Marker of therapeutic response	Level III[71]
	miRNA	Up-regulation is possibly prognostic	Preclinical

Evidence levels are based on those established by the US Preventative Services Task Force and are denoted as follows: level I, evidence obtained from at least one properly designed randomized controlled trial; II-1, evidence obtained from well-designed controlled trials without randomization; II-2, evidence obtained from well-designed cohort or case-control analytic studies, preferably from more than one center or research group; II-3, evidence obtained from multiple time series designs with or without the intervention (dramatic results in uncontrolled trials might also be regarded as this type of evidence); and III, opinions of respected authorities, on the basis of clinical experience, descriptive studies, or reports of expert committees.

*Designations of levels of evidence are based on the listed citations and were agreed to by the authors. No other authority provides the basis for these designations.

cfDNA, cell-free DNA; cfRNA, cell-free RNA; CTC, circulating tumor cell; ctDNA, circulating tumor DNA.

relatively high levels of cell-free DNA (cfDNA) in their plasma, although the overlap with patients with benign diseases, such as inflammatory bowel disease, was substantial enough to undermine clinical utility.[81] The identification of *RAS* oncogene mutations in circulating DNA of patients with pancreatic cancer and myelodysplastic syndrome/acute myeloid leukemia was the initial hint that tumor-specific nucleic acids were present in the circulation.[82,83] Subsequently, other forms of tumor-specific abnormalities, such as loss of heterozygosity (LOH) of microsatellites[84] and methylation of CpG islands, confirmed that circulating tumor DNA (ctDNA) was present in the blood.[85,86]

The origin of cfDNA is presumed to be dead cells (necrotic, apoptotic, or phagocytosed).[87] Apoptosis has been proposed as the leading contributor of cfDNA on the basis of the nucleosomal size multiples of the DNA, with the size of most plasma cfDNA fragments in both cancer patients and healthy controls being <300 bp, as measured by electron microscopy, with no significant difference between the two.[88] Evidence is abundant enough at this point to exclude CTCs as the origin of ctDNA; however, even after decades of study, the origin is uncertain. An active release of cellular DNA from living cells has been proposed.[89] This possibility has served, in turn, as support for the theory of genometastasis, which postulates that DNA is transferred between cells via transfection and actively transcribed.[90,91] cfDNA is not particle associated and, thus, is not related to exosomes or a variety of microparticles.[92,93] This is not believed to be true of cell-free RNA (cfRNA).[94,95] cfDNA likely does circulate bound to histone proteins.[96] Among normal research subjects, most cfDNA (between 60% and 95%) is contributed by hematopoietic cells, as demonstrated by studies of sex-mismatched bone marrow transplantation subjects.[92] In the case of cancer patients, the contribution by tumor cells to cfDNA is varied. LOH studies suggest that almost all of the cfDNA is attributable to the tumor cells.[84] By contrast, extensive enrichment for tumor-specific mutations via selective PCR strategies may be needed to reveal rare tumor molecules, suggesting, in this case, that tumor cells contribute a minority of the cfDNA.[97] The variables influencing the relative amounts of tumor and normal cfDNA (eg, gene amplification, selective loss, and tumor heterogeneity) are not understood.

The clearance of cfDNA from the blood appears to proceed through organ-based mechanisms, predominantly the liver and kidney in mouse models,[98] with liver being the primary uptake site. Organ uptake is more rapid for single-stranded DNA than for double-stranded DNA. DNA >15 bp does not measurably persist in the mouse blood stream >20 minutes for single-stranded DNA or 40 minutes for double-stranded DNA. Although blood contains several DNases, they are not active against protein-bound DNA, providing further support for the concept of histone-DNA complexes.[99] The half-life of cfDNA has been best studied in the context of pregnancy. The mean half-life of fetal DNA in post-partum women is 16 minutes, with a range of 4 to 30 minutes and a complete absence by 2 hours.[100] The situation with cancer appears to be analogous, because post-surgical resection patients have similarly short half-lives of tumor-specific DNA, although such cancer studies may be confounded by occult metastases or residual disease.[101]

Methods to extract nucleic acids from plasma or serum were initially varied, which led to inconsistent results. Indeed, the preferred substrate (plasma or serum) was also unsettled for several years. Currently, most investigators use plasma derived from EDTA anticoagulated blood. Although larger amounts of nucleic acids appear in the serum, these have been demonstrated to be from lysed residual white blood cells.[102] The peripheral blood white cell DNA can present a significant problem for identification of ctDNA, because it is present in great excess and, thus, can decrease sensitivity of detection of ctDNA. Several commercial blood collection tubes made specifically for the collection and stabilization of extracellular DNA and RNA are available, but they are not used in most protocols.[103,104] The initial processing step after plasma is obtained is usually a

pair of centrifugations, first a low-speed spin, followed by a higher-speed spin to remove remaining blood cells without lysis.[105] Nucleic acids are then extracted from the remaining supernatant by one of a variety of methods. Extraction kits intended for intact blood cells or tissues are commonly used, but these have the drawback of not capturing all of the nucleic acid, which tends to be of relatively low molecular weight.[106] Lack of standardization and optimization of processing methods are ongoing limitations in the field.[107]

The quantification of cfDNA has been performed by a variety of methods, mostly spectrophotometrically or by dye intercalation. Normal reference ranges are not well established. On occasion, >1000 ng cfDNA/mL of blood has been reported in healthy controls, but the mean seems to be approximately 1 to 10 ng/mL.[108] Patients with a variety of diseases, ranging from infection to autoimmunity to cancer, generally have higher levels of cfDNA, but there is substantial overlap between the populations, and most reports do not show a statistically significant difference from healthy people. Complicating any conclusion is the fact that the size of the patient cohorts in most studies is small. Even large studies are relatively poorly controlled.[109] Although quantification itself may not be informative for tumor diagnosis, a decrease in level after therapy may be useful for residual disease detection, as has been demonstrated for Epstein-Barr virus DNA and nasopharyngeal carcinoma.[110]

Clinical Utility of Circulating Cell-Free and Tumor Cell DNA

Because extracellular nucleic acids are presumably released from most or all cells in the body, their detection has been referred to as a whole body biopsy or liquid biopsy. Although there are hundreds of articles on the topic, most have small study populations and only a few thousand patient samples in aggregate have been assayed for tumor nucleic acids. A wide variety of malignancies, from breast adenocarcinoma to gliomas, have been studied, but particular emphasis has been placed on colorectal and pancreatic cancer, in large part because *KRAS* mutations have been a frequent biomarker. The performance characteristics of these assays have been modest. Correlation between tumor abnormalities (point mutation, microsatellite instability, methylation, and translocation) and the same or similar abnormalities in the plasma or serum cfDNA is not absolute, and discordances happen in both directions (Esteller et al[85] provide a tabular summary of these comparisons). Overall clinical sensitivity of these serum and plasma markers may be as high as 90% in known cancer patients, but more commonly runs between approximately 30% and 80%, and less for methods like loss of microsatellite heterogeneity, which are expected to have a lower discrimination threshold.[104] Specificity is also varied, and complicated by the fact that there is reasonable evidence of either subclinical mutations or premalignancy in clinically normal individuals.[97] There are many caveats to these numbers: studies are small and underpowered, they are often poorly controlled or uncontrolled, they examine one or a few markers, they use a variety of marginally validated methods, often with poor analytic sensitivities, and they examine mixed populations (eg, stages) of patients.[104] Clearly, more assay validation studies are needed before these protocols can be brought into the clinic. It is possible that technical improvements in assays, in particular the advent of digital or massively parallel sequencing, will lead to more sensitive and reproducible biomarker detection.[111] These techniques are already being optimized for detection of multiple markers from small samples, sometimes at the single-cell level. The ability to reproducibly examine small amounts of DNA would allow detection of multiple targets, such as known cancer-associated mutations.

Perhaps more promising or more advanced than initial diagnosis or screening for cancer is the possibility of tumor monitoring through the use of ctDNA biomarkers. This has the benefit of providing a known target for mutation screening, because the tumor itself can be sequenced before cfDNA analysis and common, disease-specific abnormalities can be targeted. An early example of this approach was the identification of minimal residual follicular lymphoma by searching for the characteristic *BCL2/IGH* translocation.[112] Such minimal residual disease detection may also benefit from next-generation methods.[113] Of course, detection of a few mutant molecules (as little as 0.01% of the total ctDNA) requires stringent measures to avoid false-positive results.[101]

The combination of cfDNA concentration with other blood tumor biomarkers may also prove to be useful,[114,115] as would the correlation studies between tumor and blood biomarkers. Clinical validation involving multicenter studies is critically needed to further define these relationships.

Tumor-Specific Gene Mutations

Pancreatic Cancer

Pancreatic cancer has the distinction of being the first solid tumor associated with a specific mutation in ctDNA.[116] This is due, in part, to the availability of a frequently mutated and easily assayed target, the *KRAS* gene. Sorenson et al[116] used allele-specific amplification to assay for mutations in codon 12 in the plasma or serum of pancreatic adenocarcinoma patients. Only three patients with metastatic disease were tested, and each showed mutation in codon 12.[116] This article essentially launched the field of ctDNA.

The liquid biopsy aspect of ctDNA holds particular attraction for pancreatic cancer. It is relatively common, presents late in its course, and is challenging to biopsy. The differential diagnosis with pancreatitis is a common one, and several studies have focused on discriminating between these, with mixed success. The sensitivity of detecting primary pancreatic adenocarcinoma on the basis of ctDNA is generally low (approximately 30% to 50%), but the specificity is generally higher (approximately 90%).[82,117,118] A variety of detection methods have been used, including restriction

digestion and single-stranded conformational polymorphism. In one case, sensitivity was improved when a serum protein marker (CA19.9) was measured in concert with DNA measurements.[117] Disappointingly, pancreatitis cases also exhibited mutations in the *KRAS* gene, although at a lower frequency (5% to 15%) than adenocarcinoma.[82,117] In all of these studies, however, the number of cases and controls was limited and the follow-up was brief, which could be particularly pertinent given the long lead time of pancreatic cancer.[119] In at least one study, however, the presence of mutated *KRAS* DNA was an independent, poor prognostic factor.[82]

Although most studies have focused on *KRAS* mutations in pancreatic cancer because of their prevalence, other approaches have been tried. A DNA integrity assay dependent on the relative length preservation of repetitive genomic elements in tumor cells compared to normal blood cells showed promise as a biomarker for pancreatic cancer.[120] Methylation studies of promoter regions were reportedly able to discriminate pancreatic cancer from pancreatitis, although this type of multianalyte approach requires independent confirmation.[121] The advent of higher-throughput methods, such as next-generation sequencing and digital PCR, may have a profound effect on the field; for instance, one recent study showed that pancreatic duct cancer, compared to other malignancies, had a relatively high rate of ctDNA, more so in metastatic disease than nonmetastatic disease.[70] In summary, for clinical and biological reasons, pancreatic cancer is an ideal candidate for the diagnostic and prognostic use of ctDNA. ctDNA has shown as much promise in this tumor type as any, and the ability to look more broadly and in greater depth at the tumor genome may finally unlock this promise.

Colorectal Carcinoma

Several types of DNA alterations have been detected with a variable frequency in ctDNA of patients with colorectal carcinoma, including mutations of oncogenes and tumor-suppressor genes, DNA microsatellite instability, LOH, hypermethylation of gene promoters, and mutations of mitochondrial DNA.

The detection of *KRAS*, *APC*, and *TP53* mutant DNA in plasma or serum of patients with colorectal cancer has been correlated with diagnosis, prognosis, and therapeutic response in several studies.[122–125] The analysis of cfDNA for specific gene mutations, such as those in *KRAS* and *TP53*, is desirable because these genes have a high mutation frequency in many tumor types and contribute to tumor progression. The overall detection rate of *KRAS* mutations in serum or plasma of patients with colorectal cancer ranges from 25% to 30% up to 50% in different studies when considering only tumors harboring these same genetic alterations.[126] *KRAS* mutations in ctDNA have been detected in different stages of colorectal carcinoma and in premalignant disease, with the highest level found in the more advanced stage.[97] Preoperative detection of *KRAS* mutations in ctDNA has been highly specific for the presence of colorectal neoplasia, and associated with a higher risk of recurrence.[122,125,127,128] Postoperatively, persistence or reappearance of circulating mutant DNA has been shown to be a strong predictor of disease recurrence and poor prognosis.[5,129,130] Analysis of circulating mutant DNA has also shown utility in monitoring patients with colorectal carcinoma who are receiving anti-EGFR therapy.[131] This approach is highly relevant for choosing a treatment with efficacy, and provides an opportunity to repeatedly monitor patients during treatment without having to resort to repeated biopsies. However, agreement between detection of *KRAS* mutations in plasma samples and colorectal samples is not 100%, demonstrating a potential for false-positive results.[97]

In patients with tumors harboring a *TP53* mutation, the same mutation has been identified in ctDNA in approximately 40% of cases.[126] Most studies published so far have focused on portions of *TP53* between exons 4 and 8, where the most commonly encountered *TP53* mutations in colorectal cancer are located.[122,125,128,132–136]

In addition, clinically relevant mutations in *BRAF*,[135] *EGFR*, and *APC*[122,125,134,135] have now been studied in ctDNA from colorectal cancer patients. The search for *APC* mutations in ctDNA has focused on exon 15, which is a hotspot for *APC* mutations in colorectal cancer. The rate of APC mutation detection in primary ctDNA is approximately 45%.[127]

The major challenge with mutation analysis of circulating mutant DNA has been assay sensitivity and specificity. Currently, most assays target ctDNA alterations located in mutational hotspots of certain genes. Wild-type cfDNA sequences and heterogeneity of primary and metastatic tumors can also interfere with detection of ctDNA mutations. In this setting, multigene panel analysis of ctDNA would be expected to increase test sensitivity. However, available evidence does not support this assumption.[122,125] The overall detection rate for mutations in the serum or plasma of patients with colorectal cancer has been reported as approximately 35% by different groups using multigene panels.[94,125,136–138] Recently, a panel targeting mutations of the *KRAS*, *TP53*, and *APC* genes enabled the identification of at least one genetic alteration in tumor tissue from approximately 75% of patients with colorectal cancer.[122,125] Disappointingly, those same mutations were only detected in the serum of 45% of these patients. A recently devised massive parallel sequencing approach (Safe-SeqS) that can accurately detect mutations in a small fraction of DNA templates containing variant bases may improve mutation detection in ctDNA.[139]

Microsatellite alterations in ctDNA from the plasma or serum of colorectal cancer patients have shown variable detection rates across studies.[140] In addition, concordance between LOH findings in ctDNA and LOH found in DNA isolated from matched primary tumors has been variable.[132]

Although the analysis of methylated *SEPT9* DNA in plasma has been shown to be a sensitive (up to 90%) and specific (up to 88%) approach for detection of all stages of

colorectal carcinoma,[141–144] other targets appear not as sensitive. Grady et al[145] studied *MLH1* promoter hypermethylation in preoperative serum and matched tumor samples of 19 patients with microsatellite unstable colon cancers. Of those, 47.4% of tumors were positive for *MLH1* promoter methylation, and only three (33%) of these cases also demonstrated a positive result in DNA from their preoperative serum samples. Subsequent studies have reported a similar detection rate for *MLH1* promoter methylation.[123,124]

The reported detection rate of mutations in targeted regions of cell-free circulating mitochondrial DNA corresponding to primary tumors has been low (14%), which limits the application of this marker in the clinical setting.[132]

Lung Carcinoma

Circulating DNA has been detected in body fluids of patients with lung cancer,[146] and circulating DNA from lung cancer patients was shown to contain tumor-specific genetic and epigenetic alterations, including mutations, microsatellite alterations, and gene promoter hypermethylation.[147] Elevated concentrations of circulating DNA have been associated with tumor stage, prognosis, and response to chemotherapy.[148–154] The detection of *KRAS* mutations in plasma of patients with NSCLC correlates with poor prognosis.[154,155] Plasma *KRAS* mutation status is also associated with a poor tumor response to EGFR–tyrosine kinase inhibitors in NSCLC patients and may be used as a predictive marker in selecting patients for such treatment.[155] Furthermore, *EGFR* mutation analysis in tumor-derived DNA from pleural effusion fluid is potentially practical for predicting the response to gefitinib treatment in advanced NSCLC.[156,157]

Aberrant hypermethylation of *CDKN2A* has been reported to be an early event in lung carcinogenesis and a potential biomarker for early diagnosis.[85,158,159] Hypermethylation of *CDKN2A* can be detected in the serum and/or plasma of patients with lung cancer,[160] even before clinical evidence of malignancy.[161,162] No statistically significant differences have been observed among histological types (adenocarcinoma versus squamous cell carcinoma) or clinical stages, indicating that *CDKN2A* hypermethylation is a common and early event during lung carcinogenesis in general. In addition, positive tumor and circulating *CDKN2A* indicates advanced stage in NSCLC,[163] and patients with plasma and preresection pleural lavage *CDKN2A* tend to have shorter survival.[163] Aberrant hypermethylation of *CDKN2A* has also been associated with tumor dissemination, and metastatic potential and poor prognosis in NSCLC.[163–165] Overall, *CDKN2A* methylation detection in plasma or serum is a specific marker for detection of NSCLC.[160] More recently, serum detection of methylation of 14-3-3σ was shown to be a new independent prognostic factor for survival in NSCLC patients receiving platinum-based chemotherapy.[166]

LOH has been detected in cfDNA and tumor cell DNA from patients with small cell lung cancer (SCLC) and NSCLC.[167–169] In a study by Bruhn et al,[167] 31% of the SCLC patients had microsatellite alteration(s) or LOH in at least one locus analyzed in chromosomes X, 6, and 21. In 40% of the cases, the identical alteration was detected in the plasma DNA. In the group of patients diagnosed with NSCLC, a microsatellite alteration or LOH was detected in at least one locus in 33% of the patients. In all but two patients, the identical alteration observed in the DNA from tumor cells was also detected in the DNA isolated from blood plasma. The high prevalence of microsatellite alterations of 3p, even in stages I and II of lung cancer, in independent series of lung cancer patients,[150,169,170] suggests that this feature is also associated with the early phase of the disease and can thus be used as an independent marker, possibly improving the diagnostic potential.

Emerging technologies, such as next-generation sequencing, are expected to facilitate the discovery of clinically relevant genetic biomarkers for diagnosis, prognosis, and personalized therapeutics of lung cancer. And these findings can potentially increase the informativeness of tumor molecular signatures in plasma or serum.

Other Tumor Types

ctDNA has been evaluated for most common tumor types. The trends described for the exemplar tumor types described above (pancreatic, colorectal, and lung carcinoma) hold true for most cancers: sample sizes are relatively small, follow-up times are relatively brief, and test parameters are relatively modest. For example, in the area of testing ctDNA in melanoma patients, the earliest of studies showed that LOH of anonymous microsatellite markers correlated well between tumor and plasma.[171] This study was performed on 76 patients. Thirteen years later, a multiparameter study hypothesized a correlation with biopsy-proven melanoma focused on ctDNA quantity, integrity, *BRAF*-mutated DNA, and methylation of *RASSF1A*. This study was performed on 76 patients and 63 healthy controls, had <5 years of follow-up, and reported that a multiparameter approach to identify melanoma was needed to overcome the non-specificity of each component individually.[172] In the interval, one group demonstrated the clinical utility of testing plasma for *BRAF*-mutated ctDNA in predicting chemotherapy response.[173] Across these studies, the lack of a consistent set of analytes and small study size, as well as the case-control approach, have greatly hampered unequivocal meta-analysis.

One exception to a small-scale approach is a test for methylation of the septin 9 gene (*SEPT9*), which has been examined in two large prospective colorectal cancer screening trials as part of a premarket approval submission to the U.S. Food and Drug Administration. In the PRESEPT Study, a large cohort of 1544 colonoscopy patients was screened prospectively with a quantitative PCR of the *SEPT9* promoter region after bisulfite conversion. The sensitivity and specificity for detection of carcinomas from average-risk patients were 48.2% and 91.5%, respectively.[174] In a study comparing the *SEPT9* assay to the commercial fecal immunochemical

test, 290 average-risk people undergoing screening endoscopy were tested with paired stool and plasma samples. The plasma test was not inferior to the fecal blood test with regard to sensitivity, but the same could not be shown with regard to specificity (Epi proColon Test; Epigenomics, Inc., Germantown, MD). Thus, when large cohort studies are performed, they have demonstrated good, but by no means stellar, results for ctDNA as a screening tool.

Although it is outside the scope of this review, similar molecular approaches are being taken on other specimen types, such as urine, sputum, and stool.

Epigenetic Alterations

The detection of methylated ctDNA represents one of the most promising approaches for risk assessment in cancer patients.[175] Assays for the detection of promoter hypermethylation may have higher sensitivity than microsatellite analyses, and can have advantages over mutation analyses.[176] Aberrant DNA methylation, which seems to be common in cancer, occurs at specific CpG sites. There are particular tumor-suppressor genes that are frequently methylated and down-regulated in certain cancers. There are several advantages to the detection of aberrant DNA methylation over the detection of genetic mutations. Hypermethylation of multiple tumor-suppressor genes is frequently observed in cancers. Thus, the sensitivity of a cancer detection test can be enhanced by simultaneous detection of the hypermethylation of multiple genes. To improve assay conditions and the clinical relevance, the selection of appropriate genes from a long list of candidate genes that are known to be methylated in cancer is essential. Several studies have reported the presence of aberrant methylation in tumor tissues, and similar changes were also detected in the plasma/serum samples.[177] Such studies have indicated a good correlation between restricted expression at the tissue level and the occurrence of detectable levels of candidate biomarkers in serum/plasma DNA. In this connection, the circulating methylated DNA approach has been applied as a biomarker in various forms of cancer, including pancreatic cancer,[178] ovarian cancer, prostate carcinoma,[179] hepatocellular carcinoma, esophageal adenocarcinoma, colorectal carcinoma, breast cancer,[180] head and neck squamous cell carcinoma, testicular cancer,[181] and lung cancer.

To evaluate whether degree of methylation measurement could be used as a useful serum-based biomarker of breast cancer, Sturgeon et al[182] used pyrosequencing to define methylation status of a panel of 12 breast cancer–related genes (*APC*, *BRCA1*, *CCND2*, *CDH1*, *ESR1*, *GSTP1*, *HIN1*, *CDKN2A*, *RAR*, *RASSF1*, *SFRP1*, and *TEIST*). For all genes, median levels of methylation were higher in lymph node–positive breast cancer cases than the controls. The most significant findings were for *TWIST*, *SFRP1*, *ESR1*, *CDKN2A*, and *APC*; however, the differences in methylation levels were still not sufficiently distinct to be able to distinguish between cases and controls in a clinical setting.

In a study by Chimonidou et al,[183] *SOX17* methylation was examined in 79 primary breast tumors, 114 paired samples of DNA isolated from CTCs and ctDNA, and 60 healthy individuals. The *SOX17* promoter was highly methylated in primary breast tumors, in CTCs isolated from patients with breast cancer, and in corresponding ctDNA samples. Although there was significant correlation between *SOX17* methylation in ctDNA and CTCs in patients with early breast cancer, this was not observed in patients with verified metastasis.[184]

cfRNA

Circulating gene transcripts are also detectable in the plasma of cancer patients.[184] Extracellular human mRNA was first described in 1999 in the circulation of melanoma patients, where the relatively melanocyte-specific tyrosinase mRNA was shown to exist in a particulate or packaged form.[94] This was followed by identification of other forms of RNAs, in particular miRNAs, in the plasma or serum.[185–187] Cancer cells often have distinct gene expression patterns different from normal tissues. This difference can be used diagnostically through detecting the tumor-specific transcripts in the circulation of cancer patients.[188] It is known that RNA released into the circulation is surprisingly stable despite the fact that increased amounts of RNases circulate in the blood of cancer patients.[94] This implies that RNA may be protected from degradation by its packaging into exosomes, such as microparticles, microvesicles, or multivesicles, which are shed from cellular surfaces into bloodstream. The detection and identification of RNA can be performed using several methods, including microarray technologies or quantitative real-time RT-PCR.[189]

Studies are emerging in which blood mRNA signatures could be used as prognostic or predictive markers, or both. Results from two studies published in *The Lancet Oncology* suggest that transcript levels of a few selected genes in blood samples from cancer patients can significantly improve outcome prediction. In a study by Ross et al,[190] a panel of 168 inflammation- and prostate cancer–related genes was assessed with optimized quantitative PCR to assess biomarkers predictive of survival. A six-gene model separated patients with castration-resistant prostate cancer into two risk groups: a low-risk group with a median survival of >34.9 months and a high-risk group with a median survival of 7.8 months. In a separate study, Olmos et al[191] used microarray-based expression profiling of whole blood samples from 64 patients with advanced castration-resistant prostate cancer and 30 patients undergoing active surveillance to identify expression patterns specific for aggressive disease. A nine-gene signature was developed that was significantly associated with poor overall survival. The biological relevance of these prognostic signatures is largely unknown. Just as with ctDNA, studies of cfRNA lack a large scale and a correlation between tumor behavior and findings in blood biomarkers.

The predictive value of mRNA signatures was examined in 98 rectal cancer patients,[71] in whom plasma levels of cfRNA

and telomere-specific reverse transcriptase mRNA were found to predict therapy response. Although the detection of gene expression patterns in blood of cancer patients sounds promising, questions have been raised as to how much of the circulating RNA in cancer patients is derived from tumor cells, and how much comes from the hematopoietic system, possibly as a response of blood cells to a disease condition.[192] Issues such as optimal timing of blood sample collection and influence of therapy still need to be addressed.

Circulating miRNA

miRNAs represent a class of naturally occurring, small, noncoding RNAs. The secretory mechanism and biological function of extracellular miRNAs remain unclear. It is speculated that miRNAs in the blood of cancer patients could play the same important roles as miRNAs in tissues, and some studies have tried to correlate miRNA expression in solid tumors with that in blood.[186,193] Circulating miRNAs are not cell associated but seem to escape degradation by endogenous RNase activity by residing in microvesicles,[194] exosomes, microparticles, and apoptotic bodies, and recently protein-miRNA complexes[195] have also been proposed. miRNAs remain stable after being subjected to harsh conditions, including boiling, low/high pH, extended storage, and freeze-thaw cycles. Measurement of circulating miRNA levels is made challenging because of contamination by varying levels of cellular miRNAs of different hematopoietic origins. Protocols for isolation and stabilization of circulating miRNAs will need to be standardized and to include approaches to selectively detect miRNAs, possibly at the single molecule level, from plasma of cancer patients.[196] Techniques such as next-generation sequencing and expression profiling might allow generation of miRNA profiles in blood that would correlate with tumor progression.[197,198] Measurements obtained from plasma and serum were not always correlated, and it appears that plasma samples will be more suitable for investigations of miRNAs as blood-based biomarkers for noninvasive diagnosis in various tumor entities.

Several studies have tried to define correlation between circulating miRNAs and clinical variables in different types of cancer.[199–201] In their study of metastatic and localized prostate cancer, Nguyen et al[202] observed that miR-375 and miR-141 were significantly up-regulated in prostate cancer specimens and their release in blood was further associated with advanced cancer.

When levels of vesicle-related miRNAs were correlated with patient survival in NSCLC, it was observed that levels of let-7f and miR-30e-3p were associated with poor outcome. A separate study looked at transforming growth factor-β signaling pathway–related serum miRNAs as predictors of survival in advanced NSCLC.[203] Survival analysis identified 17 miRNAs significantly associated with 2-year patient survival, and 17-miRNA risk score was generated that was able to identify patients at the highest risk of death.

It has been consistently demonstrated that expression of miRNAs is altered in tumor compared to normal tissue, and that these changes may be reflected in the plasma/serum of cancer patients compared to healthy individuals. However, when small differences are observed, the significance of findings cannot always be determined.[204] Larger studies are needed to better define clinical utility of these blood biomarkers.

Exosomes and Circulating Microvesicles

In addition to CTCs, cfDNA and that subclass denoted ctDNA, exosomes, and circulating microvesicles are small membrane-bound cell fragments (sizes between 30 and 1000 nm diameter), and they may find clinical use in the near term.[205,206] Exosomes overlap with circulating microvesicles in size on the smaller part of the range (30 to 100 nm in diameter). Exosomes arise from a somewhat distinct mechanism: they are released either from the cell when multivesicular bodies fuse with the plasma membrane or directly from the plasma membrane. Like circulating microvesicles, evidence is accumulating that exosomes have specialized functions and play a key role in coagulation, intercellular signaling, and waste management.[207,208] Both of these circulating cell parts are found in a variety of body fluids and interstitial spaces.[209] Although many investigators initially thought these were cellular debris, circulating microvesicles and exosomes were recently shown to have roles in cell signaling and intercellular molecular communication.[210–212] This rubric currently comprises a heterogeneous population of exosomes and shed microvesicles, with distinct mechanisms of formation. Circulating microvesicles are actively released into the extracellular space to interact with specific target cells and have been demonstrated to deliver bioactive molecules.[213] In many carcinomas, circulating microvesicle levels increase.[214] This alteration in circulating microvesicle burden may find future use as a surrogate for disease severity.[61,215] In addition, microvesicle biochemistry may provide biochemical or molecular markers for tumor severity.[216,217]

Conclusions

Isolation and characterization of CTCs and ctDNA are likely to improve cancer diagnosis, treatment, and minimal residual disease monitoring. Examination of peripheral blood has the advantage of providing a minimally invasive method for early and serial assessment of multiplex predictive and prognostic markers during multistage disease progression. Because higher CTC burden has been shown to predict poor prognosis in metastatic disease, efforts at aggressive chemotherapeutic intervention are being tested.[218] The initial results from these studies are promising. However, exploratory trials will need to take into account the possibility of tumor heterogeneity, within both the tumor and CTCs. Emerging evidence suggests that CTC heterogeneity for tumor-related mutations exists

and that it may be clinically important.[219–222] Yet, investigators are going to require a more complete understanding of the phenotypic aspects of a tumor that can be inferred from CTCs. Specifically, detection of cancer-related molecular alterations in CTCs and ctDNA may provide an advantageous substrate for precise information about a patient's disease. However, more trials are required to validate the clinical utility of precise molecular markers for a variety of tumor types.

Epigenetic alterations, cfRNA, and miRNA are each in the early stages of biomarker development. Each has shown promise in breast cancer and prostate cancer, with some limited success for using cfRNA in rectal cancer prognosis through telomere-specific PCR. Although some miRNA species have been implicated in high-profile public discussion as biomarkers, their clinical utility has not yet been established. It would, therefore, be premature for molecular pathology validation.

Finally, although exomes and microvesicles have not yet found application in the clinical laboratory, pharmacological assay of exosomes and nanoparticles may eventually permit a gauge for the efficacy of delivery of targeted agents via novel techniques.

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Acknowledgments

The Circulating Tumor Cell Working Group took the following approach to summarize relevant research results in this active area of clinical investigation: i) The group subdivided topics appropriate to each member's area of expertise for initial research and draft. ii) Sections were then assembled as a rough draft document. iii) The rough draft document was critiqued and edited by each member of the working group. iv) The document was then circulated to the Clinical Practice Committee publication's subcommittee for critique. v) A revised document was then submitted for publication.

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