Circulating Tumor Cells and Nucleic Acids for Tumor Diagnosis

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Introduction

"There are no free lunches" is an assertion popularized by the economist Milton Friedman. A blood based biomarker for solid tumors that can access the entire tumor genome/transcriptome could potentially guide treatment selection, sharpen prognosis, monitor treatment response, detect minimal residual disease (MRD), and possibly enable population screening. This seems to offer a "free lunch" for molecular pathology. The analysis of circulating tumor cells (CTC) and circulating tumor nucleic acids (CNA) each hold the promise of meeting these objectives.[1] These are not the first promising cancer biomarkers but unlike biochemical and immunological biomarkers, CTC and CNA methods do not target a single moiety and provide a wide range of pan-tumor and patient-specific markers.[2-5]

The first observation of CTC was reported in 1869,[6] and the first observation of circulating tumor nucleic acids was reported in 1972.[7] Advances in immunohistochemistry, image cytometry, and molecular biology have converged to the point where circulating tumor cells can be analyzed by methods ranging from morphology[8] through 10-color immunohistochemistry[9] and FISH[10,11] to whole genome analysis of single cells.[12] Cell-free circulating tumor nucleic acids (genomic DNA, mRNA, miRNA) can be quantified and analyzed for changes like mutation and methylation using common methods.

A better understanding of CTCs *might* also offer insights into the biology of tumor progression and metastasis.[13-15] The majority of CTC do NOT form metastases, dormant or active. The problem of determining when CTC should be worrisome will be familiar to pathologists: a similar question is raised by detection of isolated tumor cells in tissues. Complicating interpretation is that before resection, CTC arise in metastases and reseed the primary.[16-18] CTC can persist following resection of a primary tumor in the absence of detectable metastases; this could present a window into tumor "dormancy".

Most work on circulating tumor cells has been directed at breast and prostate cancer. Colon cancer, in contrast, uncommonly presents with metastasis; nonetheless, disseminated tumor cells are present in the bone marrow of many or most patients with colon cancer. CTC and tumor CNA are present in a substantial proportion of patients at all stages of primary gastrointestinal malignancies and in the majority with stage IV.[19-24]

Most studies have been small scale with casual rigor in design. A few have studied CTC for chemosensitivity.[25,26] A specific assay format for CTC has received FDA approval following large prospective trials: the change in CTC number following therapy *may* be used as one of the several criteria for changing therapy for metastatic colon, breast and prostate cancer.[27] Three exciting studies have shown the successful application of CTC/CNA analysis to monitor minimal residual disease (MRD) by targeting tumor-specific mutations and gene rearrangements.[3,4,28] The rationale is attractively simple. Profiles of circulating microRNA show promise for robust detection of primary epithelial tumors. The NIH directory of clinical trials lists 80 approved or active trials which include analysis of CTC/CNA, in sixteen CTC/CNA is the main focus (http://www.cancer.gov/search—accessed 01-06-11). Whether or not CTC and CNA assays will add a "free lunch" to the lab test menu awaits the outcome of large prospective clinical trials.

Terminology. "Circulating tumor cells" (*CTC*) can originate in EITHER primary OR metastatic tumors.[16,17] "Disseminated tumor cells" (*DTC*) are individual tumor-derived cells found in any site other than the circulation, such as lymph nodes or bone marrow. "Isolated tumor cell" (*ITC*) is defined in the TNM classification so as to include "micrometastasis": "single

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malignant cells or a few tumor cells in microclusters", not more than 0.2 mm in diameter, present within a lymph node.[29] "Circulating nucleic acids" (*CNA*) refer to nucleic acids found in serum or plasma free of cells, the prefix "cf" will be used to denote "cell free" for clarity as needed (e.g., cf-mRNA). Context should make clear if CNA refers to baseline levels or tumor-derived CNA.

Circulating Tumor Cells

Historical Background

The origin of the term "metastasis" traces to an 1829 publication by Jean Claude Recamier.[30] It was natural to speculate on the existence of circulating tumor cells but it was not until 1869 that circulating tumor cells were described, albeit in an autopsy.[6]

Biology

A Circulating Epithelial Cell Is Presumed to Be Neoplastic. Many studies are designed to determine the effect of CTC number on prognosis and so do not include a "healthy subject" arm. Early studies took pains to demonstrate that circulating epithelial cells, putative CTC, had karyotype abnormalities consistent with those of the primary.[31] CTC occur at low levels if at all in "healthy" subjects, or those with adenomas or inflammatory disease.[24,32,33] The presence of genomic alterations in the CTC in an apparently healthy subject would raise concern, but would not be proof of malignancy rather than dysplasia.

The Earliest Tumor Stage Can Generate CTC. There is a modest correlation of stage with increasing incidence of CTC and number of CTC. A low level has been reported in association with colonic adenomas.[32,34]

Not All Tumors Generate CTC. CTC have been identified up to 80% of many tumors types including gastrointestinal malignancies. The level of CTC varies widely, possibly all tumors shed CTC but some below the level of detection. The determinants are unclear. Anatomic position and size of tumor are NOT determinants in any obvious way. Variation in neoangiogenesis, altered motility, altered adhesion, and predisposition to apoptosis are all considerations.[35] Although gene signatures of primary tumors have been proposed to correlate with propensity for metastasis they have not been assessed with respect to presence or levels of CTC.[36-38]

The Mechanisms by Which Tumor Cells Intravasate to Become CTC Are Uncertain. Bockhorn et al, in a paper entertainingly subtitled "Do cancer cells crawl into vessels, or are they pushed?", presented evidence that most CTC are apoptotic.[39] This might explain, in part, how so many CTC give rise to so few DTC even though other work shows that most CTC do enter the tissues. Although several xenograft studies show high levels of apoptosis, however, most studies of CTC in humans do not *report* on apoptotic cells.[40-43] This might reflect a difference from the animal models, but *might* also reflect a bias against apoptotic cells during enrichment or in analysis.[41,44] An antibody against a neo-epitope of cytokeratin 18, which is generated in apoptosis, can be shown to mark apoptotic epithelial cells, benign and malignant. It can be used for example with the CellSearch system but is NOT part of the FDA-approved system.[41,44]

Investigation of the mechanism of CTC generation requires animal studies of human tumor xenografts (or murine tumor transplants). The study of CTC from spontaneous tumors even in animals genetically predisposed to tumors would face challenges presented by the uncertain time course and the small blood volume available to sample. Studies using intravital microscopy have helped unravel how tumor cells intravasate (and extravasate).[45-48] Chang and Tomaso implicated the development of mosaic blood vessels within tumors as providing an egress. Access to the vasculature would seem to be a requirement[49-53] but connections from the lymphatics are much less studied and their use as an alternative exit cannot be dismissed.[54-56] Whether CTC come from the periphery, the central region of a primary, or both is unknown. There is evidence that they show an altered response to hypoxia.[57]

CTC Are Rare. The concentration of CTC varies widely as a function of the patient tested and the analytical method. Typical measurement ranges from extremely rare (1 per billion nucleated blood cells) to merely very rare (1 per 10,000,000). Assay sensitivity and specificity is often determined by assaying patient samples "spiked" with cells from a tumor cell line. While a useful indicator, this probably overestimates performance in practice. There is no gold standard, so it is hard to assess the rate of false negatives with clinical specimens. Given the low concentration, the total number of CTC generated daily might seem surprisingly high after factoring in the half-life and blood volume. One of the few studies to address this experimentally in mice calculated $3–4 \times 10^6$ cells shed/g tumor/24 h.[58]The commonly cited estimate of 10^9 tumor cells/g (1 cm diameter) has been criticized as too large, but even so millions of CTC traverse the vasculature every day in many patients.[59]

CTC Half-Life Is on the Order of Hours. Measurement of CTC before and after resection of a tumor gave an estimate of 1–2 h in circulation in one study and a more global estimate of 24 h to reach an undetectable level (at most ten

half-lives).[60,61] An animal study in which labeled tumor cells were injected intravenously (not exactly mimicking the clinical scenario) showed 80% removed within 2–4 h. Many "settle out" in the vasculature. Many extravasate but most do NOT become a long-lived DTC). Many are actively removed in the liver or possibly lung.

No single operational definition of circulating epithelial tumor cells identifies all ctc, not all circulating malignant epithelial tumor cells appear epithelial. Because of their rarity, CTC have to be enriched from samples. Immunoselection is the most common method; the EpCam antigen and cytokeratins (after the cells have been permeabilized) are the most common target antigens. Neither marker is ideal.[62-65] In epithelial–mesenchymal transformation (*EMT*) epithelial cells take on a mesenchymal phenotype. If one believes EMT is a real process[66] one must consider the possibility it is reflected in CTC.[67,68] Immunoselection or immunodetection for epithelial markers could miss CTC altered by EMT. Size can be used to separate blood cells (small) from CTC (large), some implementations are rapid and simple; however, epithelial tumor cells can be smaller than the typical cut-off.[69] In contrast negative selection, in which only hematopoietic cells are removed, captures both EMT-modified and aberrant epithelial cells providing considerable enrichment for analysis.[70]

CTC often do not travel alone. Early reports described "tumor emboli" consisting of epithelial tumor cells, platelets, and neutrophils.[71-73] The evidence suggested that tumor emboli formed metastases more efficiently than did isolated CTC. Konstantopoulos reviewed recent literature on the role of platelets and of fibrinogen in metastasis.[74] Most CTC-centric studies take little note of platelet or neutrophil fellow-travelers. This might reflect that most methods to isolate CTC do not show emboli. Filter-based size selection methods like ISET can show mixed CTC clusters. It is not clear if other methods disrupt clusters, preferentially select single cells or discount clusters in image analysis.

CTC show genomic heterogeneity. A macroscopic tumor can be composed of a single dominant clone or of several divergent clones with both common and distinct genomic changes.[75,76] Navin et al reported a detailed study of heterogeneity in breast cancers and a discussion of the conceptual issues in analysis.[77] The size of the genomically homogenous region will vary inversely with the resolution of the method for genetic analysis. This picture is complicated by the recent finding that CTC *from* metastases can home to the primary.[16,17] It follows that CTC at any given time *could* vary widely with respect to genomic sequence. A small number of studies using FISH analysis has shown significant variation among CTC (and a low but still surprising level of abnormalities in the few CTC from "normals".[10,78] DTC, which arise at different times over the lifetime of the primary tumor also show prominent genetic divergence from each other and from the primary.[12,79]

DTC must originate as CTC, most CTC do not become DTC (sleeper cells, sleepwalking cells, and zombie cells). "Tumor dormancy" refers to an inferred state of disseminated tumor cells to account for the late appearance of metastatic disease years after resection of the primary.[60,80-82] Since CTC can be observed long after resection of a primary tumor, they must originate from a population of proliferating disseminated tumor cells in an equilibrium state, perhaps held in check by the microenvironment and/or the immune system. There is minimal evidence for a separate long-lived CTC compartment.

Dormant tumor cells can be likened to secret agents, planted years ahead of time ("sleeper cells"). The activating influences are unknown. Whether every DTC could potentially be activated is unknown. Presumably all DTC originate as CTC but the sheer number of CTC compared to DTC implies that most CTC do not become DTC even if the CTC extravasate. Whether or not most CTC *could* give rise to DTC if only they were to lodge in a receptive location is unknown: these CTC could be likened to "sleepwalking" cells. Many CTC are apoptotic ("zombie cells"?). The inefficient nature of this process is illustrated by an arresting *clinical* study.[83] In the study by Tarin 29 subjects with both malignant ascites AND peritoneovenous shunts were monitored, some for months. 15 subjects underwent autopsy. Some subjects were free of remote metastases even at autopsy, and most metastases identified were small.

Most DTC Are Probably not CTC in Transit. Techniques like Ki67 staining show that most DTC are not proliferating ("dormant"). This does NOT exclude the possibility that the DTC found in any given biopsy is actually a nonproliferating CTC caught in transit as by freeze-frame. In one animal model study, cells of a breast cancer line were loaded with fluorescent nanospheres and injected i.v. into mice.[84] Most cells settled into the liver. DTC with high levels of fluor could be identified for months, showing that the cells were not proliferating; however quiescent is not the same as "immobile".[84,85] CTC were not assayed.

CTC: Methods

The methods can be categorized with respect to the three phases of the process—collection, enrichment, and detection/analysis.

Collection

There have been few explicit studies of preanalytical variables such as the time to processing.[86,87] The only parameter uniformly reported is the collection volume, with typical values ranging from 5 to 20 ml. The most published work is for the CellSearch method which uses collection tubes containing a proprietary fixative as well as an anticoagulant.[24,86,88,89] Fixation provides flexibility in work flow; this is important for multi-institutional trials with a common laboratory and for reference labs.

Enrichment

Red Cell Lysis

Most protocols require removal of RBC prior to analysis. Typically this is achieved either by osmotic RBC lysis or density gradient centrifugation, followed by multiple washes. Each step carries the risk of CTC loss. The CTC-chip *flows* 5 ml whole blood past thousands of microscale "pillars" decorated with antibody, avoiding lysis and centrifugation.[2] The lack of RBC lysis *might* explain in part the high levels of CTC found with this system relative to other systems.

The 2009 N.I.H. conference "Circulating Tumor Cells: Emerging Technologies for Diagnosis, Prognosis, and Treatment" highlighted a wide variety common as well as uncommon methods such as dielectric cell separation[90] and photoacoustic flowmetry.[91] This does not exhaust the variety described in the literature.[46,92-96] An intriguing functional selection method plates peripheral cells on chorioamniotic membrane coated tissue culture plates.[97,98] The DEPArray system employs dielectrophoretic separation using tens of thousands of programmable "cages" which can separate individual cells followed by imaging and permits individual cell recovery, all on a silicon chip integrated with a microfluidic cartridge.[99]

The most common approaches fall into three categories:

- Flow cytometry/sorting
- Selection by size (filtration)
- Immunomagnetic selection (positive or negative)

Flow Cytometric/Cytometric Methods

Current flow sorters can process 50,000 cells/s so it is feasible albeit nontrivial to look for CTC as is shown in the studies by Low et al.[46] Low et al also describe a novel marker for CTC: a fluorescently labeled folate analog which binds tightly to the high-affinity folate receptor commonly expressed on epithelial tumors.[100] Sorting permits post-detection analysis by methods like FISH.[101,102]

Size Selection

Circulating epithelial tumor cells are *generally* larger than hematopoietic cells.[69] Filter-based size selection using an 8 μm opening is typical. Microfluidic size selection methods have also been described.[93] The large cells which are retained can be transferred on the filter to a slide for IHC or FISH studies or pooled and studied by PCR. Commercial systems are available: ISET (Isolation by Size of Epithelial Tumor cells).[69] The system could underestimate CTC by missing small tumor cells, especially those undergoing EMT. Occasional large nonepithelial cells which are captured should be flagged by the subsequent detection method. ISET is the *only* method so far shown to consistently capture clusters of CTC, keeping them intact. The limited evidence indicates that the process does NOT *produce* CTC clusters.

Positive Immunoselection

Immunoselection can be performed manually using magnetic beads or with automated instruments. A tissue-agnostic approach is to perform immunocapture with an antibody directed at a pan-epithelial target. EpCAM and cytokeratins (typically 7, 18, 19 and 20) are the most common targets for positive selection. EpCAM (CD326) is implicated in adhesion, it is NOT universally expressed on CTC.[62,103] There are differences in performance among the several EpCAM specific monoclonals. Antolovic et al investigated the effects of different EpCAM specific antibodies in CTC enrichment.[104] mAb BerEP4 and mAb KS1/4 recognize different epitopes. Detection was performed using anti-CK20 IHC. Of 39 patients 11 were positive with BerEP4 enrichment, 5 positive with KS1/4, none with both antibodies. Compounding problems, EpCAm expression can be lost in EMT. The cytokeratins (CK) are intracellular antigens so a permeabilization step is necessary prior to selection or detection. CK can be upregulated on granulocytes in inflammation, so immunoselection for CK will be problematic as will detection if selection does not exclude granulocytes.[105,106]

Antibodies specific for a particular tissue, such as CEA, have also been used. In most such studies the selected cells were pooled and analysis was limited to PCR (mRNA for expression or DNA for mutations) rather than cell-by-cell analysis as with IHC. The commercial AdnaTest uses a mixture of anti-EpCAM and anti-MUC1 antibodies; however, this selects any cell positive for EpCAM OR MUC1 (or both). Peripheral blood cells recovered by nonspecific interactions should be invisible if the gene target is tissue-specific (for mRNA) and/or mutations are only present in CTC (for DNA) both of which are plausible assumptions. This also presupposes that normal epithelial cells do NOT circulate or at least not at the level of CTC.

Negative Immunoselection

Antibodies such as anti-CD45 can be used to enrich by negative selection.[70,107] CD45 is expressed on most peripheral blood cells but NOT on epithelial cells, malignant or benign. Lara et al typically reduce the number of blood cells from 4×10^9 to 8×10^3 cells per mL, at which point analysis by IHC/FISH is feasible. Using this approach CTCs were detected in 20 of 32 head and neck cancer patients.[107] The average number of CTCs detected was 22 per mL of blood with the number ranging from 1 to 282 CTC/ml. This method should also capture and possibly detect EMT altered CTCs.

CTC Methods of Analysis

Morphology. Surprisingly few studies have reported morphologic assessments.[8,108] One study asserts that CTC reflect the morphology of the primary tumor in the sense that inspection of the CTC permitted differentiation among breast/colon/prostate/lung in many cases. The cytologic appearance of EMT has not been described.

Ploidy. DAPI staining is widely used primarily to ensure only intact cells are studied. Although there is a long tradition of studying DNA ploidy in tumors by image cytometry, this has received little attention with respect to CTC.

IHC. This is the most common technique for selection and enumeration of CTC. A CTC is typically defined as an EpCAM positive (or cytokeratin positive), CD45-negative cell with an intact DAPI-stained nucleus. Image analysis with carefully matched highly multiplexed fluorescent antibodies is feasible. Uhr et al described a 10-plex system.[9] Negative immunoselection requires scanning more cells than does positive immunoselection. Although Balasubramanian et al[70] describe using confocal microscopy there are higher throughput systems such as the imaging cytometer which permits morphologic imaging of cells and can incorporate both IHC and cytogenetic FISH images (cells are labeled before flow). Samples would have to be enriched.[109] In the FAST system the entire specimen is spread on a large slide. The cells are fixed and then stained. The entire slide is scanned by a laser cytometer.[110,111]

Cytogenetics. Karyotyping was important in early studies to confirm the relationship of CTC to the corresponding primary tumors. More recently FISH has been applied both to trace clonal evolution and to look at pharmacogenetic predictors like HER2 amplification in breast cancer. Patient/tumor-specific translocations could be suitable for FISH as well as RT-PCR analysis of CTC to detect MRD.[28]

Gene Expression. Numerous tumor specific targets (e.g., CEA, guanyl cyclase) as well as epithelial markers (EpCAM, keratins) have been tested by RT-PCR in CTC. Most reports have relied on qualitative detection (present, absent) even when using real-time PCR. Quantitative PCR could provide greater reliability as would validation of cut-offs.

Mutation Detection. Detection of specific mutations, such as Kras2 in a metastasis generally but not invariably reflects the status of the primary tumor.[112] A similar disagreement can be seen when CTC are analyzed but is much less studied.[10] As with gene expression, validation of sensitivity and specificity is often limited.

Whole Genome/Transcriptome Amplification. CTC and CNA yield little nucleic acid for analysis. Whole genome or whole transcriptome amplification can be applied, enabling use of microarrays or NextGen sequencing.[113-116] Whole-transcriptome amplification of CTC by Smirnov et al generated a five-gene profile which could distinguish among CTC of colon, prostate and breast cancer as well as from normal profiles with 94% accuracy.[113]

In Vivo Detection (Animal Models). Tumor xenografts either expressing GFP or preloaded with a fluor-like FITC-dextran are injected into an immunodeficient mouse, then monitored by fluorescent microscopy through temporary skin flaps or permanent "surgical windows". This approach can offer insight into the generation of CTC within the tumor as well as on circulation kinetics.[117]

CNA

History of CNA Analysis

The earliest demonstration of cell-free DNA in blood was in 1948.[118] The first report of CNA in association with solid tumors was, arguably, in 1972 or 1977.[7,119] An increased level of *total* DNA in the serum of patients with malignancy was seen relative to serum of healthy patients. Elevated levels of CNA in autoimmune disorders, especially lupus, had already been reported. It was much more recently shown that mRNA, microRNA, and methylated DNA can be routinely measured in cell-free serum and plasma.

The advent of PCR enabled demonstrations that the same mutation in a primary tumor could often be found in the corresponding CNA. Kras-2 was by far the most commonly studied gene. In a prospective study Kras2 mutations were identified in the CNA of 3.8% (of 1,098 subjects) and TP53 mutations in 5.5% (of 550 subjects) of patients with bladder cancer, but Kras and TP53 mutations were ALSO found in CNA from 1% and 3% respectively of healthy controls.[120] Mutations were found in smokers without known tumors.

Detection of mutations in cfDNA occasionally presaged the clinical detection of cancer.

Fleischacker et al provides an exhaustive summary of the literature on CNA through 2006.[121] The survey covers literature on point mutations, microsatellite variation, loss of heterozygosity, methylation, and RNA expression. As a result of the limited experimental design, the early studies mainly serve as proof of principle—that alterations can be identified in the CNA of many cancer patients.

"Personalized medicine" is a current touchstone of optimal medical care. Genomic/transcriptomic analysis of a tumor can identify point mutations, insertions–deletions, and fusion transcripts which are present in the tumor (or at least the dominant clone) and then used for literally "personalized" monitoring of minimal residual disease by analysis of CTC, CNA, or both.[2-4,28]

Much current work is directed at the measurement of circulating methylated promoter sequences, microRNA profiles, and tumor-specific mutations. MicroRNA expression signatures show robust performance in classifying epithelial cancers including "tumors of unknown origin".[122,123] Profiling of circulating miRNA appears very promising for detection of solid tumors.[124-128] Whole genome amplification of tumor CNA has been successful for moderate scale SNP/LOH analysis.[129] The few published NextGen Sequencing studies of CNA for copy number are technically unsatisfactory.[130,131] Next Generation Sequencing of fetal DNA in the maternal circulation (an example, after all, of circulating neoplastic DNA) *has been* analyzed successfully by Next Generation Sequencing for copy number variation.[132]

CNA: Biology

Source of CNA Circulating DNA, mRNA, and miRNA are present at low levels in all people. The basal level of CNA reflects normal cell turnover primarily from hematopoietic cells. A study of a sex-mismatched bone marrow transplant recipient showed that most cfDNA came from the donor.[133] It is a leap to generalize from a result in a bone marrow transplantation patient, but this conclusion supports expectations. Elevated levels of cfDNA have been reported in conditions such as stroke and lupus and after radiation therapy.[134-138] In many but not all cancer patients, the level of total circulating DNA is elevated.[139-143] The proportion of CNA derived from CTC (as distinct from primary tumor) has not been determined. In some cases a rise in total cfDNA has preceded clinical detection of cancer.[140,141]

That some cancer patients had elevated cell-free DNA did NOT prove that the cell-free DNA came from the tumor rather than immune or stromal cells. The demonstration that the primary tumor and CNA often contained the same mutation or loss of heterozygosity supported the conclusion that some increased DNA came from the tumor cells.[144,145] Xenograft studies of human tumors in immunosuppressed mice showed the cfDNA is overwhelmingly of human origin. The reported fraction of tumor DNA ranges from 3 to 93%, a summary statistic cited in the review by Ziegler et al.[146] Jahr undertook a novel approach, measuring levels of rearranged T-cell receptor gene sequences and of methylated selectin promoter in cfDNA, reasoning that these would reflect the inflammatory and endothelial (stromal) components.[147] Failing to see signals for the study concluded that most cfDNA in cancer patients is tumoral in origin.

Whether cfDNA originating from tumor cells is generated by apoptosis or necrosis has been decided both ways. DNA from necrotic cells is suggested to be high MW (>10,000 kb) whereas DNA from apoptotic cells is shorter, typically showing a ladder pattern with "rungs" 180 bp apart, reflecting nucleosomal packaging.[148,149] Jahr et al looked at CNA in subjects with a variety of tumors and found both short and long DNA fragments.

DNA. Circulating DNA is thought to be carried in nucleoprotein complexes. Circulating nucleoproteins, including the specifically methylated histones have been implicated, but it has not been shown that the bulk of cfDNA is present in such complexes.[149-152] The fate of various CNA is uncertain. cfDNA disappears rapidly, with a half-life of 4–30 min, as judged by study of circulating fetal DNA.[153]

Methylated DNA. Methylation of DNA in promoter sequences can turn gene expression down or off. Methylation of the promoter for MLH1 in many sporadic microsatellite unstable colon cancers is a classic example. Methylated DNA promoter sequences, including MLH1, can be detected in CNA.[154,155] Conditions, such as aging and inflammation are associated with increased methylation of varying combinations of promoters; whereas some drugs can decrease methylation.

mRNA. Measureable cell-free mRNA is present in the circulation despite the reputation of RNA for "fragility". Serum has potent RNAse activity: exogenous RNA added to serum shows a half-life on the order of seconds.[156] The leading explanation is that the cf-mRNA is present in either a proteolipid complex or an intact microvesicle.[157,158]

miRNA. Mature miRNAs are 20–25 nucleotide long RNA molecules. Each miRNA includes a "seed sequence" which matches, imperfectly, corresponding sequences in tens or hundreds of mRNA. Mature miRNAs regulate these mRNA either by inhibiting translation or promoting degradation. The stability of miRNA in blood, serum, and plasma is striking. After incubation of aliquots of whole blood at 4°, 25°, or for up to 4 days "housekeeping" microRNAs were readily demonstrated with modest change (unpublished). As for circulating mRNA, experimental evidence shows that circulating miRNA is present in micro-vesicles, proteolipid

complexes (perhaps from microvesicles), and in exosomes.[159,160] Analysis of circulating miRNA in cancer was reported in 2008.[124] Kosaka has already reviewed 20 studies which primarily correlate cf-miRNA expression profiles with the presence of specific solid tumors.[126]

CNA Methods

Collection of Sample. The level in serum is invariably higher than in plasma, the difference attributable to the progressive release of nucleic acids from hematopoietic cells lysed in the evolving clot. The difference increases with the delay in separation. Many current studies still underestimate the effect of preanalytic variation in boosting apparent cfDNA (probably all CNA) levels.[161-164] Platelets, which do not contain genomic DNA, do contain both mRNA and miRNA.

Purification of CAN. Standard methods for purification of nucleic acids from blood cells can be used provided they do not loose low-molecular weight CNA. Commercial kits specific for cfDNA are available. Molecular diagnostic laboratories routinely isolate viral RNA and DNA from plasma.

Size of CNA. Notwithstanding the debate over apoptotic versus necrotic origins, much cfDNA is less than 500 bp.[165] As noted, mature miRNAs are already small. Accurate sizing and concentration measurement in this range can be obtained using microfluidic instruments such as the BioAgilent 2000.

Nucleic Acid Quantitation. UV spectrophotometric absorption is insufficiently sensitive. Fluorescent dye-binding methods, using agents like PicoGreen™ (DNA) and Ribogreen™ (RNA) are satisfactory.[166] The typical diploid human cell contains 6.6 pg of total DNA. 1,000 cells would correspond to 6.6 ng, this is near the lower limit reported for cfDNA/ml blood. Contrast this with intact CTC: 1,000 CTC/ml is a high level.

Jung compared 7 reports and found means ranging from 6 to 650 ng/ml of plasma.[167] The largest study, including 776 controls, showed a median plasma cfDNA of 26 ng/ml, a mean of 67 ng/ml but a standard deviation of 405 ng/ml. Fleischhacker gives an exhaustive listing in various cancers and controls.[121]

Mutation Detection/DNA Copy Number/Gene Expression/miRNA Expression. As for any DNA or RNA source, if the region of interest can be amplified, it can then be studied by various methods including real-time PCR and sequencing. The often small amount of CNA limits direct use of high-throughput methods like microarray and Next Generation Sequencing but as noted above whole genome/transcriptome/miRNome amplification can overcome this, albeit at risk of distorting relative levels. Digital PCR methods like BEAMING, can provide quantitative analysis of mutations, copy number, or expression level with greater sensitivity and accuracy than does typical real-time PCR or microarrays but with limited multiplexing.[168,169]

Methylation Detection of methylated CpG sites is challenging. Numerous methods are in use.[170,171] A common feature is bisulfite treatment of the DNA sample prior to PCR amplification. Traditional protocols destroy up to 90% of the input DNA which makes working with small amounts of cfDNA especially challenging. Auwera et al showed correlated levels of methylated cfDNA and CTC numbers.[172] This leaves open the question of whether the methylated sequences derive from CTC or from the primary tumor. Auwera did NOT test for methylated sequences *in* the CTC. The highly parallel BEAMING method noted above has been modified and shown to work with circulating methylated DNA sequences.[169] The authors describe a bisulfite treatment protocol which leaves 99.4% of the DNA intact.

CTC and CNA in Specific Gastrointestinal Malignancies

The following sections summarize recent findings, organized by anatomic site, for CTC, circulating methylated DNA (promoters), mRNA, and microRNA.

Esophageal Cancer and CTC

A tour-de-force study by Stocklein et al examined the question of how closely DTC reflect the primary tumor and each other at the genomic level. The data impacts the uncertainty over whether DTC arise "early" or "late" in the evolution of the primary tumor. A parallel study of CTC could help dissect the relationship of CTC and DTC. This study looked at paired primary tumors and bone marrows from 104 consecutive patients with esophageal cancer (adeno and squamous) and disaggregated lymph node preparations from 18 of these patients. DTC were identified by staining for cytokeratins or EpCAM. 38 bone marrows and 9 lymph node preps demonstrated 1 or more DTC. Sixty DTC were individually selected by micromanipulation. The DNA from each cell underwent whole genome amplification and was then assayed by comparative genomic hybridization (CGH) (not by array). Twenty-two DTC showed amplification of the region 17q12–21, which encompasses HER2. Quantitative PCR confirmed amplification of the HER2 locus in 11 of the 22 cells. Focal amplifications including HER2 but less than 5 Mb in extent would have been missed by CGH. Amplification of 17q12–21 could also encompass a region short of HER2 but still contain other significant genes. In cases where more than one DTC came from the same case, the two or three cells showed similar CGH profiles including HER2 status. In the several cases with one DTC from LN and one from bone

marrow, the profiles shared the 17q12–21 amplification but were otherwise distinct. Perplexingly (or intriguingly) amplification was seen in only about 15% of the primaries without close correlation with the finding in DTC.

Fourteen studies since 2000 have examined CTC in patients with esophageal cancer, none examined CTC morphologically or immunohistochemically.[22,173-186] Eleven used RT-PCR to detect RNA transcripts of epithelial-specific genes in mRNA prepared from the mononuclear population of peripheral blood after density gradient centrifugation. In total these studies encompassed 883 patients, most were squamous carcinoma. The most frequently target was CEA. Other genes used to infer the presence of esophageal CTC were survivin, deltaNp63, SCC antigen 1, SCCA2, Eya4 (eyes absent 4) hTERT, and cytokeratin 20. One study looked p16 deletion and cyclin D1 amplification at the DNA level in CTC (and plasma).

CEA expression has been observed in activated lymphocytes.[187] Although some studies[178] report a narrow window between patients and healthy controls, most find minimal CEA mRNA in the healthy controls. The likeliest explanation (other than PCR contamination) is that the stringency of the cut-off varies among studies. Ito et al find CK20 transcripts present in the PBMC of most healthy controls, with considerable overlap with the level in patients with esophageal cancer.[178] Contamination with activated lymphocytes has been described several times.[106] Six reports do not report quantitative results even when they use real-time PCR.

Survivin is of uncertain function beyond ability to inhibit apoptosis in selected settings. Survivin mRNA is present in the basal layer of the epidermis (and in other epithelia) but not in the intermediate or superficial layers. SCCA1 (Squamous Cell Carcinoma Antigen 1) is a member of the ovalbumin family of serine protease inhibitors. SCCA2 is a homologue found in tandem with SCCA1. SCCA1 mRNA is present at the mRNA level in lymphocytes at a very low level compared to that in epithelial cells but this could be significant relative to the level in CTC.[179] "deltaNp63" is an isoform of p63, a homologue of p53, implicated as a marker of epithelial stem cells.[188] In normal subjects expression of deltaNp63 is confined to the basal layer of stratified epithelium. In normal cells p53 targets deltaNp63 for degradation. Eya4 ("eyes absent 4") not surprisingly was first identified in drosophila. The protein has tyrosine and ser/thr phosphatase activity, beyond that little is known of its function.

The highest rate of positivity for CTC was found with survivin, with the proportion of positive cases ranging from 51 to 88%.[22,174] Grimminger did a short-term study looking only at the response to neoadjuvant therapy.[175] In that study expression of survivin in CTC was associated with a higher likelihood of a minor response but no incidence of a major response. In other studies elevated survivin levels were loosely correlated with worse outcome.

Li et al show increasing rates of positivity in correlation with worsening esophageal histology (normal, hyperplasia, dysplasia, cancer) for hTERT—24%, 30%, 52% and 80% respectively. Eya4 showed inferior discrimination at each stage.

Kaganoi et al looked at SCCA mRNA in CTC preoperatively and intraoperatively in 70 patients. 23/70 patients were positive for CTC preoperatively and 24/70 were positive for CTC collected intraoperatively but only 13 of these had also been positive in the pre-op sample. Of the patient's positive for CTC at admission, 17/23 recurred; of those negative 11/47 recurred. For those positive intraoperatively 16/24 recurred, and of those negative intraoperatively, 12/46 recurred. Of those negative in both assays, only 4/36 recurred.

These studies differ not only in the targets chosen but also in the clinical parameters: volume of blood sample, time of sampling (pre-op, intra-op, post-op), and treatment prior to surgery (for example in the Kaganoi study some subjects had chemo which might affect CTC levels), distribution of stages and length of follow-up.

Esophageal Cancer and CNA

Esophageal cfDNA. Takeshita et al measured CCND amplification (11q13) in plasma DNA in 96 patients using the level of the dopamine receptor locus as a control (11q22–23).[189] Although there was a trend toward increased recurrence in patients with a high ratio, it did not reach statistical significance but in multivariate regression analysis it did reach statistical significance.

Esophageal Cancer and Methylated CNA

Four other reports have examined CNA in esophageal cancer. All four looked at methylation markers.[177,190-192] Kawakami measured methylated APC promoter sequences in peripheral blood.[190] 13 of 52 patients with methylated APC in their primary tumor had methylated sequences detected in the plasma. Of the 13, 11 were adenocarcinomas. None of the controls (20 healthy subjects, 23 with gastritis, 11 with Barrett's metaplasia) showed circulating methylated APC promoter. The authors calculate a "hypermethylation" index, the ratio of methylated to nonmethylated sequences and determined a cut-off. Six of 52 plasma samples were considered "hypermethylated," all six had survival of less than 6 months, the non-"hypermethylated" subjects had a 50% survival rate of approximately 2 years.

Hoffmann et al looked at methylation of the APC and DAPK (Death associated protein kinase) promoters.[191,193,194] Of 59 patients, 61% were positive for one or both markers. Use of both makers led to significant discrimination with respect to survival (<2.5 years) with a p-value of 0.03.

Presurgical neoadjuvant chemoradiation was seen to significantly decrease levels of methylated promoter detected in plasma (an encouraging sign of treatment effect but an obstacle to developing a useful biomarker). Pretreatment DAPK promoter methylation had by far the strongest independent effect on survival but might be functioning as a surrogate marker for adenocarcinoma).

The study by Ikoma et al is the only study so far to look at CTC and plasma.[177] 44 patients were studied by RT-PCR for CEA and for specific methylated sites in p16, E-cadherin and RAR-beta. Mononuclear cells were isolated by density gradient from 5 ml of whole blood to measure CEA by RT-PCR. Another 5 ml aliquot was centrifuged sequentially three times to obtain cell-free plasma for detection of methylated DNA by qualitative PCR. CEA was detected in 12 patients (27%). Fourteen patients (32%) showed methylation in one or more promoters. Twenty-three patients had an abnormality in one of the assays. Methylation status of the primary tumors was not provided; no information was given regarding clinical parameters such as outcome.

Esophageal Cancer: Circulating miRNA

Xie tabulates the results of four miRNA profiling studies of esophageal cancers including adenocarcinomas and squamous cell carcinomas.[122] At the time of this writing there are no published studies of circulating miRNA in patients with esophageal cancer.

Gastric Cancer: CTC

Literature review identified 20 studies in the period 2000–2010.[195-215] These encompass 1,546 subjects (excluding controls), 13 had fewer than 100 subjects. Mimori et al looked at 810 patients (and 29 controls), extracting total RNA from whole blood (the discussion implies this was interpreted as reflecting CTC) and performing real-time PCR for CEA, CK-7, CK-19, and VEGFR-1.[203] 30% of all subjects were positive for one or more markers in peripheral blood (48% in bone marrow). Kolodziejczyk screened 268 consecutive patients with gastric cancer under consideration for chemotherapy followed by gastrectomy.[200] Samples were drawn before starting chemotherapy and just before surgery. Flow sorted CD45(−) cells screened for CTC by IHC for cytokeratins. Only 32 subjects showed CTC or DTC (bone marrow) (12%). The level of DTC but NOT CTC showed a response to chemotherapy, but the DTC "responders" showed a lower 3 year survival. Fourteen studies report correlation of one or more clinical parameters with the level of CTC.

Three studies looked at CTC directly; the others applied RT-PCR to detect gene expression or miRNA expression. Matsusaka et al used the CellSearch System to follow 52 patients with metastatic gastric cancer, each of whom had demonstrable CTC, to determine if the CTC level could stratify patient responses to therapy.[202] Patients were tested at baseline, then 2 weeks and 4 weeks after initiation of chemotherapy. The analysis concluded that a CTC level >= 4 CTC (per 7.5 ml) at either 2 weeks or 4 weeks correlated with worse outcomes (OS of 3.5 and 4.0 months respectively) than for subjects who had <4 CTC at those time points (OS 11.7 and 11.4 months). The differences in outcome, stratified by CTC, were held to be significant with $p<0.001$ independent of other parameters by univariate and multivariate analysis.

Some of the gene expression studies of CTC which do not use IHC or other imaging modality give ambiguous descriptions of the source of RNA. "RNA from the blood" could mean cells, plasma, or whole blood. If just red cells are lysed and the resulting supernatant processed for RNA it is possible that CNA will be processed and mistaken for RNA from CTC. The targets of gene expression studies included cytokeratins, survivin, MUC1, c-MET, MAGE-1 and -2, uPAR, VEGF, VEGFR-1, CEA, MT1-MMP, and CD44v6. Several studies compared CTC and DTC. Kita et al analyzed the presence of uPAR, CEA, CK-7, and CK-19 mRNA in DTC (bone marrow) and CTC (blood) in 846 patients with gastric cancer, by far the largest study of DTC/CTC in gastric cancer.[199] uPAR was the most discriminating marker. Using a stringent cut-off uPAR positive DTC were present in 51% and positive CTC in 48% of all patients. uPAR expression in both DTC and CTC showed statistically significant correlation with depth of invasion, stage, and distant metastases. Only CTC uPAR was an independent prognostic factor for distant metastasis by multivariate analysis. What appears to be the same large patient group was also studied for the utility of MT1-MMP and VEGFR-1 as biomarkers. Each gene reflects a potentially distinct role and each was found in DTC and CTC of patients with gastric cancer, but the proportion of positive patients was much smaller than for uPAR.

Chen et al provide one of the few comparative evaluations measuring CTC by both IHC and by RT-PCR.[196] Cells isolated on a ficoll gradient were subsequently either immunoselected with magnetic beads coated with antibody to CK20. IHC was performed for CEA, hTERT, CD34, and CD45. The methods section does not explicitly state the source of RNA, exegesis of the discussion suggests that it was from "whole blood" with the assumption that free circulating mRNA is negligible. Real-time PCR was performed for CK20 and beta-actin. IHC detected CTC in 25/60 (42%) of cases, none in gastritis controls ($n=20$). QPCR detected transcripts for CK20 in 32 of the 60 subjects (53%) and none in the gastritis controls. All 25 cases identified by IHC were also positive by QPCR. No clinical associations such as outcome were presented.

Gastric Cancer and CNA

Methylation. Despite several surveys of methylation in gastric cancer so far only one study of methylated sequences in plasma was identified for gastric cancer.[216,217] Bernal *et al* evaluated methylation of 24 genes in primary gastric carcinoma tissues from 32 cases. 11 genes were hypermethylated in at least 50% of cases. Of these seven genes were in a statistically significant association with the signet cell variant which could be validated in a second set. Of these only APC and Reprimo promoters showed significant methylation in plasmas paired with the tumors, and only Reprimo methylation was seen in plasma from asymptomatic cases. Reprimo participates in the p53 mediated cell cycle arrest at G2.[218] Methylation of Reprimo has been demonstrated in gastric cancer in an independent study by Luo et al as well as in other malignancies, both in the primary tumors and in the plasma (pancreas, prostate, bladder, lung)[218-221]

Gastric Cancer: miRNA. Xie et al reviewed miRNA profiling studies for all gastrointestinal malignancies, including nine studies of gastric cancers.[122] They list all discriminating miRNA from the individual studies.

Zhou et al analyzed miRNA expression in circulating *tumor cells* of patients with gastric carcinoma.[215] Zou et al looked at 90 patients—41 had pre-op samples, 49 had post-op samples drawn within 3 weeks of surgery, and 29 controls. The pre-op and post-op samples are from DIFFERENT sets of patients! miR-17 and miR-106a levels were each increased (normalized to the small RNA RNU6) in the patients with tumors relative to the controls but with moderate overlap. Both miRNA levels were decreased after surgery but remained higher than controls.

Tsujiura et al looked at several miRNA (miR-21, 17-5-p, -106a, -106b, and let-7a) in the *plasma* of 69 patients with gastric cancer, sampling both pre and post-op, and 30 healthy controls.[222] miRNA was also extracted from matching primary tumors where possible. Each of the five miRNA distinguished patients from controls ($p=0.006$) with let-7a decreased and the others increased in concentration. The miRNA(s) used for normalization is not given. Of various formulae tested by the authors, the miR-106a/let-7 ratio gave the highest AUC, 0.8979. No mention is made of confirming this conclusion with an independent validation set.

Colon Cancer and CTC

Sergeant et al reviewed all studies of CRC in which CTC were measured by *quantitative* RT-PCR.[223] Only 12 studies in the period 1999–2007 met their criteria for adequate study design. The sample size ranged from 27 to 168. The studies varied widely with respect to the cell selection method, gene(s) analyzed, and the time points sampled (preoperative, intraoperative, postoperative). Three studies did NOT indicate the collection time. Given the range of study designs any conclusions must be limited. Sergeant et al concluded that in the four studies with adequate published information, there IS evidence of an association of increased numbers of CTC (inferred from RT-PCR) with stage but NO evidence for an effect on disease-free or overall survival.

A comprehensive review, by Rahbari et al, employed a complex meta-analysis of the entire literature through June 2009.[224] The review included studies of peripheral blood CTC and bone marrow DTC. Studies were required to have more than 20 subjects and to provide sufficient information that a hazard ratio could be calculated for relapse-free survival and/or overall survival. The initial scan retrieved 1,864 studies. Of these 1,825 did NOT meet the criteria. The remaining 36 studies, including only five of the reports accepted by Sergeant, were analyzed in detail. The 36 studies encompass 3,094 subjects with sample sizes ranging from 20 to 438 patients (median of 67). Twenty-nine studies used only RT-PCR for detection of CTC. Target genes included cytokeratin 20 ($n=15$ studies), CK19 ($n=4$), CK18 ($n=2$), Kras2 ($n=4$), CEA ($n=14$), survivin, EphB4, Laminin, MAT, GalNAc, MAGE-A3, c-Met, EGFR, IL10, p63, and hTERT. The review assessed six categories of bias, the only category in which a significant number of reports failed (16/36) was lack of control for confounding. Undeterred, the reviewers performed subgroup and factor interaction analysis, finally drawing two conclusions:

- If CTC were present the recurrence free survival (RFS) as well as overall survival (OS) were each significantly decreased (hazard ratios 3.24 [95% CI: 2.06–5.10]) and 2.28[1.55–3.38]). DTC positivity was of marginal significance.
- The perioperative time point was the most significant for both RFS and OS.

To identify relevant publications available after the period covered by Rahbari et al, PubMed was searched for the single Mesh term “neoplastic cells, circulating”. Eight substantive studies were identified.[20,25,104,108,225-228]

Miller et al reviewed three *prospective* large multicenter studies CTC in metastatic disease using the CellSearch system, one each for metastatic breast, prostate, and colon cancer, which form the core for the FDA approval of CTC measurement using a specific reagent kit and a specific instrument. This system uses magnetic nanoparticles coated with anti-EpCAM antibodies for selection. The fixed cells are permeabilized and labeled with DAPI (to stain nuclei), anti-CK-Phycoerythrin, directed at the *intra*cellular cytokeratins 8, 18, and 19 (characteristic of epithelial cells), anti-CD45-Allophycocyanin to highlight retained leukocytes. The cells are scanned and the images analyzed semiautomatically with operator interaction. This system require that the CTC express BOTH EpCAM and cytokeratins. FISH analysis is possible but NOT part of the FDA-approved application.

FDA approval is for measurement of change in CTC number as an acceptable guide to changing therapy in patients with metastatic CRC..[19,24,229] Patients were serially monitored. For CRC the cut-off for "positivity" was >3 CTC/7.5 ml of whole blood. Of 295 normals, 3% had 1 or more putative CTC and none had greater than 5 CTC. Of 255 subjects with various benign diseases (all sites) 7% had one or more CTC, 0.5% had more than 10 CTC. Of 413 patients with metastatic CRC, 47% had one or more CTC, 18.2% had 5 or more CTC, 11.6% had 10 or more, 2% had 50 or more (the table does NOT list 3 CTC as a stratification cut-off). Patients with greater than 3 CTC showed an overall survival of 8.5 months, patients with less than 3 CTC showed an OS of 19.1 months. The discussion notes that results are significantly operator dependent despite the partially automated image analysis. Normal subjects showed a mean of 53 unclassified cells, patients with metastatic CRC showed a mean of 223 unclassified cells. These are large numbers in an assay where a change of one CTC/sample can have major impact on clinical action. Patients who converted from positive to negative even weeks after treatment had significantly improved outcomes. The authors recommended confirmation of a trend prior to clinical action. CTC measurement outperformed CEA measurement.

Operative manipulation can transiently increase the number of CTC.[230,231] A seemingly natural concern is that a large efflux of CTC might increase risk of metastases. Given the large number of CTC circulating daily in some subjects this would not seem a compelling concern unless there is a qualitative difference in the CTC released or if surgical stress changed the systemic or microenvironmental response to the CTC. The limited evidence is inconclusive.

Peach et al reviewed nine studies colon cancer in which sampling of peripheral CTC took place 24 h or more after surgery, when most CTC released by trauma, should be absent.[232] Six of nine studies showed the CTC count was an independent adverse prognostic factor.

Wong et al studied 462 patients using a manual bead-based immunoselection for EpCAM followed by manual IHC for CK20.[34] 62% of 132 patients with CRC were positive, 6% of 50 patients with adenomas were positive, and none of 160 patients with benign or no disease. The authors note that chromosome 17 aneusomy was seen in 90% of the CK20 positive cases. Hardingham et al using immunoselection and RT-PCR found CTC in 3/30 patients with adenomas and in 4/34 patients with ulcerative colitis.[32]

Colon Cancer: CNA

Methylated CNA. Numerous methylated promoter sequences have been identified in colon cancer.[233] Methylated hMLH1 promoter sequences have been detected in serum of patients with microsatellite unstable colon cancer.[154] In nine cases with hMLH1 promoter hypermethylation, three cases showed promoter methylation in the serum. Methylated hMLH1 promoter sequences were NOT identified in the serum of patients in whom the primary did not show hypermethylation. p16 promoter hypermethylation occurs in colorectal (as well as esophageal and gastric cancers) with the incidence often increasing with stage. Two studies reported detection of p16 methylation in serum of colorectal cancer patients. Zou et al looked at matching tissue and serum samples from 52 patients with cancer, 34 with adenomatous polyps, and 10 healthy individuals.[234] p16 hypermethylation was present in 20/52 (38%) of the cancer tissues. Of those 20 cases, 14 matching sera showed p16 hypermethylation (70%). No methylated p16 promoter sequences were detected in the sera of the other cancer cases, patients with adenomas, or healthy controls. Nakayama et al looked at matched tumor, remote normal mucosa, and serum samples in 168 cases of colorectal cancer. The text appears to assert that in all 99 positive tumors, the matching serum also showed at least weak p16 promoter hypermethylation whereas controls showed no methylation in serum, but a figure in the text suggests only 30–40% of sera from patients showed substantial methylation.[235,236]

Among the interesting recently identified genes methylated in colorectal cancer is HPP1 (Hyperplastic Polyposis Gene 1, aka TMEFF2, ["transmembrane containing epidermal growth factor and follistatin domains"]).[237] HPP1 is expressed in epithelium along the GI tract and in pericryptal myofibroblasts. The function is unclear. Sabbioni and Wallner have each shown that methylated HPP1 promoter sequences can be detected in serum of patients with colon cancer. Sabbioni found hypermethylation in 83% of tumors and none in control tissues.[238] Methylated HPP1 promoter sequences were identified in serum. Wallner et al identified HPP1 promoter methylation in the sera of 13% of 24 patients with local CRC, 36% of 14 with metastatic disease, and none in 20 healthy controls.[239]

Model et al used methylation-specific microarrays to assay colorectal samples: 115 metastatic cancers, 89 adenocarcinomas, 55 polyps, 31 inflammatory bowel mucosa, and 67 healthy mucosae.[240] Promising candidates included TMEFF2 (HPP1), ZDHHC22, SLITRK1, SLC32A1, DLX5, GSK3B, NGFR, and PCDH17 all of which distinguished colon neoplasia not just from normal colon but also from other tissues, especially blood (important for application to CNA). Ten markers were confirmed by real-time PCR on an independent set of 149 adenocarcinomas. Methylation of an additional marker, ALX4, showed high specificity for colon tissue BUT in both normal and malignant colon.

A second large survey used "methylation microarrays" followed by quantitative PCR confirmation.[241] This time candidates were chosen not just for minimal expression in normal tissue but also for minimal expression in blood cells. The three top candidates still included NGFR and TMEFF2 but now also SEPT9. A member of the septin protein family,

SEPT9 was discovered as a fusion partner in leukemia. The function of this class of proteins is uncertain but involves, in part, binding to the metaphase plate during mitosis. Loss of SEPT9 leads to loss of chromosome segregation. SEPT9 was present not only in the plasma of 69% of CRC patients but also in 14% of controls.

Subsequent work extended analysis of the performance of SEPT9 in plasma, looking at 379 CRC patients and 179 controls split over several test and validation groups, with comparable performance to the initial report.[242] This study included ALX4, identified earlier as a potential biomarker. ALX4, also known as Aristaless 4, is a homeobox gene with a known target sequence. ALX4 was also flagged by Zou et al in a survey of primary tumors.[243] He et al described a triplex Methylight assay for TMEFF2, SEPT9, and ALX4 in analysis of peripheral blood.[244] The combined assay showed 84% sensitivity and 87% specificity for primary tumors and 81% sensitivity and 90% specificity for cell-free methylated DNA in peripheral blood.

Zou et al identified methylation of several genes in addition to ALX4 in a high proportion of primary tumors: BMP3, EYA2, and vimentin, each methylated in approximately 70% of cases.[243] Detection in adenomas ranged from 48% for EYA2 and 72% for vimentin to 89% for ALX4. Frequency in normal epithelial ranged from 5% to 10%. This group also extensively studied fecal DNA markers and shown that methylation of the first (untranslated) exon of vimentin is an analytically robust marker. A methyl-binding protein domain immobilized on a column was used to enhance detection in the discovery phase. Without the enrichment column methylated vimentin sequence was only detected in a single cancer

Li et al used MethylBEAMING in an elegant high tech study of methylated vimentin promoter sequence in circulating DNA and in fecal DNA from patients with colorectal cancer: plasmas from 110 normal controls and from 81 patients with colorectal cancer, evenly distributed over all four Duke's stages.[168,169] Methylation of the vimentin promoter had been already demonstrated in primary tumors and fecal samples in colorectal cancer.[245,246] Fecal samples were from 38 normal controls, 20 patients with adenomas, and 22 from patents with colorectal cancer (against multiple stages). In 2 ml of peripheral blood from normal subjects a mean of 3,170 DNA vimentin exon 1 fragments were detected. Of these, on average, 0.6 vimentin exon 1 fragments were methylated per sample (only eight samples had >1 methylated molecule). The CRC patients showed a mean of 8,240 total vimentin exon 1 fragments and a mean of 335 methylated fragments. In fecal DNA, normal subjects showed a mean of 47,3000 total vimentin exon 1 fragments with 1% methylated, subjects with an adenoma showed 69,600 total fragments with 3.8% methylated, and cancer patients (all stages) showed 236,000 fragments with 7.3% methylated. Viewed differently the authors give the sensitivity for plasma as 59% overall and specificity as 93%. For Dukes A and B, curable stages, sensitivity was 52%. For the fecal study, using the optimal cut-off, 45% of patients with adenomas and 41% of patients with colorectal cancer were positive, only 5% of healthy subjects were positive.

The sensitivity and specificity of several of the tests for circulating methylated sequences might not seem high enough to be clinically useful for screening populations but they appear significantly better than the statistics for fecal occult blood testing.

Colon Cancer and Circulating miRNA

There are numerous miRNA profiles of primary colon cancer tumors, but none of CTC or plasma.

Conclusion

The role of circulating tumor cells (CTC) in the cancer ecosystem (the primary, the stroma, the metastases, the disseminated tumor cells, and immune cells) could remain elusive even as a clinical role is defined. It is tempting to dismiss circulating tumor nucleic acids as debris; however, uptake of tumor miRNA has been shown for normal cells and free DNA can activate elements of the immune system. Several findings merit emphasis:

- Circulating tumor cells can number in the millions per day but very few become disseminated tumor cells, even fewer give rise to metastases.
- Circulating tumor cells can originate in the primary or in a metastasis but can also persist after complete resection even in the absence of known metastases.
- Determinants of the level of circulating tumor cells and/or nucleic acids are unknown.
- The half-life of CTC, based on limited data, is on the order of several hours. Many are apoptotic.
- Direct detection of CTC is complicated by epithelial–mesenchymal transformation as well as the lack of uniform marker expression even in the absence of EMT.
- Limited data shows genomic heterogeneity among CTC and with respect to the primary.

With respect to future clinical applications one can, with some confidence or foolhardiness, commit to print a few predictions:

Expanded Applications for Monitoring the Effectiveness of Therapy

This is the one area for which the FDA has already approved a test. Because of genomic heterogeneity it is not clear that genomic analysis of CTC/CNA alone will dictate therapy.

Monitoring Minimal Residual Disease

As whole genome/transcriptome analysis of primary tumors becomes commonplace, data on translocations and deletions will enable sensitive detection of persisting or recurrent disease by analysis of CTC or CNA. Because of genomic heterogeneity and continued "evolution" more than one abnormality will have to be assayed. Development and validation of such personalized diagnostics will be costly at first. Proving clinical benefit will require long-term clinical studies.

Screening Populations at Risk

Analysis of CTC and CNA, especially microRNA and methylated DNA, will each be evaluated as screening tests for populations at risk. None of these tests will be perfect biomarkers but should offer significant improvements on current biomarkers like fecal occult blood (or "none" in the case of esophageal cancer).

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