The Future of Precision Medicine in Oncology

Lori M. Millner, PhD, NRCC[a,b], Lindsay N. Strotman, PhD[a,c,*]

KEYWORDS

• Liquid biopsy • Targeted therapy • Circulating tumor cell (CTC)
• Cell-free nucleic acid (cfNA) • Exosomes • Extracellular vesicle (EV)

KEY POINTS

- Precision medicine in oncology is focused on identifying therapies that are based on genetic characterization of a patient's tumor.
- One of the newest additions to the precision medicine arsenal is the liquid biopsy, which is based on circulating tumor cells (CTCs), cell-free nucleic acid (cfNA), and exosomes.
- Liquid biopsies allow longitudinal information regarding a patient's tumoral genotype to be obtained through peripheral blood draws and are less invasive than traditional tissue biopsies.

NEED FOR PRECISION MEDICINE IN ONCOLOGY

Precision medicine in oncology is focused on identifying which therapies will be most effective for each patient based on genetic characterization of their cancer. Traditional chemotherapy is cytotoxic and destroys all cells that are rapidly dividing. The foundation of precision medicine is targeted therapies, first developed in the late 1990s. Targeted therapies inhibit specific molecules involved in tumor growth and dissemination of cancer cells. Studies have also been performed to discover targets that predict effectiveness in radiation therapy. The proportion of clinical trials requiring a genetic alteration for enrollment has increased dramatically over the past several years, and many studies have demonstrated benefit of targeted therapies over cytotoxic therapies in both progression-free survival (PFS) and overall survival (OS).[1,2]

Precision medicine requires an understanding of the vast heterogeneity and complexity of tumors as well as the processes that drive heterogeneity. Mechanisms of tumoral heterogeneity acquisition include somatic mutations, C > T transitions at CpG sites, activity of apolipoprotein B mRNA editing enzyme, therapy, chromothripsis,

Disclosure Statement: The authors have nothing to disclose.
[a] PGXL Technologies, 201 East Jefferson Street, Suite 306, Louisville, KY 40202, USA; [b] Department of Pathology and Laboratory Medicine, University of Louisville, Louisville, KY, USA; [c] Department of Engineering, University of Louisville, Louisville, Kentucky, USA
* Corresponding author. PGXL Technologies, 201 East Jefferson Street, Suite 306, Louisville, KY 40202.
E-mail address: lindsay.strotman@pgxlab.com

Clin Lab Med 36 (2016) 557–573
http://dx.doi.org/10.1016/j.cll.2016.05.003 **labmed.theclinics.com**
0272-2712/16/$ – see front matter

and whole-genome doublings.[3] The result of these processes is a diverse tumor that can harbor many different subclonal populations. Currently, targeted therapies are often effective against only a subclonal population of cells; therefore, other methods that can better assess heterogeneity are needed. This review discusses roles that biomarkers serve, including predictive, prognostic, pharmacodynamic, and diagnostic purposes. The discussion includes targeted therapies already in use for many cancers as well as biomarkers in development. Special attention is paid to the rise of the use of liquid biopsies in the field of precision medicine in oncology, including CTCs, circulating cfNA, and extracellular vesicles (EVs).

TYPES OF BIOMARKERS: DIAGNOSTIC, PREDICTIVE, PROGNOSTIC, AND PHARMACODYNAMIC

"A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathologic processes, or biological responses to a therapeutic intervention is the definition given by the Biomarkers Working Group."[4] This biomarker should serve as a sign of a normal or abnormal process or of a condition or disease.[5] The Food and Drug Administration (FDA) defines biomarkers into 4 general categories, mainly related to uses in therapy development. Several biomarkers can fall within multiple categories, because the surrogate endpoint can vary (**Fig. 1**).

1. Diagnostic biomarker: categorizes patients by presence or absence of a specific physiologic or pathophysiological state or disease
2. Prognostic biomarker: indicates degree of risk for disease occurrence or progression, distinguishing "good" and "poor" outcomes
3. Predictive biomarker: categorizes patients by the likelihood of response to a particular beneficial or harmful therapy

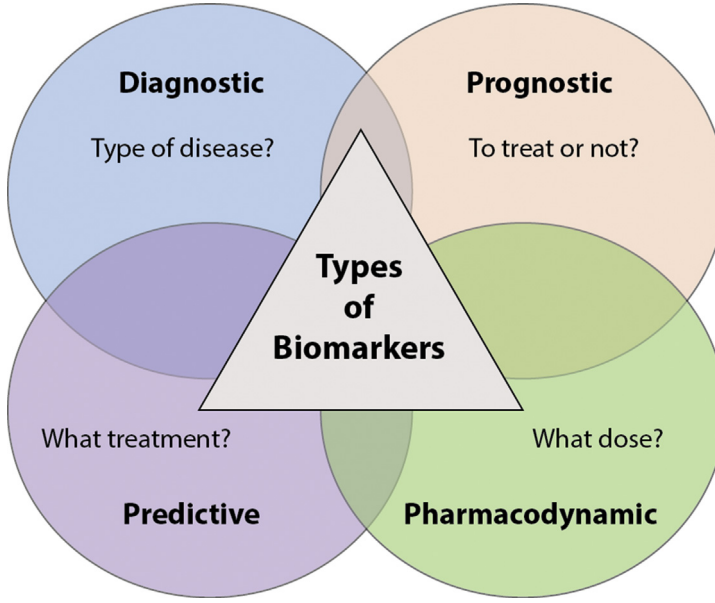

Fig. 1. Types of biomarkers.

4. Pharmacodynamic biomarker: shows a biological response has occurred to therapy prior to or after treatment; can be considered treatment-specific or informative of disease response

Due to the substantial risk of adversely affecting public health if a biomarker is falsely accepted as a surrogate endpoint, robust scientific evidence is needed to justify qualification of a biomarker before validation can even begin. Therefore, FDA-cleared assays using a biomarker in cancer have been limited.

FOOD AND DRUG ADMINISTRATION–CLEARED TESTS VERSUS LABORATORY DEVELOPED TESTS

In this discussion, tests that have been cleared by the FDA (FDA) and laboratory developed tests (LDTs) are included; however, there are distinctions between them. An LDT is a type of in vitro diagnostic test that is designed, manufactured, and used within a single laboratory. LDTs currently are not required to go through premarket review and other stringent requirements of FDA-cleared tests because they were considered simple tests.[6] Due to an increase of LDT use and complexity, on September 30, 2014, the FDA presented a new draft guidance to congress indicating increased FDA oversight of LDTs. The landscape is currently changing and laboratories must stay up to date regarding these changes and how they may affect the approval process of tests developed by laboratories.

The major types of cancer and how precision medicine is having an impact both on biomarker identification and treatment are examined. This discussion is not meant to be an exhaustive list of all cancer subtypes but, rather, examples of how precision medicine is influencing each discipline.

PRECISION MEDICINE IN BREAST CANCER

Breast cancer (BCa) is the most commonly diagnosed cancer for women both in the United States and globally. BCa is the second ranking cancer responsible for female mortality in the United States, causing an estimated 40,730 deaths in 2015.[7,8] The major types of targeted therapy in BCa include endocrine therapy, anti-HER2 therapy, PI3K, BRCA1/2, and CK4/6 inhibitors (**Table 1**). Some of the first targeted agents in oncology were developed against hormone receptor (estrogen receptor and

Table 1
Targeted therapies in breast cancer

Therapy	Drug	Type
Endocrine therapy	Tamoxifen	Selective estrogen receptor modulator
	Anastrozole	Aromatase inhibitor
	Exemestane	Aromatase inhibitor
	Letrozole	Aromatase inhibitor
	Fulvestrant	Estrogen receptor down-regulator
HER2 therapy	Trastuzumab	Monoclonal antibody
	Ado-trastuzumab emtansine	Antibody conjugated to cytotoxic agent (T-DM1)
	Lapatinib	Small molecule inhibitor
	Pertuzumab	Monoclonal antibody
CDK4/6 inhibitors	Palbociclib	Small molecule inhibitor
PI3K-mTOR inhibitors	Everolimus	Small molecule inhibitor

progesterone receptor)–positive BCa. Estrogen-focused therapies have remained part of the standard of care for more than 30 years. Tamoxifen modulates the estrogen receptor[9] and aromatase inhibitors decrease amount of estrogen produced,[10] whereas other agents, like fulvestrant, degrade the estrogen receptor.[11]

DIAGNOSTIC, PROGNOSTIC, OR PREDICTIVE TESTS

There are several prognostic and predictive assays using breast tumor tissue. Some of the most well-known predictive assays are Oncotype DX (Genomic Health, Redwood City, CA), MammaPrint (Agendia, Uíge, Angola), and Prosigna PAM50 (NanoString Technologies, Seattle, WA) and have been recently reviewed.[12] Likely the most well-known prognostic assay is the Oncotype DX, which uses reverse transcriptase (RT)–polymerase chain reaction (RT-PCR) to analyze expression of 16 cancer-related genes and 5 housekeeping genes. An algorithm analyzing expression of these genes generates a recurrence score that indicates which patients will benefit most from chemotherapy in addition to hormone therapy.[13] Both the American Society of Clinical Oncology (ASCO) and the National Comprehensive Cancer Network (NCCN) guidelines include Oncotype DX testing in their recommendations for estrogen receptor–positive, HER2-negative, and node-negative patients.[14] An FDA-cleared prognostic assay, MammaPrint, is included in some international recommendations.[15] This assay is based on microarray analysis of a 70-gene signature used to accurately select early-stage BCa patients who are highly likely to develop distant metastases and would benefit the most from adjuvant chemotherapy.[16] The Prosigna PAM50 is another FDA-cleared prognostic assay that uses RT-PCR to examine 50 genes identifying BCa subtypes. The PAM50 also determines a risk of recurrence score to determine prognosis and add significant information to commonly available immunohistochemistry markers.[17]

FDA-cleared tests

1. MammaPrint
2. Prosigna PAM50
3. GeneSearch BLN Test Kit (Janssen Diagnostics, Raritan, NJ): detects spread of BCa to lymph nodes by measuring 2 gene targets abundant in BCa tissue and scarce in lymph nodes
4. HER2 fluorescence in situ hybridization (FISH) assays
   a. INFORM HER2 Dual ISH DNA Probe Cocktail (Ventana, Oro Valley, AZ)
   b. *HER2* CISH pharmDxTM Kit (DAKO, Carpinteria, CA)
   c. Dako TOP2A FISH pharmDx Kit (DAKO, Carpinteria, CA)
   d. DakoCytomation Her2 FISH pharmDx Kit (DAKO, Carpinteria, CA)

LDTs

1. Oncotype DX
2. Genomic Grade Index (MapQuant Dx, Ipsogen, Marseille, France)
3. Breast Cancer Index (BioTheranostics, San Diego, CA)
4. EndoPredict (Sividon Diagnostics, Köln, Germany)

PRECISION MEDICINE IN NON–SMALL CELL LUNG CANCER

Lung cancer is the leading cause of cancer mortality. Lung cancer can be divided into small cell lung cancer (10%–15%) and non–small cell lung cancer (NSCLC) (85%).[8] The main NSCLC predictive biomarkers found in NSCLC include ALK fusion oncogene (anaplastic lymphoma kinase) and sensitizing epidermal growth factor receptor (EGFR) mutation. Currently, there are 3 FDA-cleared companion biomarker assays

used to detect these biomarkers that are associated with different targeted therapies (Table 2). Other targeted therapies that have arisen include angiogenesis inhibitors and immune checkpoint inhibitors (see Table 2); however, there are currently no associated biomarkers.

DIAGNOSTIC, PROGNOSTIC, OR PREDICTIVE TESTS

Other emerging biomarkers in NSCLC as listed in the 2016 NCCN guidelines include KRAS, HER2, and BRAF V600E mutations; ROS1 and RET gene arrangements; MET amplification; and MET exon skipping mutation. For example, KRAS mutations can serve as a prognostic biomarker indicative of patient survival independent of treatment received[18] and as a predictive biomarker showing lack of therapeutic efficacy to EGFR targeted therapies.[19] Although FDA-cleared KRAS mutational detection assays have been approved for use in colorectal cancer (ie, cobas KRAS Mutation Test [Roche, Basel, Switzerland] and *therascreen* KRAS RGQ PCR Kit [Qiagen, Hilden, Germany]), they have not yet been approved for use in NSCLC. Additionally, the multiple biomarkers discovered in NSCLC have led to the development of mutational screening assays designed to detect multiple biomarkers simultaneously. Although a majority of these assays are usually performed using multiplex PCR, next-generation sequencing (NGS) panels have rapidly begun to appear, because both mutations and gene rearrangements can be detected simultaneously. No matter the detection scheme, the 2016 NSCLC NCCN guidelines are strongly endorsing use of these multiplexed assays.

FDA cleared

1. *therascreen* EGFR RGQ PCR Kit: a real-time PCR test for the qualitative detection of exon 19 deletions and exon 21 substitutions mutations in EFGR using DNA derived from formalin-fixed paraffin-embedded tumor tissue; received approval with gefitinib
2. cobas EGFR Mutation Test: a companion diagnostic for mutations in EGFR using DNA derived from formalin-fixed paraffin-embedded tumor tissue; received approval with osimertinib
3. Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular, Santa Clara, California)

Table 2
Targeted therapies in non–small cell lung cancer

Therapy	Drug	Type
Angiogenesis inhibitors	Bevacizumab	Monoclonal antibody targets VEGF-A
	Ramucirumab	Recombinant humanized monoclonal antibody targets VEGFR2
Tyrosine kinase inhibitors	Gefitinib	Small molecule EGFR inhibitor
	Erlotinib	Small molecule EGFR inhibitor
	Afatinib	Small molecule irreversible covalent EGFR and HER2 inhibitor
	Osimertinib	EGFR inhibitor
	Cetuximab	EGFR inhibitor
	Crizotinib	Small molecule ALK, ROS1, and MET inhibitor
	Ceritinib	Small molecule ALK and IGF-1 inhibitor
	Alectinib	Small molecule ALK and RET inhibitor
Immune checkpoint inhibitor	Nivolumab	Monoclonal antibody acting as immunomodulatory blocking PD-1 ligand activation on activated T cells
	Pembrolizumab	Humanized antibody to target PD-1

LDTs

1. Sequenom MassARRAY System (Agena Biosciences, San Diego, CA): a nonfluorescent detection platform using mass spectrometry to accurately measure PCR-derived amplicons. They have also developed the LungCarta® and LungFusion™ panel.
2. SnaPshot Multiplex System (Thermo Fisher Scientific, Waltham, MA): uses a primer extension-based method to multiplex up to 10 SNPS and detects using capillary electrophoresis
3. NGS or massively parallel sequencing
   a. Sentosa SQ NSCLC panel (VELA Diagnostics, Kendall, Singapore)
   b. Pervenio Lung NGS (Thermo Fisher Scientific): uses Ion AmpliSeq Lung Cancer Research Panel. Thermo Fisher also offers RNA Fusion Lung Cancer Research Panel.
   c. Lung Cancer Comprehensive Mutation and Translocation Panel (ARUP Laboratories, Salt Lake City, UT)
   d. Lung Cancer Mutation Panel (Quest Diagnostics, Madison, NJ)

PRECISION MEDICINE IN PROSTATE CANCER

Prostate cancer (PCa) is the most common solid tumor in men in the United States, ranking 5th in mortality and more than 230,00 cases diagnosed each year.[20] The main historical PCa biomarker is prostate-specific antigen (PSA). PSA screening has, however, garnered criticism over the years due to the possibility of overdetection, which has led to efforts to find other improved biomarkers.[7,12,21] The majority of effort in PCa biomarker development is to distinguish PCa from benign prostatic conditions with better sensitivity and specificity than PSA and to differentiate aggressive from nonaggressive PCa. Although there are currently 3 FDA-cleared tests and several other LDTs, new predictive and pharmacodynamic biomarkers are being pursued due to the development of targeted PCa therapies (**Table 3**). The main target of these therapies is the androgen receptor (AR), which is a main driver of PCa.[22]

DIAGNOSTIC, PROGNOSTIC, OR PREDICTIVE TESTS

FDA-cleared tests

1. Prostate Health Index (Beckman Coulter, Brea, CA; in partnership with the National Cancer Institute Early Detection Research Network): measures 3 forms of PSA to better distinguish PCa from benign prostatic conditions

Table 3
Targeted therapies in prostate cancer

Therapy	Drug	Type
Androgen synthesis inhibitors	Abiraterone acetate	Small molecule CYP17 inhibitor
	Ketoconazole	Small molecule CYP450 inhibitor
Antiandrogen	Enzalutamide	Small molecule AR inhibitor
	Flutamide	Small molecule AR inhibitor
	Bicalutamide	Small molecule AR inhibitor
	Nilutamide	Small molecule AR inhibitor
Immunotherapeutic	Sipuleucel-T	Autologous Cancer Vaccine

2. Prosensa (Gen-Probe, San Diego, CA): nucleic acid amplification test measuring PCa antigen 3 expression in urine to better distinguish PCa from benign prostatic conditions
3. CELLSEARCH Circulating Tumor Cell Kit (Janssen Diagnostics)

LDTs

1. Oncotype DX: measures expression of 12 cancer-related genes from small formalin-fixed paraffin-embedded tissue samples to discriminate PCa patients into very low, low, or intermediate risk groups that should be under active surveillance
2. Prolaris Score (Myriad Genetics, Salt Lake City, UT): measures tumor cells growth characteristics and expression of 46 different genes from prostate biopsy core specimens to stratify disease risk of progression
3. Prostarix (performed at Metabolon and offered by Bostwick Laboratories, Glen Allen, VA): measures signature of 4 metabolites from urine samples post–digital rectal examination using liquid chromatography–mass spectrophotometry
4. Mi-Prostate Score (University of Michigan Health System, Ann Arbor, MI): combines urine test for PCa antigen 3 from Progensa, TMPRSS2:ERG fusion, and serum PSA levels to produce risk assessment of PCa and aggressiveness
5. ConfirMDx (MDxHealth, Irvine, CA): detects an epigenetic specific profile (ie, DNA methylation) in prostate biopsy core specimens
6. Prostate Core Mitomic Test (MDNA Life Sciences, West Palm Beach, FL): detects mitochondrial DNA deletions in prostate biopsy core specimens
7. 4Kscore Test (Opko Lab, Nashville, TN): combines free PSA and total PSA, human kallikrein 2, and intact PSA measurements and considers age, digital rectal examination results, and prior biopsy status to determine the percent likelihood of finding high-grade cancer on biopsy

PRECISION MEDICINE IN MELANOMA

Rates of melanoma have risen faster than any other cancer over the past 2 decades with 75,000 new cases diagnosed each year.[23] These figures are thought to be underestimated, because many superficial and in situ melanomas treated in outpatient setting are not reported.[24]

Unlike other cancers, melanoma does not respond to chemotherapy or radiation, which has led to the development of immune and targeted therapies (**Table 4**). Approximately 50% to 60% of patients with metastatic melanoma have an activating mutation in V600 of BRAF (serine/threonine-protein kinase B-raf), an intracellular signaling kinase.[25] Although patients initially respond to BRAF inhibitors with increased PFS and OS, resistance usually arises due to alternative activation of other downstream targets in the same signaling kinase pathway. Therefore, other inhibitors

Table 4
Targeted therapies in melanoma

Therapy	Drug	Type
BRAF V600E inhibitors	Dabrafenib	Small molecule inhibitor
	Vemurafenib	Small molecule inhibitor
Immune checkpoint inhibitor	Ipilimumab	Monoclonal antibody
	Trametinib	Small molecule inhibitor
Tyrosine kinase inhibitor	Imatinib	Monoclonal antibody

(ie, MEK1 and MEK2) have been developed for downstream MAPK signal transduction pathways targets. Finally, immunotherapeutic agents have been developed that target immune checkpoint inhibitors, which harness the individual's own immune system to target and destroy cancerous cells.[26,27] These immune and targeted therapies are also summarized in **Table 4**.

DIAGNOSTIC, PROGNOSTIC, OR PREDICTIVE TESTS

Other kinase inhibitors are also being developed against additional mutations found in Melanoma as listed in the 2016 NCCN guidelines, including BRAF, NRAS, c-kit, GNA11 and GNAQ. KIT is the most notable example because there is already an FDA-cleared tyrosine kinase inhibitor used against Bcr-Abl in chronic myelogenous leukemia and gastrointestinal stromal tumors harboring a KIT mutation. A phase II study of 43 patients with KIT-mutated metastatic melanomas demonstrated a 23% overall response rate with this therapy, although response was of limited duration.[28] Finally, 10% of melanomas are attributed to hereditary predisposition resulting in the development of LDTs to identify individuals at risk, which is important for implementing strategies to reduce the burden of early disease.

FDA cleared

1. cobas® 4800 BRAF V600 (Roche): a companion diagnostic for V600 that received approval with BRAF inhibitor vemurafenib
2. THxID-BRAF (bioMėrieux, Marcy-l'Étoile, France): a companion diagnostic for V600E or V600K that received approval with BRAF inhibitors dabrafenib and trametinib

LDTs

1. myPATH (Myriad): measures expression of 23 different genes from melanoma lesions
2. NGS (includes only melanoma targeted panels performed in clinical laboratories and is only a brief, not comprehensive view of all the current panels available)
   a. IVD Sentosa SQ Melanoma Panel (Vela Diagnostics)
   b. Melanoma NGS Panel (Fulgent Diagnostics, Temple City, CA)
   c. Melanoma Targeted Gene Panel by NGS (Mayo Medical Laboratories, Rochester, MN)
   d. Hereditary Melanoma Panel (Baylor Miraca Genetics Laboratories, Houston, TX)
   e. Melanoma Hereditary Cancer Panel (ARUP Laboratories)

PRECISION MEDICINE IN COLORECTAL CANCERS

Colorectal cancer is the third most common cancer in both men and women, with an estimated 49,190 expected US deaths in 2016.[29] Much effort has been put into targeted therapies and diagnostic tests for this disease. Three main classes of targeted therapies have been approved to treat metastatic colorectal cancer, including multikinase inhibitors, angiogenic inhibitors, and anti-EGFR antibodies. These therapies are summarized in **Table 5**. Multikinase inhibitors include regorafenib, dabrafenib, and vemurafenib. Targets of regorafenib include angiogenic (VEGF1–3 and TIE2), stromal (PDGFR-β and FGF1), and oncogenic (KIT, RET, and B-RAF) protein kinases.[1,30] Clinical trials have demonstrated a modest increase in OS for patients on regorafenib. Dabrafenib and vemurafenib both inhibit the V600E mutation in BRAF, which is mutated in up to 15% of colorectal cancer cases and signifies poor prognosis.[31] Angiogenic inhibitors include bevacizumab and aflibercept. Bevacizumab inhibits vascular endothelial growth factor (VEGF), which plays a central role in the tumor

Table 5
Targeted therapies in colorectal cancer

Therapy	Drug	Type
Angiogenesis inhibitors	Bevacizumab	Monoclonal antibody – targets VEGF
	Aflibercept	Fusion protein – targets VEGF
Anti-EGFR therapy	Panitumumab	Monoclonal antibody
	Cetuximab	Monoclonal antibody
Multikinase inhibitors	Regorafenib	Small molecule inhibitor – angiogenic and oncogenic protein kinases
	Dabrafenib	Small molecule BRAF V600E inhibitor
	Vemurafenib	Small molecule BRAF V600E inhibitor

angiogenic pathway. When used in combination with chemotherapy, bevacizumab was shown to increase PFS and OS.[32] Aflibercept also targets VEGF and has demonstrated increased OS and PFS when used in combination with FOLFIRI.[29] EGFR signaling results in cell proliferation and migration. Anti-EGFR monoclonal antibodies include cetuximab and panitumumab. Studies have demonstrated that patients with nonmutated KRAS have increased PFS and OS when treated with either cetuximab or panitumumab in combination with FOLFIRI or FOLFOX compared with chemotherapy alone.[5,7,8]

DIAGNOSTIC, PROGNOSTIC, OR PREDICTIVE TESTS

In April of 2015, the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society for Clinical Oncology released a new set of recommendations and consensus opinions in anticipation of new guidelines for the Evaluation of Molecular Markers for Colorectal Cancer. The consensus guidelines strongly recommend RAS mutational testing for patients who are being considered for anti-EGFR therapy, KRAS, and NRAS (codons 12, 13 of exon 2; 59, 61 of exon 3; and 117 and 146 of exon 4). They also recommend BRAF V600 and deficient mismatch repair/microsatellite instability testing.

FDA-cleared tests

1. *therascreen* KRAS RGQ PCR Kit (Qiagen): a qualitative real-time PCR assay for the detection of specific mutations in the KRAS oncogene
2. cobas KRAS Mutation Test (Roche): PCR-based diagnostic test intended for the detection of mutations in codons 12 and 13 of the KRAS gene to select appropriate patients for treatment with cetuximab or panitumumab
3. Cologuard (Exact Sciences, Marlborough, MA): collects stool and examines elevated levels of altered DNA and/or hemoglobin associated with cancer/precancerous lesions

LDTs

1. KRAS Mutation Detection Kit (Trimgen, Sparks Glencoe, MD): based on a shifted termination assay andused to help select patients for panitumumab and cetuximab
2. ColoVantage (Quest Diagnostics): a blood test that detects methylated Septin9 DNA, which is a proved marker for colorectal cancer. This test is associated with an overall 70% sensitivity and 89% specificity.
3. Colox (Novogenix, Los Angeles, CA): measures the gene expression profile of 29 biomarkers by real-time PCR in peripheral blood mononuclear cells and uses an algorithm to diagnose colorectal cancer in a noninvasive manner

INTRODUCTION TO LIQUID BIOPSIES

A new precision medicine strategy in the field of oncology is the liquid biopsy. The use of liquid biopsies allows physicians increased longitudinal access to genetic material, providing greater opportunity for biomarker detection. Traditionally, tissue from a tumor is evaluated for malignancy using dyes, microscopes, and highly trained pathologists. Some tissue micrographs yield equivocal results due to poor staining or poor sample selection. In most cases, surgeons have only a single opportunity to obtain tissue from a patient. Liquid biopsy materials include CTCs, cfNA or EVs that are derived from primary and metastatic tumors and allow physicians access to longitudinal time points of a patient's disease (Fig. 2). This is accomplished because liquid biopsies are obtained from a peripheral blood draw that is much less invasive to the patient than traditional tumor tissue biopsies. The first commercial liquid biopsy test was offered in 2000, the CELLSEARCH. The CELLSEARCH assay enumerates CTCs that are known to be predictive in several epithelial cancer types. In recent years, the liquid biopsy field has matured beyond CTCs to include cfNA and EVs. cfNA typically constitutes a very small portion of a person's total circulating nucleic acids but can be useful for monitoring tumoral genetic variations. EVs, on the other hand, encapsulate nucleic acids, providing a protected environment, thus providing better stability specifically of RNA. This discussion provides information on CTCs, cfNA, and EVs for use as liquid biopsies.

CIRCULATING TUMOR CELLS

CTCs are cells that have been shed from a tumor and may be capable of forming metastases. Metastasis is the most dangerous and deadly facet of cancer and is responsible for 90% of cancer related deaths.[33] The metastatic process is accomplished by a CTC successfully carrying out a series of processes. First, the CTC must detach from the primary tumor and intravasate into the blood stream (see Fig. 2). Once in circulation, the CTC must survive a journey in the blood stream and extravasate into microvessels of a distant tissue. It must then adapt and survive in the microenvironment of the distant tissue. Finally, the CTC must colonize to form a metastatic lesion. The CELLSEARCH system for detection of CTCs is the only FDA-cleared assay for CTCs and is used as a predictor of OS and PFS in metastatic BCa patients. This method is based on detection of the epithelial markers, epithelial cell adhesion marker (EpCAM) and cytokeratins. Using this technology, however, CTCs are not detected in

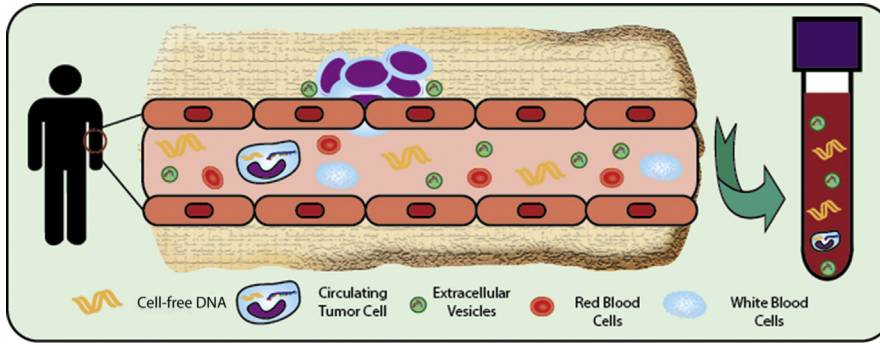

Fig. 2. Sources of material that represent liquid biopsies, including cell-free DNA, CTCs, and EVs.

40% of patients with metastatic BCa.[34–36] There are several other non–FDA-cleared methods for enumerating or isolating CTCs.[37] The methods for enriching or isolating CTCs include size-based isolation, nucleic acid–based detection, magnetic-activated cell separation (MACS), microfluidic-based isolation, and immunomagnetic isolation. These methods and companies pursuing them are listed in **Table 6**.

CELL-FREE NUCLEIC ACIDS

cfNAs circulating in blood were first described by Mandel and Metais in 1948[38]; however, it was not until 1994 when NRAS gene fragments were detected in cancer patients' blood, that the scientific community recognized cfNA's potential importance.[39,40] Since then, cfNA has become an essential tool in the liquid biopsy arsenal, gaining popularity over CTCs because cfNA isolation is deemed easier to obtain. cfNA is hypothesized to be released passively during apoptotic or necrotic events, with macrophages unable to clean-up the cell debris due to overproduction.[41] The average cfNA fragment is 180 to 200 base pairs, the same length that is released during cell death.[42] Secretion by cancer cells is also suggested as a mechanism of cfNA release. cfNA half-life is approximately 2 hours and levels have been reported to decrease dramatically after tumor removal and increase again if metastasis occurs.[43] The sensitivity and specificity of cfNA rely on the detection of common somatic alterations found in cancers. But cfNA's main limitation in cancer is that cfNA may not necessarily reflect physiologic and pathologic processes but can be associated in patients with tissue trauma, inflammatory disease, and so forth.[43] Benefits of cfNA include increased access to tumor heterogeneity compared to traditional tissue biopsy that often results in sample bias due to

Table 6
Methods for circulating tumor cell isolation

Method	Principle for Detection	Vendor
AdnaTest	RT-PCR of tumor-related transcripts	AdnaGen
ApoStream	Microfluidic-dielectric and morphology-based	ApoCell
Ariol	Immunodetection of cytokeratins 8, 18, and 19	Leica Microsystems
Biocept blood test	Microfluidic-biotinylated antibody capture	Biocept (San Diego, California)
BioFluidica CTC system	Microfluidic-antibody or aptamer capture	BioFluidica
CELLSEARCH	Immunodetection of cytokeratins 8, 18, and 19 with flow cytometry	Janssen Diagnostics
CTC-iChip	Microfluidic immunocapture	Janssen Diagnostics/Massachusetts General Hospital
CytoTrack CT11	Automated imaging	CytoTrack
DEPArray	Dielectrophoresis	Silicon Biosystems
Ikoniscope imaging system	Magnetic antigen expression	Ikonisys
ISET	Immunodetection of EpCAM	Rarecells
Parsortix	Microfluidic-size and deformability selection	Angle

spatial constraints. Lastly, liquid biopsies may be able to detect a tumor before it can be seen by imaging.

One challenge with cfNA compared with other types of liquid biopsy, is a background noise–to-signal problem requiring sensitive technologies. To this end, several investigators have pursued digital PCR technologies, which have a sensitivity of approximately 0.01% (ie, 1 mutated allele in presence of 10,000 wild-type alleles) or less.[44] Digital PCR differs from conventional in that the sample is divided into single molecules before amplification and detection, enabling more accurate quantification. The most notable companies are Raindance Technologies and Bio-Rad, which use droplet-based digital PCR by performing the PCR reactions in individual oil-water emulsion droplets. Life Technologies foregoes droplets, instead relying on thousands of nanofluidic wells to separate individual biomolecules. Targeted NGS represents the other sensitive technology being pursued; however, it is still a burgeoning technology because workflows are laborious and expensive. Additionally, bioinformatics teams are developing new algorithms to help with the signal-to-noise problem and ensure the signal is real (**Table 7**).

Table 7
Companies using cell-free nucleic acid to perform biomarker interrogation

Method	Principle for Detection	Vendor
Multiplex PCR, MassARRAY detection	Capture mutant amplicons on magnetic beads and detect by mass spec	Agena Biosciences (San Diego, CA)
Repetitive transient hybridization in alternating current field	Can detect 100 mutations down to 0.01% allelic frequency	Boreal Genomics (Vancouver, BC Canada)
Digital PCR	—	Chronix Biomedical (San Jose, CA)
Various PCR, NGS	Oncotype Seq Liquid Select	Genomic Health (Redwood City, CA)
NGS	Sequencing of ctDNA	Grail (Redwood City, CA)
Digital sequencing (PCR, barcoding, NGS)	LDTs cover 54 relevant oncogenes. Former Illumina research and development managers	Guardant Health (Redwood City, CA)
Tagged amplicon sequencing (Tam-Seq)	Identification of rare cancer mutations using NGS	Inivata (Research Triangle Park, NC)
Various PCR methods and NGS	Offer a wide variety of liquid biopsy services	MolecularMD (Cambridge, MA)
Multiplex PCR and NGS	Noninvasive prenatal test	Natera (San Carlos, CA)
PCR and NGS	Recently introduced METDetect-R plasma test	Personal Genome Diagnostics (Baltimore, MD)
Beads, emulsion, amplification, and magnetics (BEAMing) and NGS	Circulating tumor DNA allelic frequency 0.02%	Sysmex Inostics (Baltimore, MD)
Digital droplet PCR and NGS	Determine mutations in KRAS and BRAF from circulating tumor DNA; developing EGFR, NRAS, and PIK3CA	Trovagene (San Diego, CA)

EXTRACELLULAR VESICLES

EVs are the newest and least developed of liquid biopsy sources. EVs are membranous structures released by cells to the external environment. Their main function is intercellular communication, serving as vehicles to transfer cell of origin specific proteins, lipids, and nucleic acids between cells.[45] EVs can be distinguished into 4 categories mainly based on their size, cell origin, and secretion mechanisms. This nomenclature is still hotly debated (see Strotman LN, Linder M: Extracellular Vesicles Move Towards Use in Clinical Laboratories, in this issue) and in flux because tools to isolate the specific EV populations have not been developed.[46]

The traditional EV isolation method is ultracentrifugation, but it is laborious and time consuming and requires expensive instrumentation that is not amenable to most clinical laboratories. Therefore, new EV isolation methods are being developed. Although still in their infancy, these methods are being marketed as commercial kits for research and development. These kits are based on EVs' physical properties and include affinity chromatography, size exclusion chromatography, and precipitation.[47] Characterization of isolated EV is currently performed using biochemical analysis (ie, ELISA or Western blots), mass spectrophotometry, and various imaging techniques.[48] The main markers used in biochemical analysis include a 4 transmembrane domain family, tetraspanins (CD63, CD81, CD82, CD53, and CD37).[48]

Most assays being developed use mass spectrophotometry as the downstream analysis; however, nucleic acid based assays are rapidly becoming available because their sensitivity improves (ie, digital PCR and NGS). But the main advantage of EVs over other liquid biopsies types is that entrapped biomolecules seem to remain more stable because they are protected from ubiquitous RNases, avoiding issues of shipping and sample handling. Additionally, it has been shown that EVs serve as reservoirs containing enriched material specific to origin cell. Despite limited EV biology knowledge, clinical assays are rapidly being developed around EVs (**Table 8**). Exosome Diagnostics is at the forefront, having established collaboration and distribution agreements with Eli Lilly and Qiagen. Additionally, in 2015 and 2016 they plan to launch 2 LDT tests, for PCa—ExoIntelliScore Prostate—and for NSCLC—ExoDx *Lung*(ALK). They are also in the initial stages of trying to achieve FDA regulatory approval.

FUTURE OF PRECISION MEDICINE IN ONCOLOGY

The idea of using liquid biopsies is appealing due to several limitations of tissue biopsies:

- Biopsies are invasive and not practical for monitoring progression and recurrence overtime.

Table 8
Companies using extracellular vesicles to perform biomarker interrogation

Method	Principle for Detection	Vendor
Exosome extraction, RNA mutational analysis	Exosome extraction, RNA mutational analysis	Exosome Diagnostics (Cambridge, MA)
Enzyme-linked lectin-specific assay (ELLSA)	ELLSA	Exosome Sciences (Monmouth Junction, NJ)
ELISA for capture and assay	ELISA for capture and assay	HansaBioMed OU (Tallinn, Estonia)
Exosome extraction	Exosome extraction	System Biosciences (Palo Alto, CA)

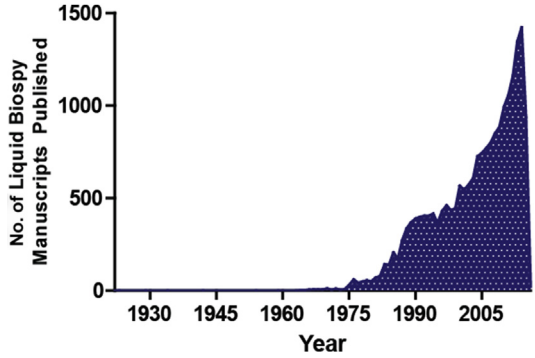

Fig. 3. Number of publications using the term, *liquid biopsies,* as quantified on PubMed.

- Tumor sample size can be limited due to its size and location, limiting the molecular testing that can be done.
- Cancer is a heterogeneous disease with molecular signatures that differ spatially and evolve over time as the disease progresses or due to therapy selective pressures.

These limitations drive the excitement behind liquid biopsies that include CTCs, cfNA, and EVs because they are cheaper, less laborious, and less invasive for the patient. The excitement is evident when examining the number of liquid biopsy publications which is increasing every year (**Fig. 3**).

With liquid biopsies, the stage can be set for diagnostic applications that include

- Early disease detection
- Monitoring treatment response and emerging molecular resistance
- Assessment of molecular heterogeneity and monitoring of tumor dynamics

To date the only FDA-cleared liquid biopsy platform is the CELLSEARCH system for CTC isolation, which serves as a prognostic indicator for breast, prostate, and colorectal cancer. Although CTCs are slightly ahead of the curve in technological development as a liquid biopsy, cfNA and EV are quickly catching up. For further liquid biopsy, however, implementation into clinical laboratories requires hurdles to be overcome including:

- Lack of standardization of sample collection, processing, and analysis
- Lack of large clinical trial validations

Although debate is focused heavily around the clinical utility of the different liquid biopsy samples, it is likely to remain until the field and technologies further mature. There is no reason to say, however, that the different types of liquid biopsy will not be complementary. For example, Exosome Diagnostics is using both cfNA and exosomes to increase the sensitivity of detecting EML4-ALK gene fusion in lung cancer patients. Cynvenio is also examining cfNA and CTCs simultaneously. As liquid biopsy sample preparation and detection become more robust and larger clinical trials are performed to prove clinical utility, it is likely that liquid biopsies will start to transform oncology treatment paradigms.

REFERENCES

1. Tobin NP, Foukakis T, De Petris L, et al. The importance of molecular markers for diagnosis and selection of targeted treatments in patients with cancer. J Intern Med 2015;278(6):545–70.

2. Roper N, Stensland KD, Hendricks R, et al. The landscape of precision cancer medicine clinical trials in the United States. Cancer Treat Rev 2015;41(5):385–90.
3. McGranahan N, Swanton C. Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. Cancer Cell 2015;27(1):15–26.
4. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 2001; 69(3):89–95.
5. Sawyers CL. The cancer biomarker problem. Nature 2008;452(7187):548–52.
6. Administration, U.S.F.a.D. 2015. Available at: fda.gov. Accessed November 17, 2015.
7. Lee YJ, Park JE, Jeon BR, et al. Is prostate-specific antigen effective for population screening of prostate cancer? A systematic review. Ann Lab Med 2013;33(4): 233–41.
8. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65(1):5–29.
9. Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365(9472):1687–717.
10. Gibson L, Lawrence D, Dawson C, et al. Aromatase inhibitors for treatment of advanced breast cancer in postmenopausal women. Cochrane Database Syst Rev 2009;(4):CD003370.
11. Robertson JF, Llombart-Cussac A, Rolski J, et al. Activity of fulvestrant 500 mg versus anastrozole 1 mg as first-line treatment for advanced breast cancer: results from the FIRST study. J Clin Oncol 2009;27(27):4530–5.
12. Wong LM, Neal DE, Johnston RB, et al. International multicentre study examining selection criteria for active surveillance in men undergoing radical prostatectomy. Br J Cancer 2012;107(9):1467–73.
13. Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 2004;351(27): 2817–26.
14. Lang JE, Wecsler JS, Press MF, et al. Molecular markers for breast cancer diagnosis, prognosis and targeted therapy. J Surg Oncol 2015;111(1):81–90.
15. Senkus E, Kyriakides S, Ohno S, et al. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2015; 26(Suppl 5):v8–30.
16. Mook S, Van't Veer LJ, Rutgers EJ, et al. Individualization of therapy using Mammaprint: from development to the MINDACT Trial. Cancer Genomics Proteomics 2007;4(3):147–55.
17. Wallden B, Storhoff J, Nielsen T, et al. Development and verification of the PAM50-based Prosigna breast cancer gene signature assay. BMC Med Genomics 2015; 8:54.
18. Slebos RJ, Kibbelaar RE, Dalesio O, et al. K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. N Engl J Med 1990;323(9):561–5.
19. Eberhard DA, Johnson BE, Amler LC, et al. Mutations in the epidermal growth factor receptor and in KRAS are predictive and prognostic indicators in patients with non-small-cell lung cancer treated with chemotherapy alone and in combination with erlotinib. J Clin Oncol 2005;23(25):5900–9.
20. Sartori DA, Chan DW. Biomarkers in prostate cancer: what's new? Curr Opin Oncol 2014;26(3):259–64.
21. Vickers AJ, Lilja H. We need a better marker for prostate cancer. How about renaming PSA? Urology 2012;79(2):254–5.

22. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev 2004; 25(2):276–308.
23. Karagiannis P, Fittall M, Karagiannis SN. Evaluating biomarkers in melanoma. Front Oncol 2014;4:383.
24. Linos E, Swetter SM, Cockburn MG, et al. Increasing burden of melanoma in the United States. J Invest Dermatol 2009;129(7):1666–74.
25. Long GV, Menzies AM, Nagrial AM, et al. Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. J Clin Oncol 2011;29(10): 1239–46.
26. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363(8):711–23.
27. Robert C, Long GV, Brady B, et al. Nivolumab in previously untreated melanoma without BRAF mutation. N Engl J Med 2015;372(4):320–30.
28. Guo J, Si L, Kong Y, et al. Phase II, open-label, single-arm trial of imatinib mesylate in patients with metastatic melanoma harboring c-Kit mutation or amplification. J Clin Oncol 2011;29(21):2904–9.
29. American Cancer Society. Cancer Facts & Figures 2015. Atlanta: American Cancer Society; 2015. Available at: http://www.oralcancerfoundation.org/facts/pdf/Us_Cancer_Facts.pdf.
30. Wilhelm SM, Dumas J, Adnane L, et al. Regorafenib (BAY 73-4506): a new oral multikinase inhibitor of angiogenic, stromal and oncogenic receptor tyrosine kinases with potent preclinical antitumor activity. Int J Cancer 2011;129(1):245–55.
31. Safaee Ardekani G, Jafarnejad SM, Tan L, et al. The prognostic value of BRAF mutation in colorectal cancer and melanoma: a systematic review and meta-analysis. PLoS One 2012;7(10):e47054.
32. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004; 350(23):2335–42.
33. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nature reviews. Cancer 2003;3(6):453–8.
34. Cristofanilli M, Hayes DF, Budd GT, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol 2005; 23(7):1420–30.
35. Witzig TE, Bossy B, Kimlinger T, et al. Detection of circulating cytokeratin-positive cells in the blood of breast cancer patients using immunomagnetic enrichment and digital microscopy. Clin Cancer Res 2002;8(5):1085–91.
36. Hayes DF, Cristofanilli M, Budd GT, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 2006;12(14 Pt 1):4218–24.
37. Millner LM, Linder MW, Valdes R Jr. Circulating tumor cells: a review of present methods and the need to identify heterogeneous phenotypes. Ann Clin Lab Sci 2013;43(3):295–304.
38. Mandel P, Metais P. Les acides nucléiques du plasma sanguin chez l'homme. C R Seances Soc Biol Fil 1948;142(3–4):241–3 [Article in Undetermined Language].
39. Vasioukhin V, Anker P, Maurice P, et al. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br J Haematol 1994;86(4):774–9.
40. Sorenson GD, Pribish DM, Valone FH, et al. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomarkers Prev 1994;3(1):67–71.

41. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11(6):426–37.
42. Jiang P, Chan CW, Chan KC, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. Proc Natl Acad Sci U S A 2015;112(11): E1317–25.
43. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14(9):985–90.
44. Sausen M, Parpart S, Diaz LA Jr. Circulating tumor DNA moves further into the spotlight. Genome Med 2014;6(5):35.
45. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 2013;200(4):373–83.
46. Gould SJ, Raposo G. As we wait: coping with an imperfect nomenclature for extracellular vesicles. J Extracell Vesicles 2013;2.
47. Witwer KW, Buzás EI, Bemis LT, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles 2013;2:20360.
48. Lotvall J, Hill AF, Hochberg F, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles 2014;3:26913.