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Liquid Biopsy to Identify Actionable Genomic Alterations

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OVERVIEW

Liquid biopsy has been used extensively in solid malignancies to detect actionable driver mutations, to monitor treatment response, to detect recurrence, to identify resistance mechanisms, and to prognosticate outcome. Although many liquid biopsy sequencing platforms are being used, only five test kits have received government approval. We review representative literature on these government-approved liquid biopsy kits, which are primarily used to detect *EGFR* mutation in lung cancer and *RAS* (*KRAS, NRAS, BRAF*) mutations in colorectal carcinoma. Another emerging use of single-gene liquid biopsy is to detect *PIK3CA* mutations and to understand resistance to hormonal blockade in breast and prostate cancers. The two most commonly used next-generation sequencing (NGS) liquid biopsy tests (Guardant 360, Guardant Health; Foundation-ACT, Foundation Medicine Inc.) are discussed. The ability and the applicability of NGS platform to detect tumor mutation burden are also addressed. Finally, the use of circulating tumor DNA (ctDNA) to detect minimal residual disease may be the most important use of ctDNA in the setting of tumor heterogeneity. The ability to identify “shedders” and “nonshedders” of ctDNA may provide important insight into the clinicopathologic characteristics of the tumor and portend important prognostic significance regarding survival.

The use of liquid biopsy (primarily from blood) in solid malignancy provides a convenient and safe way to detect the presence of actionable driver mutations, to assess the resistance mechanisms to actionable driver mutations, to monitor treatment response, to detect early recurrence, to serve as an adjuvant to radiologic imaging as post-treatment surveillance, and to prognosticate the outcome of cancer treatment. Cell-free DNA, including ctDNA, circulating tumor cells, and exosomes containing tumor microRNAs can all be detected by liquid biopsy. The biologic nature of ctDNA, the various sequencing platforms used in liquid biopsy, and the various utilities of liquid biopsy have recently been expertly and comprehensively reviewed by Wan and colleagues.[1] The many sequencing platforms used in liquid biopsy can be broadly summarized as nondigital, digital, and NGS. The performances of these individual platform have been reviewed extensively.[2–10] However, only five liquid biopsy test kits are approved by government agencies.

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DETECTION OF SPECIFIC ACTIONABLE GENOMIC ALTERATIONS BY LIQUID BIOPSY

Detection of Activating *EGFR* Mutations in Lung Cancer

Cobas EGFR mutations test version 2 (*EGFR* del 19, L858R, T790M).—The Cobas test (Roche Molecular Diagnostics, Pleasonton, CA) is the only U.S. Food and Drug Administration (FDA)–approved liquid biopsy to detect the two most common activating epidermal growth factor receptor (EGFR) mutations (*EGFR* del19 and *EGFR* L858R) for the selection of EGFR tyrosine kinase inhibitor. It was subsequently approved for the detection of the most common acquired resistance mutation, *EGFR* T790M, after progression with first- or second-generation EGFR tyrosine kinase inhibitors for selection of osimertinib to treat patients with *EGFR* T790M–positive non–small cell lung cancer (NSCLC; Table 1).[11–14] ENSURE, a randomized phase III trial comparing erlotinib to platinum/gemcitabine chemotherapy as first-line treatment of *EGFR*-positive NSCLC provided the basis for the approval of detection of *EGFR* del19 and *EGFR* L858R.[15] Additional large-scale real-life prospective trials (ASSESS, Europe and Japan; and IGNITE, Russia and China/South Korea/Taiwan) studying the feasibility and testing the concordance of using Cobas liquid biopsy versus tumor have been completed.[16,17] In the IGNITE study, the concordance between 2,561 matched tissue/cytology and plasma samples was 80.5%, sensitivity was 46.9%, and specificity was 95.6%.[17] In the ASSESS study, the concordance of mutation status in 1,162 matched samples was 89%, sensitivity was 46%, specificity was 97%, positive predictive value was 78%, and negative predictive value was 90%. Two combined single-arm phase II studies of osimertinib provided the basis for the approval for the detection of *EGFR* T790M.[18] A European study (APPLE) investigating the use of liquid biopsy to detect *EGFR* T790M mutation is ongoing.[19]

Therascreen EGFR plasma RGQ PCR kit.—The Therascreen EGFR Plasma RGQ PCR test (Qiagen Inc., Venlo, the Netherlands) is approved in the European Union (E.U.; Conformité Européene-In Vitro Diagnostics) for detection of the two most common *EGFR* mutations (*EGFR* del19 and *EGFR* L858R) but not T790M (Table 1).[20–23] The IFUM study provided the basis for adoption of this test in European Union.[24,25]

AmoyDx Super-ARMS EGFR mutation test kit.—The newest government-approved liquid biopsy, the AmoyDx Super-ARMS EGFR mutation test kit (AmoyDx, Xiamen, China), was approved on January 22, 2018, by the Chinese Food and Drug Administration to detect *EGFR* del19, *EGFR* L858R, and *EGFR* T790M mutations (Table 1).[26–28] AURA17 is a single-arm study of osimertinib in China that provided the supporting evidence for the approval of this test kit.[28]

Role in diagnosis.—Although all three tests can detect the three “uncommon” *EGFR* mutations (L861Q, G719X, and S768I)—for which afatinib was approved for use in the United States on January 16, 2018—none of them is officially approved to detect them.[29] Table 1 shows that across all three test kits, the sensitivity for detecting T790M seems to be lower than that for detecting the two common activation mutations. The higher the prevalence of *EGFR* mutations (e.g., approximately 40%–50% in Asian populations), the

lower the negative predictive value of the liquid biopsy (Table 1); this is based on the Cobas EGFR version 2 calculations. Thus, a negative *EGFR* plasma test result in a high-endemic region for *EGFR* mutations should be followed by a tumor tissue biopsy.

In addition, the National Institute of Health and Care Excellence in the United Kingdom recently published their technology summary of evidence of seven different liquid biopsy platforms.[30] The four additional tests reviewed, in addition to the three government-approved tests, were the AmoyDx EGFR 29 mutation detection kit, AmoyDx Super-ARMS EGFR T790M mutation detection kit, Droplet Digital PCR (ddPCR) Dx system (Bio-Rad, Hercules, CA), and PANA Mutyper R EGFR kit (Panagene, Daejeon, Korea). The major summary finding is that plasma *EGFR* detection has specificity similar to but sensitivity lower than those of tumor biopsy. The resource impact to the health care system is similar to that for standard of care, but plasma testing could be cost-effective if this led to fewer tissue biopsies[30]

Going forward, increasing evidence suggests that the ability to identify detectable circulating *EGFR* mutations from liquid biopsy ("shedders" compared with "nonshedders") has prognostic implications. In the FLAURA and AURA3 phase III trials (Table 2), although both shedders and nonshedders benefited from osimertinib, progression-free survival (PFS) in the osimertinib arm was numerically better for nonshedders than for shedders; this finding indicates that shedders in general have a higher tumor burden (Table 2).[14,31] Additionally, among patients treated at National Taiwan University Hospital who progressed while receiving osimertinib, a retrospective genomic analysis that used plasma BEAMing technology (Sysmex Inostics, Hamburg, Germany) to detect *EGFR* mutations showed that, again, shedders had significantly shorter median PFS while receiving osimertinib, shorter PFS after progression on osimertinib, and shorter overall survival (OS) after osimertinib treatment (Table 2).[32] Furthermore, 42.5% (17/40) of the shedders compared with only 7.7% (1/13) of nonshedders had brain metastases in this study, again indicating that shedders had more extensive/poor-prognosis disease than nonshedders. Finally, the "disappearance" of *EGFR* T790M at the time of progression during osimertinib treatment was associated with significantly shorter PFS after osimertinib therapy (Table 2), indicating more recalcitrant non–*EGFR*-mediated resistance mechanisms.[32] Shedders of *EGFR* T790M usually have higher tumor burden with late-stage disease. Similarly, shorter PFS has been reported in shedders with *EGFR* L858R mutation than in nonshedders from a retrospective analysis of the EURTAC trial.[33,34]

Detection of *RAS* and *BRAF* Mutations in Colorectal Cancer

Retrospective analysis of two randomized phase III trials in metastatic colorectal cancer (mCRC; PRIME, CRYSTAL) comparing the addition of anti-EGFR antibody to chemotherapy versus chemotherapy alone indicated that multiple *KRAS*, *NRAS*, and *BRAF* mutations modulate the clinical response to anti-EGFR antibodies.[35,36] In particular, an additional 14.7% (CRYSTAL) to 17% (PRIME) of non–exon 2 *KRAS* mutations were detected, which also did not benefit from EGFR monoclonal treatment, hence the detection of KRAS mutation should be extended to non–exon 2 *KRAS* mutations. The E.U. has approved three sets of liquid biopsy kits from two different companies that detect an

extended spectrum of *RAS* mutations to select for patients with mCRC who will potentially benefit from anti-EGFR therapy and avoid the unnecessary expense and additional side effects of anti-EGFR antibody treatment.

OncoBEAM RAS CRC kit (*KRAS* and *NRAS*).—Several studies using different tissue comparator tests provided the evidence for approval of OncoBEAM RAS detection kit (Sysmex Inostics; Table 3) for the detection of *KRAS* and *NRAS* mutations in mCRC.[37–42] Not surprisingly, when a much more sensitive tissue comparator test, such as NGS, is used, the sensitivity and specificity of BEAMing technology are not as high (e.g., with the Sanger sequencing method as the comparator; Table 3).[43] The additional use of BEAMing to detect *KRAS* mutations includes real-time monitoring of treatment, detection of resistance mechanisms, and early detection of relapse and/or residual disease; these have been concisely reviewed by an expert task force panel.[44]

Idylla ctKRAS mutation test and Idylla ctNRAS-BRAF mutation test.—The RASANC study provided the supporting evidence for the approval of the Idylla tests (Biocartis, Inc., Jersey City, NJ) for the detection of *KRAS*, *NRAS*, and *BRAF* in mCRC.[45,46] Primary tumor removal, metachronous status, absence of liver metastases, and peritoneal carcinomatosis were significantly associated with mutant *RAS* tumor and negative plasma status, indicating the difference between shedders and nonshedders.[45]

In head and neck cancer, additional use of liquid biopsy to detect *RAS* mutations has been reported to identify resistance to cetuximab (anti-EGFR antibody)-based therapy.[47] However, the resistance to anti-EGFR antibody therapy is not limited to *RAS* mutations based on liquid biopsy.[48–50]

BRAF V600E

BRAF V600E mutation is a validated and actionable driver mutation in melanoma and NSCLC.[51,52] Surprisingly, to date there is little published literature on the clinical use of liquid biopsy to detect *BRAF* V600E mutation in melanoma and NSCLC.[53] Most of the literature on liquid biopsy for *BRAF* V600E is in mCRC, especially when used in extended-spectrum *RAS* mutational analysis to select patients for anti-EGFR treatment (Table 3). Not surprisingly, a greater amount of *BRAF* V600E detected at liquid biopsy was associated with worse prognosis for survival in mCRC (hazard ratio [HR], 7.33; 95% CI, 1.04–2.89; p = . 002) in a multivariate analysis that included age, tumor location, carcinoembryonic anti-gen (CEA), *KRAS* mutational status, and amount of circulating cell-free DNA.[54]

PIK3CA Mutations

Although no approved drug specifically targets *PIK3CA* mutations, and *PIK3CA* mutations have not been conclusively shown to be a driver mutation, *PIK3CA* mutations may confer sensitivity to PIK3CA inhibitors. Two randomized phase III trials have investigated the potential clinical benefit of the addition of buparlisib (BKM210), a class 1 pan-PIK3CA inhibitor, to fulvestrant in hormone receptor–positive, HER2-negative patients whose disease is refractory to aromatase inhibitors (AIs; BELLE-2) and additionally refractory to mTOR inhibition (BELLE-3).[55,56] Both trials investigated the presence of plasma *PIK3CA*

mutations, established by using BEAMing technology, and its implication for clinical outcome. Overall, the most common *PIK3CA* mutations identified from ctDNA from BELLE-3 were H1047R (39.7%), E545K (36.8%), E542K (18.4%), and H1047L (5.1%).

The sensitivity of the plasma *PIK3CA* mutations compared with tissue *PIK3CA* mutations in BELLE-2 was 71.2% (99/139), specificity was 79.2% (244/307), and overall concordance was 76.7% (342/446). Similarly, for BELLE-3, sensitivity was 80% (70/87), specificity was 87% (142/163), and concordance was 84.8% (212/250). The lower concordance in BELLE-2 probably results from the fact that detection of *PIK3CA* mutation in tumor is performed by using Sanger sequencing, which is not as sensitive as BEAMing technology. The presence of *PIK3CA* mutations in plasma corresponded to improved PFS in patients who received buparlisib in addition to fulvestrant compared with patients who received only fulvestrant in both BELLE-2 and BELLE-3 trials (Table 4).[55,56] However, neither trial analyzed the prognostic significance of shedders versus nonshedders of *PIK3CA* mutations.

The role of *PIK3CA* mutation in breast cancer is being further evaluated in two phase III trials: SOLAR-1 () is comparing alpelisib (a PI3KA inhibitor) and fulvestrant versus fulvestrant alone, with a prospective analysis of *PIK3CA* mutations in ctDNA, and SANDPIPER () is comparing taselisib (GDC-0032) and fulvestrant versus fulvestrant alone in *PIK3CA*-mutant breast cancer as determined by Cobas testing. Otherwise, the eligibility criteria for both trials are similar to those of BELLE-2 (disease that is refractory to AI). If these trials will lead to the eventual approval of PIK3CA inhibitors, then *PIK3CA* mutations detection will become very important.

The prognostic significance of pretreatment plasma *PIK-3CA* in cervical cancer was also investigated in 117 patients with cervical cancer treated in Hong Kong over a period of 10 years (1997 to 2007). ddPCR was used to detect *PIK3CA* E542K and E545K mutations.[57] Overall, 26 of 117 (22.2%) patients were identified (23.7% in squamous cell carcinoma of the cervix and 15.0% in adenocarcinoma of the cervix). The presence of plasma *PIK3CA* mutations was associated with high pathologic grade and large tumor size (mean tumor size, 4 cm) compared with 3 cm for patients negative for plasma *PIK3CA* mutations but was not associated with age, clinical stage, presence or absence of lymphovascular invasion, or pelvic lymph node metastasis. Although *PIK3CA* mutation was associated with shortened disease-free survival and OS on univariate analysis, on multivariate analysis *PIK3CA* mutation did not affect disease-free survival (HR 1.449; p = .464) or OS (HR 1.261; p = .643). Going forward, in analyses of larger cohorts of patients with cervical cancer who have uniform stage, histology, histologic grade, and treatment modality and are from a more contemporaneous treatment period, plasma *PIK3CA* mutations may turn out to be an important prognostic factor in cervical cancer. Liquid biopsy for *PIK3CA* mutation has also been investigated in small numbers of patients with bladder cancer and mCRC.[58,59]

HER2 Amplification

HER2 amplification was validated as an actionable target in breast cancer in 2001 and in gastric adenocarcinoma in 2010.[60,61] The gold standard of detecting *HER2* amplification in breast cancer is by immunohistochemistry or florescence in situ hybridization. Given the generally ready availability of tumor tissue from patients with breast cancer, there is scant

literature on the use of liquid biopsy to detect or to monitor *HER2* amplification at diagnosis or during treatment, respectively. Small studies have demonstrated that *HER2* amplification detected in plasma correlates well with tumor *HER2* amplification and that the level of *HER2* amplification can be used to monitor treatment and recurrence in gastric cancer.[62,63] *HER2* amplification was successfully identified by using a liquid biopsy NGS panel to identify driver mutations to enroll in a basket targeted therapy trial that included HER2 inhibition.[64]

USE OF LIQUID BIOPSY TO DETERMINE RESISTANCE MECHANISMS TO HORMONAL BLOCKADE IN SOLID TUMORS

Estrogen Receptor (*ESR1*) Mutation

Detection of *ESR1* mutation (primarily in the ligand-binding domain) in plasma has been investigated as a resistance mechanism to an AI in two randomized phase III trials, SoFEA (Study of Faslodex Versus Exemestane With or Without Arimidex) and PALOMA3 (Palbociclib Combined With Fulvestrant in Hormone Receptor–Positive HER2-Negative Metastatic Breast Cancer After Endocrine Failure), using the Bio-Rad QX-200 ddPCR system. In the SoFEA analysis, patients with mutant *ESR1* in the exemestane treatment arm had significantly worse PFS (p = .01) than those without mutated *ESR1.* Similarly, PFS was significantly improved (p = .02) in patients with mutant *ESR1* when they received fulvestrant-containing regimens but not (p = .77) in patients with wild-type *ESR1* (Table 5). On the other hand, not surprisingly, the retrospective analysis of the PALOMA3 showed that both *ESR1*-mutated (HR 0.43; 95% CI, 0.25–0.74; p = .002) and *ESR1* wild-type (HR 0.49; 95% CI, 0.35–0.70; p < .001) patients benefited from the addition of palbociclib to fulvestrant compared with those receiving placebo and fulvestrant because palbociclib is a CDK4/6 inhibitor (Table 6).[65]

Together, these two trials suggest that for patients who progressed while receiving a nonsteroidal AI and have detectable *ESR1* mutations, it is best to switch to fulvestrant-containing regimens, such as fulvestrant and palbociclib. For patients who progressed while receiving a nonsteroidal AI and have no detectable *ESR1* mutations, switching to an irreversible AI, such as exemestane, remains a treatment option before proceeding to fulvestrant-contain regimens. These are hypothesis-generating observations because only 161 of 723 (22.3%) patients enrolled in the SoFEA and only 360 of 551 (65.3%) patients enrolled in PALOMA3 had plasma available and successfully analyzed.

In the PALOMA3 trial, logistic multivariate analysis showed that the presence of plasma *ESR1* mutations was negatively associated with tamoxifen treatment only (odds ratio [OR], 0.06; 95% CI, 0.01–0.45; p = .01) and positively associated with sensitivity to endocrine therapy (OR, 3.95; 95% CI, 1.59–9.78; p = .003), bone metastasis (OR, 3.31; 95% CI, 1.54–6.39; p = .002), and visceral metastasis (OR, 1.74; 95% CI, 1.02–2.98; p = .04). Importantly, multivariate analysis of the PFS benefit in PALOMA3 showed a negative association with plasma *ESR1*-mutated genotype (HR 1.4; 95% CI, 1.07–2.08; p = .02), even after factoring in the significant benefit from palbociclib.[65]

Recent analysis of a large trial of first-line AI therapy in breast cancer indicated that *ESR1* mutations may play a role in AI resistance, but other resistance mechanism, such as *KRAS*, have been identified.[66] Finally, liquid biopsy using NGS indicated multiple resistance mechanisms to AI in addition to *ESR1* mutations.[67]

Androgen Receptor Variant 7

A splice variant of the androgen receptor variant 7 (*AR-V7*) that deletes the ligand domain of *AR* isolated from circulating tumor cells in the plasma is involved in de novo and acquired resistance to abiraterone (an androgen synthesis inhibitor) and enzalutamide (an *AR* ligand–binding domain antagonist), both of which have been approved for the treatment of castration-resistant prostate cancer (Table 7). In multivariable Cox regression analysis stratified by treatment type, *AR-V7* detection remained independently predictive of prostate-specific antigen–defined PFS (HR 8.2; 95% CI, 2.7–24.9; p < .001), clinical/radiographic PFS (HR 4.9; 95% CI, 1.7–13.8; p = .003), or OS (HR 5.0; 95% CI, 1.3–19.8; p = .021; Table 7).[68] Further study has indicated that *AR* amplification was associated with resistance to enzalutamide, and mutations (*H874Y, T877A, D879E, L881I, E893K*, and *M895V)* in the ligand-binding domain of *AR* are more associated with resistance to abiteratone.[69] More recently, a longitudinal multiplex targeting sequencing of plasma from a study of 65 patients with castration-resistant prostate cancer receiving enzalutamide indicated that besides *AR* amplification of *AR* mutations, *RB1* loss (p = .01) and *MET* copy number gain/amplification (p = .2) were significantly associated with shorter PFS after adjustment for the presence of ctDNA.[70] Overall mutations in the direct target of hormonal blockade can partially explain the resistance to hormonal treatment of breast and prostate cancer. However, the advent of NGS liquid biopsy has allowed the discovery a much more complex pattern of resistance mechanisms.

NEXT-GENERATION SEQUENCING PLATFORMS

Liquid biopsy using NGS will be the dominant platform, with its increased depth of coverage, simultaneous multigene sequencing, and ability to simultaneously detect all modes of genomic alterations (point mutations, insertion/deletion [indel], amplification, and gene rearrangements) to account for both tumor heterogeneity and multiple resistance mechanisms and determination of tumor mutation burden (TMB). The two most common commercially used liquid biopsies are Guardant360 (Guardant Health, Redwood City, CA) and FoundationACT (Foundation Medicine Inc., Cambridge, MA). As listed in Table 8, the limit of detection and the gene panel of Guardant360 has evolved over time. It is important to be aware of the number of exact genes included in the NGS sequencing platform by consulting with the company websites because technology advances will lead to increased complexities of the NGS panel being offered commercially. It is also important to note how the interpretation of the literature depends on the various versions of Guardant360.

Among the many other NGS liquid biopsy platforms that are available or under development are Oncomine Lung cfDNA Assay (ThermoFisher Scientific, Waltham, MA),[88] Archer Reveal ctDNA (ArcherDX, Boulder, CO),[89] Oncotype SEQ (Genomic Health, Redwood City, CA),[90] LiquidDx (MolecularMD, Portland, OR),[91] CancerIntercept Detect and

CancerIntercept Monitor (Pathway Genomics, San Diego, CA),[92] OptiSeq NGS Pan-Cancer Panel (DiCarta, Richmond, CA),[93] and PlasmaSELECT (Personal Genome Diagnostics, Baltimore, MD).[94]

USE OF LIQUID BIOPSY TO DETERMINE TUMOR MUTATION BURDEN

TMB is usually expressed as number of mutations per mega-base of DNA (MB)[95] and has been shown to be predictive to response to anti–PD-L1 therapy.[96] In the CheckMate026 trial, which compared nivolumab to platinum-based chemotherapy as first-line treatment of NSCLC, OS did not differ between nivolumab and chemotherapy. A subgroup analyzed with TMB, as determined by whole-exome sequencing, indicated that patients with NSCLC and high TMB had a marginally significantly improved PFS compared with those receiving platinum-based chemotherapy (9.7 months [95% CI, 5.1 months to not reached] versus 5.8 months [95% CI, 4.2–8.5 months]; HR 0.62 [95% CI, 0.38–1.00]). OS did not differ between nivolumab and chemotherapy regardless of high or low TMB.[96] Importantly, there is excellent concordance between TMB as determined by a commercially available (Foundation Medicine Inc.) tissue-based hybrid-capture NGS method and whole-exome sequencing of the tumor but essentially no correlation between TMB and PD-L1 expression.[96]

It is now feasible to determine TMB by liquid biopsy using whole-exome sequencing or NGS hybrid-capture method.[97,98] The blood TMB (bTMB) assay developed by Foundation Medicine Inc. uses 10 mL of plasma to deliver an account of somatic base substitutions down to 0.5% allele frequency across 394 genes from as little as 1% tumor content in a cell-free DNA sample. Computational methods differ: bTMB analyzes only single-nucleotide variants (SNVs), whereas tissue TMB also includes analysis of indels and fusions. Sensitivity, specificity, and positive predictive value were 93.9%, 100.0%, and 100.0%, respectively, when compared with tissue TMB.[98] Importantly, the bTMB assay demonstrates strong sensitivity and specificity between the cut-points of 8–20 mutations/MB. The clinical utility of the bTMB assay was retrospectively analyzed in 783 patients with NSCLC receiving second-line therapy (OAK and POPLAR); PFS and OS were compared between atezolizumab (an anti–PD-L1 antibody) and single-agent docetaxel as second-line treatment. At a bTMB cutoff of 16 or greater, PFS and OS significantly improved in the phase II randomized POPLAR study.[99] Among the 850 patients enrolled in the phase III OAK trial, 583 patients were considered as the “biomarkers evaluable population” (BEP) for bTMB. A bTMB of 16 or greater represented 27% of the BEP. There was an OS benefit for patients with both bTMB of 16 or greater (HR 0.64; 95% CI, 0.44–0.92) and bTMB less than 16 (HR 0.65; 95% CI, 0.52–0.81). Indeed, PFS (HR 0.73; 95% CI, 0.56–0.95) and OS (HR 0.69; 95% CI, 0.52–0.93) benefits were observed in patients with bTMB of 10 or less. A bTMB of 10 or less represents 43% of the BEP, and the sensitivity and specificity were both 100% when compared with tissue TMB.[99] The bTMB assay is being validated in two trials. B-F1RST () is a phase II single-arm study of atezolizumab as first-line treatment of NSCLC while prospectively collecting plasma to evaluate whether bTMB can predict for improved clinical outcome with atezolizumab. B-FAST () is prospectively enrolling patients with NSCLC receiving first-line therapy in a global, multicenter, multicohort, randomized phase II/III trial without certain targetable driver mutations (*RET* or *ALK* fusion) detected based

on blood-based assays; additionally, the trial is comparing single-agent atezolizumab therapy to standard of care based on bTMB results for those patients without RET or ALK rearrangement.[100]

USE OF CTDNA IN LIQUID BIOPSY TO DETECT MINIMAL RESIDUAL DISEASE IN SOLID TUMORS

Most solid tumors do not have a single truncal actionable driver mutation. There is vast literature on the use of nonspecific ctDNA in solid malignancies ranging from detection to monitor treatment response to detection of recurrence, and even for survival prognostication, all of which are beyond the scope of this review. However, ctDNA detection by liquid NGS has been used to identify minimal residual disease (MRD); we review two important studies here.[101,102]

In a study of 230 patients with resected stage II colon cancer conducted by Tie and colleagues, 20 (8.7%) patients had ctDNA detected by massive parallel sequencing. Most of these mutations were *TP53*, *APC*, and *KRAS*. There was no difference in clinicopathologic difference (age, sex, right-versus left-sided colon, tumor differentiation, T3 versus T4, number of lymph node resected < 12 versus ≥ 12, mismatch repair status, presence or absence of lymphovascular invasion, with or without adjuvant chemotherapy) between patients with and without detectable ctDNA. Among the 178 patients not treated with adjuvant chemotherapy, postoperative recurrence occurred in 11 of 14 (78.6%) ctDNA-positive patients compared with 16 of 164 (9.8%) ctDNA-negative patients. The 3-year relapse-free survival rate was 0% for ctDNA-positive patients compared with 90% for ctDNA-negative patients (HR 18; 95% CI, 7.9–40; $p = 2.5 \times 10^{-12}$). By multivariate analysis, ctDNA positivity (HR 28; 95% CI, 11–68; p = .0001) and T stage (T4 versus T3; HR 8.1; 95% CI, 3.1–21; p < .0001) were the two independent predictive factor for RFS after factoring in the above clinicopathologic factors. In addition, among the 230 patients, ctDNA positivity (HR 14; 95% CI, 6.8–28; p < .0001) and T stage (HR 2.6; 95% CI, 3.1–5.5; p = .001) remained the two independent factors for PFS after factoring in adjuvant chemotherapy. More importantly, postoperative elevation of CEA level was not a predictive factor for relapse-free survival, even by univariate analysis with or without adjuvant chemotherapy.[101] Furthermore, ctDNA positivity immediately after completion of postoperative chemotherapy was significantly associated with poor relapse-free survival (HR 11; 95% CI, 1.8–6.8; p = .001). However, the number of patients analyzed was 44, and only three patients had ctDNA positivity immediately after completion of postoperative chemotherapy.

Finally, during the follow-up period, ctDNA was significantly more frequently positive (23/27 [85%]) than was CEA elevation (11/27 [41%]) at the time of radiographic recurrence (p = .002). The time between ctDNA positivity (median, 167 days) was significantly longer than that between CEA elevation (median, 61 days) to radiographic recurrence (p = .04). This retrospective analysis of prospective collected data indicated that ctDNA monitoring has the ability to detect MRD and hence early recurrence, leading to the potential initiation and/or lengthening of adjuvant chemotherapy based on the presence or absence of ctDNAs.

There is a randomzied ctDNA driven adjuvant chemotherapy trial in resected stage II CRC in Australia/New Zealand (DYNAMIC, Trial ID number: ACTRN12615000381583). In the standard-of-care arm, adjuvant chemotherapy wil be given as determined by the clinicians, while in the investigational arm, adjuvant chemothrapy will be guided by the presence (adjuvant chemotherapy) or absence (no adjuvant chemotheray) of postoperative ctDNA in resected stage II CRC. The primary endpoint is to evaluate whether an adjuvant therapy strategy based on ctDNA results may affect the number of patients treated with chemotherapy and recurrence-free survival.

TRACERx is a U.K. study that developed proprietary NGS technology to link the individual patient's tumor DNA to plasma ctDNA by identifying two distinct tumor-associated mutations (usually SNV).[102] The resected tumor was further microdissected at several different areas, and all "subtumors" underwent NGS sequencing to detect tumor heterogeneity.[103] The TRACERx study group used this phylogenetic ctDNA (at least two SNVs) tracking to detect recurrence in 96 patients with resected early-stage lung cancer. A group of 24 patients were followed longitudinally with pre- and postoperative plasma ctDNA; of these, 10 had been relapse free for a median of 775 days, whereas 14 had confirmed relapse. Thirteen of the 14 patients who relapsed had ctDNA detected before or at clinical relapse compared with only one of 10 patients in remission (two SNVs). Overall, the median duration between ctDNA detection and radiographic evidence of relapse was 70 days. Relapse can involve one or more subclones. This analysis has some limitations due to lack of regularly scheduled imaging to account for potential lead-time bias of regular liquid biopsy compared with detection of recurrence by clinical practice.[104] Regardless, the TRACERx approach remains an important technology that accounts for tumor heterogeneity in the primary tumor, especially because most lung cancers do not harbor a signature actionable driver alteration.

Detection of MRD may turn out to be the most important aspect of use of liquid biopsy because it may help guide extent of adjuvant treatment,[105] similar to the use of Epstein-Barr virus load in the treatment of early-stage nasopharyngeal carcinoma.[106]

SHEDDERS VERSUS NONSHEDDERS OF ACTIONABLE GENOMIC ALTERATIONS

In the TRACERx study, 46 of 96 patients had at least two SNVs that allowed phylogenetic ctDNA tracking, including 30 of 31 (97%) lung squamous cell carcinomas, 11 of 58 (19%) lung adenocarcinomas, and five of seven other NSCLCs. Among stage I NSCLC, 16 of 17 squamous cell carcinomas (94%) compared with five of 39 (13%) adenocarcinomas had detectable ctDNA. Of note, the median necrosis rate in lung squamous cell carcinoma (40%; n = 31) was significantly higher than in ctDNA-positive lung adenocarcinomas (15%; n = 11) and ctDNA-negative adenocarcinomas (2%; n = 47; $p < .0001$). Multivariate analysis indicated that nonadenocarcinoma histology ($p = .001$), lymphovascular invasion ($p = .042$), and high Ki-67 proliferation index ($p = .022$) are independent predictors of ctDNA positivity but not tumor necrosis (10% increment increase; $p = .862$), tumor size (1-cm increment increase; $p = .134$), or amount of cfDNA ($p = .229$). Importantly, the mean plasma variant

allele frequency positively corresponded to tumor size. The TRACERx team was able to estimate that a mean clonal plasma variant allele frequency of 0.1% corresponds to a tumor burden of 10 cm^3 or a burden of 302 million tumor cells.[102]

A large-scale systemic review of 39 studies that included 4,052 patients, the detection of ctDNA was associated with a significantly worse OS in multivariable analyses [HR 2.70; 95% CI, 2.02–3.61; $p < .001$).[107] There was also a statistically significant association between high total cell-free DNA and worse OS at 3 years by multivariate analysis (HR 1.91; 95% CI, 1.59–2.29; $p < .001$).[107] Thus, the use of liquid biopsy to identify shedders and nonshedders has important clinical implication and has been proposed to be included as part of the staging workup (tumor, node, metastasis, biopsy).[10]

CONCLUSION

The advantages of liquid biopsy not only include the possibility to circumvent the need to achieve adequate tumor tissue biopsy specimens, it also reflects an aggregate of ctDNA output from potentially both primary and all metastatic sites accounting for tumor heterogeneity that cannot be evaluated by a single core tumor needle biopsy. Liquid biopsy may provide prognostic implication and help guide initiation and/or duration of adjuvant treatment by detecting MRD. Although all the current government-approved liquid biopsy test kits are designed to detect only mutations in one or a few specific genes, the future of liquid biopsy will be using NGS with increased sensitivity and ability to detect all modes of genetic alteration and TMB. The gene panel and limit of detection used by commercial NGS liquid biopsy kits change over time. It is incumbent upon the ordering physician to understand the current NGS platforms and performance characteristics and upon readers to interpret the literature with the comprehension of the changes in the gene panel and performance characteristics of commercial NGS tests as time goes by.

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PRACTICAL APPLICATIONS

- Despite the widespread use of liquid biopsy in solid malignancy, only five distinct test kits have government approval: Cobas EGFR Mutations Test v2 (Roche Molecular Diagnostics; FDA approval for detection of *EGFR del19, EGFR L858R*, and *EGFR T790M* in lung cancer), Therascreen EGFR RGQ Plasma PCR kit (Qiagen Inc; E.U. approval for detection of *EGFR* del19 and *EGFR* L858R in lung cancer); AmoyDx Super-ARMS EGFR mutation test (AmoyDx; Chinese Food and Drug Administration approval for detection of *EGFR* del19*, EGFR* 858R, and *EGFR* T790M in lung cancer); OncoBEAM RAS CRC Kit (Sysmex Inostics; E.U. approval for detection of *KRAS* and *NRAS* mutations in colorectal cancer); and Idylla ctKRAS Mutation Test and Idylla ctNRAS-BRAF Mutation Test (Biocartis, Inc.; E.U. approval for detection of *KRAS, NRAS*, and *BRAF* mutations in colorectal cancer).
- Currently, liquid biopsy is most commonly used to detect actionable *EGFR, RAS* (*KRAS, NRAS*), and *BRAF* V600E mutations, with potentially in the future for detection of *PIK3CA* mutations.
- Liquid biopsy based on NGS can detect multiple genomic alterations (mutations, indel, amplification, rearrangement) in multiple actionable genes and also determine tumor mutation burden used to potentially select for immune checkpoint inhibitors. Eventually, liquid biopsy using NGS will supersede use of single/oligo gene liquid biopsy detection kits.
- Liquid biopsy to detect nonspecific ctDNA in tumors without clear driver mutations may be most useful in detecting minimal residual disease, which can account for tumor heterogeneity in the adjuvant setting.
- Distinguishing “shedders” and “nonshedders” of ctDNA may provide insight into tumor biology and prognostic significance.

TABLE 1.

Approved Liquid Biopsy Test Kits for Detection of Activating and Resistance *EGFR* Mutations in Non–Small Cell Lung Cancer

	Therascreen EGFR Plasma RGQ PCR Kit	Cobas EGFR Mutation Test v2	AmoyDX Super-ARMS EGFR Mutation Detection Kit
PCR Kit			
Approving Agency/Region	European Union 98/79,	U.S. FDA	Chinese FDA
	Conformitée Européene-ln Vitro Diagnostics directive		
Date of Approval	January 12, 2015	June 1, 2016 (*EGFR* del 19, *EGFR* L858R) September 28, 2016 (*EGFR* T790M)	January 22, 2018 (*EGFR* del 19, *EGFR* L858R, *EGFR* T790M)
Manufacturer	Qiagen	Roche	AmoyDx
Sequencing Platform	Scorpion Amplification Refractory Mutation System	Scorpion Amplification Refractory Mutation System	Scorpion Amplification Refractory Mutation system
Detectable Technology *	Analog (real-time PCR)	Analog (real-time PCR)	Analog (real-time PCR)
MAF Quantification	Semiquantitative	Semiquantitative	Semiquantitative
No. of *EGFR* Mutations Detected	29	42	41
Major *EGFR* Mutations Detected	*EGFR* del 19 (19 different mutations)	*EGFR* del 19 (29 different mutations)	*EGFR* del 19 (29 different mutations)
	EGFR L858R	*EGFR* L858R (2 different mutations)	*EGFR* L858R
	EGFR T790M	*EGFR* T790M	*EGFR* T790M
	EGFR G719X (3 different mutations)	*EGFR* G719X (3 different mutations)	*EGFR* G719X (3 different mutations)
	EGFR S761I	*EGFR* S761I	*EGFR* S761I
	EGFR L861Q	*EGFR* L861Q	*EGFR* L861Q
	EGFR exon 20 insertions (3 insertions)	*EGFR* exon 20 insertions (5 insertions)	*EGFR* exon 20 insertions (5 insertions)
Indication	*EGFR* del 19,	*EGFR* del 19	*EGFR* del 19
	EGFR L858R	*EGFR* L858R	*EGFR* L858R
	EGFR Del 19 and *EGFR* L858R	*EGFR* T790M	*EGFR* T790M
Study(ies) Supporting Approval	IFUM	ENSURE (Y025121)	Single-center, single-arm study (First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China)
Comparator Tissue Test	Therascreen *EGFR* test (tissue use)	Cobas *EGFR* mutation test v2 (tissue test) NGS on an lllumina MiSeq	AmoyDx *EGFR* 29 mutation detection kit (tissue)
No. of Patients	859 with tumors successfully screened	601 with tumors successfully screened	109 screened
	652 with successfully paired tumor/plasma analyzed	431 with successfully paired tumor/plasma analyzed and validated	61 with tissue positive for all *EGFR* mutations
		217 enrolled	50 with plasma positive for all *EGFR* mutations

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	Therascreen EGFR Plasma RGQ PCR Kit	Cobas EGFR Mutation Test v2	AmoyDX Super-ARMS EGFR Mutation Detection Kit
	Plasma RGQ PCR Kit	Mutation on Test v2	Detection Kit
Primary Data	Screening for all patients:	Screening for all patients	Of 61 patients positive for *EGFR* mutations from tumor, 50 were also detected by plasma
	Of 105 patients positive for *EGFR* mutations from tumor, 69 (65.7%) were detected by plasma	Of 210 patients positive for *EGFR* mutations from tumor, 161 (76.7%) were detected by plasma	Of 48 patients negative for *EGFR* mutations from tumor, 48 were also negative for *EGFR* mutations by plasma
	Of 547 patients negative for *EGFR* mutations from tumor, 546 (99.8%) were negative for *EGFR* mutation by plasma	Of 221 patients negative for *EGFR* mutations from tumor, 217 (98.2%) were negative for *EGFR* mutation by plasma	
		Screening for *EGFR* del 19 patients	
		Of 120 patients positive for *EGFR* del 19 mutation from tumor, 97 (80.8%) were detected by plasma	
		Of 311 patients negative for *EGFR* del 19 mutation from tumor, 307 (98.7%) were negative for *EGFR* mutation by plasma	
		Screening for *EGFR* L858R mutation	
		Sensitivity: Of 90 patients positive for *EGFR* L858R from tumor, 61 (67.8%) were detected by plasma	
		Specificity: Of 341 patients negative for *EGFR* L858R from tumor, 338 (99.1%) were negative for *EGFR* L858R by plasma	
Performance Characteristics**	Sensitivity: 65.7% (95% CI, 55.8%–74.7%) Specificity: 99.8% (95% CI, 999%–100%) Concordance: 94.3% (95% CI, 92.3%–96.0%) PPV: 98.6% (95% CI, 92.3%–100%) NPV: 93.8% (95% CI, 91.5%–95.6%)	Prevalence: 15%	Sensitivity: 82% (95% CI, 72.3%–91.6%) Specificity: 100% PPV: 81.4% (95% CI, 71.4%–91.3%) NPV: 100%
		PPV: 88.6% (95% CI, 79.7%–96.6%)	
		NPV: 96.0% (95% CI, 94.39%–97.6%)	
		Prevalence: 40%	
		PPV: 96.8% (95% CI, 93.0%–99.4%)	Concordance: 89.9% (95% CI, 84.3%–95.6%)
		NPV: 86.4% (95% CI, 83.39%–89.4%)	
		Prevalence: 50%	
		PPV: 97.8% (95% CI, 95.0%–100%)	
		NPV: 80.8% (95% CI, 77.49%–84.8%)	
Clinical Outcome Data	ORR (*EGFR* del 19 + L858R)	PFS benefit of erlotinib over chemotherapy (ITT population, n = 217)	ORR
	Tumor+/plasma+: 72.5% (95% CI, 61.0%–81.6%); tumor+/plasma−: 63.6% (95% CI, 46.6%–77.8%)	HR, 0.33 (95% CI, 0.23–0.47); log-rank test p < .0001	All patients: 64.3% (95% CI, 48%–78%; n = 42)
	ORR [*EGFR* del 19)	PFS benefit of erlotinib over chemotherapy (tumor+/plasma+; n = 137)	Tumor+/plasma+: 65.7% (95% CI, 48%–81%; n = 35)

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	Therascreen EGFR Plasma RGQ PCR Kit	Cobas EGFR Mutation Test v2	AmoyDX Super-ARMS EGFR Mutation Detection Kit
	Tumor+/plasma+: 82.2% (95% CI, 68.7%–90.7%); tumor+/plasma−: 65.0% (95% CI, 43.5%–73.7%)	HR, 0.29 (95% CI, 0.19–0.45); log-rank test p < .0001	Tumor+/plasma−: 57.15% (95% CI, 18%–90%)
	ORR [*EGFR* L858R)	PFS benefit of erlotinib over chemo (tumor+/plasma−; n = 42)	
	Median PFS (all enrolled patients)	HR, 0.37 (95% CI, 0.50–0.90)	
	All enrolled patients: 9.7 mo (95% CI, 8.5–11.0 mo)		
	Tumor+/plasma+: 10.2 mo (95% CI, 8.5–12.5 mo)		
	EGFR del 19		
	All: 9.6 mo (95% CI, 8.0–11.0 mo)		
	Tumor+/plasma+: 10.3 mo (95% CI, 8.5–12.4 mo)		
T790M			
Validation Study	NA	Combined AURA extension study and AURA2 (*EGFR* T790M)	AURA17
Primary Data	NA	551 of 710 (78%) patients enrolled in both trials had paired tissue and plasma samples	240 patients
		416 of 551 (75.5%) patients were plasma positive for T790M, 127 (23.0%) were plasma negative for T790M, and 8 patients had invalid plasma tests	
Comparator Tissue Test		Cobas *EGFR* mutation test v2 (tissue test)	Cobas *EGFR* mutation test v2 (tissue test)
Performance Parameters	NA	*EGFR* T790M	AmoyDx Super-ARMS *EGFR* T790M mutation detection kit only
		Sensitivity: 61% (95% CI, 57%–66%)	
		Specificity: 79% (95% CI, 70%–85%)	*EGFR* T790M
		Concordance: 65% (95%: 61%–69%)	Sensitivity: 49% (95% CI, 41%–57%)
		EGFR del 19	Specificity: 78% (95% CI, 67%–86%)
		Sensitivity: 85% (95% CI, 81%–89%)	
		Specificity: 98% (95% CI, 95%–100%)	
		Concordance: 90% (95% CI, 87%–92%)	
		EGFR L858R	
		Sensitivity: 76% (95% CI, 69%–82%)	
		Specificity: 98% (95% CI, 96%–99%)	
		Concordance: 91% (95% CI, 88%–93%)	
Clinical Outcome	NA	ORR	
		T790M tumor+/plasma+: 64% (95% CI, 57%–70%; n = 235)	

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	Therascreen EGFR Plasma RGQ PCR Kit	Cobas EGFR Mutation Test v2	AmoyDX Super-ARMS EGFR Mutation Detection Kit
		T790M tumor+/plasma−: 70% (95% CI, 62%–77%; n = 155)	
References	20–25	11–13,18,23	26–28

*Analog detection (also known as real-time PCR) uses fluorescent markers that attach to specific mutation sites, making them detectable; fluorescence in the sample is detected as a whole. In digital PCR, the sample is separated into many partitions, each tested individually, providing a lower threshold of detection.

**Can detect the level of ctDNA at or above threshold limit, fully quantitative can measure the level ctDNA in a sample.

Abbreviations: FDA, U.S. Food and Drug Administration; PCR, polymerase chain reaction; MAF, mutant allele frequency; IFUM, Iressa Follow up Measure Study; NGS, next-generation sequencing; ORR, overall response rate; NPV, negative predictive value; PPV, positive predictive value; ITT, intention-to-treat; HR, hazard ratio; PFS, progression-free survival; NA, not applicable.

TABLE 2.

Survival Outcome (Progression-Free Survival, Overall Survival, and Survival After Osimertinib Progression) of *EGFR* T790M Shedders Versus Nonshedders

FLAURA[31]									
	Overall (n = 556)			**Shedders (n = 359)**			**Nonshedders (n = 124)**		
***EGFR* Variable**	**Osiemrtinib**	**Gefitinib/Erlotinib**	**HR (95% CI); p Value**	**Osimertinib**	**Gefitinib/Erlotinib**	**HR (95%CI); p Value**	**Osimertinib**	**Gefitinib/Erlotinib**	**HR (95% CI); p Value**
Patients, n	279	277		183	176		60	64	
Median PFS (95% CI)	18.9 (15.2–21.4) mo	10.2 (9.6–11.1) mo	0.46 (0.37–0.57); p < .0001	15.2 (13.7–20.7) mo	9.7 (8.4–11.1) mo	0.44 (0.34–0.57); p < .0001	23.5 (17.8–24.3) mo	15.0 (9.7–18.3) mo	0.48 (0.28–0.80); p = .0047
AURA3									
	Overall (n = 419)			**Shedders (n = 272)**			**Nonshedders**		
	Osimertinib	**Chemotherapy**	**HR (95% CI); p Value**	**Osimertinib**	**Chemotherapy**	**HR (95% CI); p Value**	**Osimertinib**	**Chemotherapy**	**HR (95% CI); p Value**
Patients, n	279	140		116	56		NR	NR	
Median PFS (95% CI)	10.1 (8.3–12.3) mo	4.4 (4.2–5.6) mo	0.30 (0.23–0.41); p < 0.0001	8.2 (6.8–97) mo	4.2 (41.–5.1) mo	0.42 (0.29–0.61)	NR	NR	NR
AURA Phase I and AURA Extension Study (National Taiwan University Hospital study)[32]									
	Shedder	**Nonshedder**	**p Value**	**Shedder Without Brain Metastases**	**Nonshedder Without Brain Metastases**	**p Value**	**T790M "Drop Out" at Osimertinib Progression**	**Nonshedders at Osimertinib Progression**	**Shedders (New or Continual) at Osimertinib Progression**
Patients, n	40	13		23	12		11	12	17
Median PFS (during osimertinib treatment)	9.4 (7.1–13.8) mo	15.4 (9.6–NR) mo	.055	12.4 (8.4–20.9) mo	15.4 (11.1–NR) mo	.26	2.6 (1.3–NR) mo	11.1 (9.4–NR) mo	12.6 (8.4–13.9) mo
Median OS (during osimertinib treatment)	13.5 (9.7–21.7) mo	36.5 (33.3–NS) mo	.0157	19.8 (11.7–NR) mo	36.5 (28.4–NR) mo	.089	9.3 (5.3–NA) mo	22.4 (15.6–NA) mo	15.4 (11.7–36.5) mo
Patients, n	38	9		21	8		11	12	17
Median survival after	5.0 (3.8–9.0) mo	14.5 (7.2–NR) mo	.008	7.7 (5.0–NR) mo	17.8 (12.2–NR) mo	.031	5.0 (3.0–NA) mo	10.8 (7.2–NA) mo	5.0 (3.1–16.5) mo

osimertinib progression							

Abbreviations: HR, hazard ratio; PFS, progression-free survival; NR, not reached; NA, not available; OS, overall survival.

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TABLE 3.

Performance Characteristics of OncoBEAM RAS CRC Kit, Idylla ctKRAS, and Idylla ctNRAS-BRAF Mutation Tests

	OncoBEAM RAS CRC Kit	Idyllact KRAS Mutation Test	Idylla ctNRAS-BRAF Mutation Test
CE Mark Approval Date	April 2016	November 2017	November 2017
Technology	BEAMing digital PCR	Idylla real-time PCR	Idylla real-time PCR
Amount of Plasma Needed, mL	3–4	1	1
Hands-on Time, min	Dedicated technician	< 1	< 1
Turnaround Time	1 wk	130 min	110 min
Gene Coverage	34 mutations: 16 *KRAS* and 18 *NRAS*	21 *KRAS*	18 *NRAS*
	Exon 2: codons 12 and 13	Exon 2: codons 12 and 13	Exon 2: codons 12 and 13
	Exon 3: codons 59 and 61	Exon 3: codons 59 and 61	Exon 3: codons 59 and 61
	Exon 4: codons 117 and 146	Exon 4: codons 117 and 146	Exon 4: codons 117 and 146
			5 *BRAF*
			Exon 15: codon 600
Sensitivity in Plasma, %	0.02–0.04	0.5	0.5
MAF Quantification	Quantitative	Semiquantitative	Semiquantitative
Clinical Validity Measures	OncoBEAM RAS CRC Kit, Instructions for	RASANC study (n = 198)[45,46]	
	Use OBMRASIVD[37]	Comparator test: plasma-based NGS analysis with sensitivity of 0.2%	
	Tissue comparator test: standard of	*KRAS*+ (n = 84)	
	care tissue testing	*NRAS*+ (n = 6)	
	Sensitivity: 92.6% (112/121)	*BRAF*+ (n = 13)	
	Specificity: 94.0% (110/117)	*RAS.* Sensitivity: 97.8% (90/92)	
	Concordance: 93.3% (222/238)	*RAS:* Specificity: 95.3% (101/106)	
	Grasselli et al, 2017 (n = 146)[38]	*RAS:* Concordance: 96.5% (191/198)	
	Tissue comparator test: PCR	*KRAS:* Concordance: 96%	
	Sensitivity: 89%	*NRAS:* Concordance: 100%	
	Specificity: 90%	*BRAF:* Concordance: 99.5%	
	Concordance: 89.7%		
	PPV: 84%		
	NPV: 93%		
	Vidal et al, 2017 (n = 115)[39]		

	OncoBEAM RAS CRC Kit	Idyllact KRAS Mutation Test	Idylla ctNRAS-BRAF Mutation Test
	Tissue comparator test: comparison: standard of care Sensitivity: 92.6% (112/121) Specificity: 94% (110/117) Concordance: 93.3% (222/238) Schmiegel et al, 2017 (n = 98)[40] Tissue comparator test: Sanger sequencing Sensitivity: 90.4% (47/52) Specificity: 93.5% (43/46) Concordance: 91.8% (90/98) Normanno et al, 2017 (n = 92)[43] Tissue comparator test: NGS Sensitivity: 69.7% Specificity: 83.1% Concordance: 78.3% (72/92) PPV: 69.7%; 83.1% Hahn et al, 2015 (n = 46)[42] Comparator tumor test: Sanger sequencing or pyrosequencing Sensitivity: 92% (23/25) Specificity: 100% (21/21) Concordance: 96% (44/46)		
Clinical Outcome	Grasselli et al, 2017 (n = 146)[38] Median OS: Tissue *KRAS*-wild-type: 39.1 mo Tissue *KRAS*-mutated: 28.7 mo Plasma *KRAS*-wild-type: 42.9 mo Plasma *KRAS* mutated: 27.8 mo	NR	

Abbreviations: CE, Conformité Européene; PCR, polymerase chain reaction; MAF, mutant allele frequency; PPV, positive predictive value; NPV, negative predictive value; NGS, next-generation sequencing; NR, not reported.

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TABLE 4.

Clinical Outcomes of BELLE-2 and BELLE-3 According to *PKI3CA* Mutation Status in Tumor and Plasma

	Overall Population		ctDNA *PIK3CA*+		ctDNA *PIK3CA*−		Tumor *PIK3CA*+		Tumor *PIK3CA*−	
Variable	**Buparlisib and Fulvestrant**	**Placebo and Fulvestrant**	**Buparlisib and Fulvestrant**	**Placebo and Fulvestrant**	**Buparlisib and Fulvestrant**	**Placebo and Fulvestrant**	**Buparlisib and Fulvestrant**	**Placebo and Fulvestrant**	**Buparlisib and Fulvestrant**	**Placebo and Fulvestrant**
BELLE-2 *										
Patients, n	576	571	87	113	199	188	21	19	123	120
ORR, % (95% CI)	11.8 (9.3–14.7)	7.7% (5.7–10.2)	18.4 (10.9–28.1)	3.5 (1.0–8.8)	11.6 (7.5–16.8)	10.6% (6.6–16.0)	NR	NR	NR	NR
Median PFS, mo (95% CI)	6.9 (6.8–7.8)	5.0 (4.0–5.2)	7.0 (5.0–10.0)	3.2 (2.0–5.1)	6.8 (4.7–8.5)	6.8 (4.7–8.6)	4.4 (1.6–NE)	10.7 (3.0–NE)	5.1 (3.5–8.5)	4.7 (3.3–8.5)
HR (95% CI)	0.78 (0.67–0.89)		0.58 (0.41–0.82)		1.02 (0.79–1.30)		1.18 (0.49–2.85)		0.98 (0.72–1.32)	
p Value	.00021 (1-sided)		.001 (1-sided)		.557 (1-sided)		NR		NR	
BELLE-3 **										
Patients, n	289	143	100	35	132	81	75	34	135	69
ORR, % (95% CI)	3.9 (2.8–4.2)	1.8 (1.5–2.8)	4.2 (2.8–6.7)	1.6 (1.4–2.8)	3.9 (2.8–4.3)	2.7 (1.5–3.6)	4.7 (2.9–6.7)	1.4 (1.4–2.2)	2.8 (1.9–3.4)	1.7 (1.4–2.9)
Median PFS, mo (95% CI)	0.67 (0.53–0.84)		0.46 (0.29–0.73)		0.73 (0.53–1.00)		0.39 (0.23–0.65)		0.81 (0.59–1.12)	
HR (95% CI)	.0030		.0031		.026		< .0001		.099	

* BELLE-2: Women who were estrogen receptor–positive or progesterone receptor–positive, human epidermal growth factor receptor 2–negative, aromatase inhibitor refractory. ctDNA *PKI3CA* determined by BEAMing polymerase chain reaction; tumor *PIK3CA* determined by Sanger sequencing.

** BELLE-3: Women who were estrogen receptor-positive or progesterone receptor–positive, human epidermal growth factor receptor 2–negative, aromatase inhibitor refractory, and refractory to mTOR inhibition. ctDNA *PIK3CA* determined by Inostics BEAMing polymerase chain reaction, covering exons 9 and 20; tumor *PIK3CA* determined by Cobas PIK3CA assay covering exons 7, 9, and 20.

Abbreviations: ORR, overall response rate; NR, not reported; NE, not evaluable; PFS, progression-free survival; HR, hazard ratio.

Table 5.

Effect of *ESR1* Mutations on Clinical Outcome in Patients Receiving Endocrine Therapies in SoFEA Trial

Variable	Exemestane		
	***ESR1* Wild-Type**	***ESR1*-Mutated**	**Hazard Ratio (95% CI); p Value**
Patients (n)	39	18	
Median PFS, mo (95% CI)	8.0 (3.0–11.5)	2.6 (2.4–6.2)	2.12 (1.18–3.81); .01
Median OS, mo (95% CI)	22.8 (17.6–32.4)	12.8 (5.7–27.0)	1.65 (0.81–3.38); .16
	***ESR1*-Mutated**		
	Fulvestrant Regimens	**Exemestane**	
Patients (n)	45	18	
Median PFS, mo (95% CI)	5.7 (3.0–8.5)	2.6 (2.4–6.2)	0.52 (CI, 0.30–0.92); .02
	***ESR1* Wild-Type**		
	Fulvestrant Regimens	**Exemestane**	
Patients (n)	59	39	
Median PFS, mo (95% CI)	8.0 (3.0–11.5)	5.4 (3.7–81.)	1.07 (0.68–1.67); .77

Abbreviation: PFS, progression-free survival.

Table 6.

Effect of *ESR1* Mutations on Clinical Outcome in Patients Receiving Endocrine Therapies in PALOMA3 Trial

	***ESR1*-Mutated**		
Variable	**Palbociclib + Fulvestrant**	**Placebo + Fulvestrant**	**Odds Ratio or Hazard Ratio (95% CI); p Value Statistical Consideration**
Patients, n	63	28	
ORR, %	17.5	3.6	5.94 * (0.78–270.5); .06
Median PFS, mo (95% CI)	9.4 (5.3–11.1)	3.6 (2.0–5.5)	0.43 ** (0.25–0.74); .002
	***ESR1* Wild Type**		
	Palbociclib + Fulvestrant	**Placebo + Fulvestrant**	
Patients, n	177	92	
ORR,%	21.5	14.1	1.66 * (0.81–3.61); .14
Median PFS, mo (95% CI)	9.5 (9.2–NE)	5.4 (3.5–7.4)	0.49 ** (0.35–0.70); ≤.001
	***ESR1* Status Available**		
	***ESR1* Wild Type**	***ESR1*-Mutated**	
Patients, n	261	91	
Median PFS (95% CI)	9.2 (7.4–10.9)	7.3 (3.7–9.1)	1.46 ** (1.06–2.02); .02

* Odds ratio (univariate analysis).

** Hazard ratio (univariate analysis).

Abbreviations: ORR, overall response rate; NE, not evaluable.

TABLE 7.

Androgen Receptor Variant 7 Detected by Quantitative Reverse Transcriptase Polymerase Chain Reaction in Circulating Tumor Cells as Both Primary and Acquired Resistance to Abiraterone and Enzalutamide

	Abiraterone (n = 31)		Enzalutamide (n = 31)	
	Pretreatment AR-V7–Positive	**Pretreatment AR-V7–Negative**	**Pretreatment AR-V7–Positive**	**Pretreatment AR-V7–Negative**
Patients, n	6	25	12	19
PSA ORR, % (95% CI)	0 (0–46)	68 (46–85)	0 (0–26)	53 (29–76)
p Value	.004		.004	
PSA PFS, mo (95% CI)	1.3 (0.9–NR)	5.3 (5.3–NR)	1.4 (0.9–NR)	6.0 (3.8–NR)
HR (95% CI)	16.1 (3.9–66.0)		7.4 (2.7–20.6)	
p Value	< .001		< .001	
PFS*, mo (95% CI)	2.3 (1.4–NR)	6.3 (6.3–NR)	2.1 (2.0–NR)	6.1 (4.7–NR)
HR (95% CI)	16.5 (3.3–82.9)		8.5 (2.8–25.5)	
p Value	< .001		< .001	
OS, mo	10.6	NR	5.5	NR
HR (95% CI)	12.7 (1.3–125.3)		6.9 (1.7–28.1)	
p Value	.006		.002	
	Pretreatment (n = 62)		**Pretreatment AR-V7–Negative (n = 42)**	
	AR-V7–Positive	**AR-V7–Negative**	**Post-Treatment AR-V7–Negative**	**Post-Treatment AR-V7–Positive**
Patients, n	18	44	36	6
ORR, % (95% CI)	0 (0–19)	61 (45–76)	68 (52–81)	17 (4–58)
p Value	< .0001		NA	
PSA PFS, mo (95% CI)	1.4 (0.9–2.6)	6.1 (5.3–NR)	6.1 (5.9–NA)	3.0 (2.2–NA)
HR (95% CI)	10.5 (4.7–23.6)		NA	
p Value	< .001		NA	
PFS*, mo (95% CI)	2.1 (1.9–3)	6.4 (6.1–NR)	6.5 (6.1–NA)	3.2 (3.1–NA)
HR (95% CI)	12.7 (5.1–31.9)		NA	
p Value	< .001		NA	
OS (95% CI)	9.2 (4.5–NR)	>11.9 (11.9–NR)	NA	NA

HR (95% CI)	8.3 (2.5–27.4)	NA
p Value	< .001	NA

*Clinical or radiographic; PSA response is defined as ≥50% decline in baseline PSA maintained for ≥4 weeks.

Abbreviations: AR-V7, androgen receptor-variant 7; PSA, prostate-specific antigen; ORR, overall response rate; PFS, progression-free survival; NR, not reached; HR, hazard ratio; OS, overall survival; NA, not available.

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TABLE 8.

Comparison of Various and Most Current Versions of Guardant360 and Foundation Medicine FoundationACT Liquid Biopsy Platforms (as of December 31, 2017)

	Guardant360 54-Gene Panel	Guardant360 68-Gene Panel	Guardant360 70-Gene Panel	Guardant360 73-Gene Panel*[71]	Foundation Medicine FoundationACT 62-Gene Panel*[72]
Commercial Launch Date	June 2014	August 2015	October 2015	November 2016	June 2016
Amount of Blood Needed	Two 10-mL Streck tubes	Two 10-mL Streck tubes	Two 10-mL Streck tubes	Two 10-mL Streck tubes	Two 10-mL tubes (Foundation ACT Specimen Collection Kit)
Minimal Amount of Cell-Free DNA	> 5 ng of cell-free DNA1	> 5 ng of cell-free DNA1	> 5 ng of cell-free DNA1	> 5 ng of cell-free DNA1	≥ 20 ng of cell-free DNA
Methods for Isolating ctDNA	QIAamp circulating nucleic acid kit (Qiagen)	QIAamp circulating nucleic acid kit (Qiagen)	QIAamp circulating nucleic acid kit (Qiagen)	QIAamp circulating nucleic acid kit (Qiagen)	
Types of Alterations Analyzed and No. of Genes	Complete exons (18 genes)	Complete exons (29 genes)	Complete exons (30 genes)	Complete exons (19 genes)**	Complete exons (27 genes)
	Critical exons (36 genes)	Critical exons (39 genes)	Critical exons (40 genes)	Critical exons (54 genes)	Critical exons (33 genes)
	CNV (3 genes)	Indel (1 gene)	Indels (3 genes)	Indels (23 genes)	Indel (27genes)
		CNV (16 genes)	CNV (18 genes)	CNV (18 genes)	Amplification (27 genes)
		Fusions (4 genes)	Fusions (6 genes)†	Fusions (6 genes)†	Fusions (6 genes)†‡ Promoter region only (TERT)
Sequencing Platform	HiSEquation 2500 (lllumina)l, 2	HiSeq 2500 or NextSEquation 500 (lllumina)	HiSeq 2500 or NextSEquation 500 (lllumina)	HiSeq 2500 or NextSEquation 500 (lllumina)	HiSeq 2500 or HiSeq 4000 (lllumina)
Depth of Coverage	?10,000× (average raw)			~15,000×(average raw)	7,503× (median, unique, actual depth of coverage reported)
Single-Nucleotide Variation	Varies between < 0.1% and 0.1%; depends on particular gene	Varies between < 0.1% and 0.1%; depends on particular gene	Varies between < 0.1% and 0.1%; depends on particular gene	≥ 0.04%; ≥ 0.25%, sensivitiy 100%, PPV 99.2%; 0.05–0.25% sensitivity 63.8%, PPV 96.3%	≥ 0.1% (0.1%–0.5%; sensitivity, 67.3%;PPV, 93.6%) ≥ 0.05% (sensitivity > 98.9%; PPV > 99.9%)
Indel	Varies between < 0.1% and 0.1%; depends on particular gene	Varies between < 0.1% and 0.1%; depends on particular gene	Varies between < 0.1% and 0.1%; depends on particular gene	≥ 0.02%; ≥ 0.02%, sensitivity 100%, PPV 98.2%; 0.05–0.2% sensitivity 67.8%, PPV 98.2%	≥ 1% (sensitivity, 99.9%; PPV, 98.8%)
CNV/Amplification	Varies between < 0.1% and 0.1%; depends on particular gene	Varies between < 0.1% and 0.1%; depends on particular gene	Varies between < 0.1% and 0.1%; depends on particular gene	≥ 2.12 copies	≥ 20% (sensitivity, 95.3%; PPV, 97.5%)

	Guardant360 54-Gene Panel	Guardant360 68-Gene Panel	Guardant360 70-Gene Panel	Guardant360 73-Gene Panel*[71]	Foundation Medicine FoundationACT 62-Gene Panel*[72]
Fusions/ Rearrangement		Varies between < 0.1% and 0.1%; depends on particular gene	Varies between < 0.1% and 0.1%; depends on particular gene	≥ 0.04%; ≥ 0.2% sensitivity 95%, PPV 100%; 0.05–0.2% sensitivity 83%, PPV 100%	≥ 1% (sensitivity > 99.9%; PPV, 98.8%) < 1% (sensitivity, 86.8%; PPV > 99%)
Turnaround Time for Results			Within 2 wk of receiving blood sample	Median of 7 calendar days of receiving blood sample	Within 2 wk of receiving blood sample
Difference in Gene Panel		ABL, CSF1R, ERBB4, FLT3, KDR (VEGFR2), PROC, RB1, and SMARCB1 genes were deleted and replaced with ARAF, ARID1A, BRCA1, BRCA2, CCND1, CCND2, CCNE1, CDK4, CDK6, CDKN2B, ESR1, GATA3, MAP2K1, MAP2K2, NF1, NTRK1, NTRK3, NFE2L2, RHEB, RHOA, RIT1, RAF1, and ROS1 genes. Copy number amplification calling increased from 3 to 16 genes (added AR, BRAF, CCND1, CCND2, CCNE1, CDK4, CDK6, FGFR1, FGFR2, KIT, KRAS, MYC, PDGFRA, PIK3CA, RAF1J Fusing calling added for 4 genes (ALK, ROS1, RET, NTRK1). Indel calling added for EGFR (exon 19/20)	RB1 and TSC1 genes were added; number of fusions detected increased from 4 to 6 (additional FGFR2 and FGFR3 fusion detection). Indel coverage for ERBB2 (exon 19/20) and MET exon 14 splice site variants wasadded	SRC and CDKN2B were deleted from panel and DDR2, MAP-K1(ERK2), MAPK3 (ERK1), MTOR, and NTRK3 were added. Copy number amplification calling was increased to 18 genes (CCND1, CCND2). Indel coverage expanded from 3 to 23 genes (ATM, APC, ARID1A,BRCA1, BRCA2, CDH1,CDKN2A, GATA3, KIT, MLH1,MTOR, NF1, PDGFRA, PTEN, RB1,SM AD4,ST-K11,TP53,TSC1,VHL)	NA
Cost	NA	NA	NA	$5,800 per test ($5,000 suggested contracted price per test)	$5,800 USD per test (contracted price may vary)
References (Selected Clinical Use of One Particular Platform)	73–76	75–79	64,75,76,80,81	82,83	84–87

*Although the difference in the number of genes is 11 between the current Guardant360 and Foundation Medicine FoundationACT, the actual difference in the number of genes analyzed is 31 because of unique genes analyzed by each liquid biopsy. Twenty-one genes were unique to Guardant360: AR, ARID1A, ATM, FBXW7, GATA3, HNF1A, MAPK3, MLH1, MPL, NFE2L2, NOTCH1, NTRK1, NTRK3, RB1, RHEB, THOA, RIT1, SMAD, STK11, TSC1, VHL. Ten genes were unique to Foundation Medicine FoundationACT: ABL, BTK, CRKL, ERRFI1, FOXL2, MDM2, MYD88, PDGFR-beta, PD-L2, VEGF-alpha.

**The 19 genes that have complete exon coverage in the Guardant 73-gene panel are as follows: APC, AR, BRAF, BRAC1, BRCA2, EGFR, ERBB2, HRAS, KRAS, NRAS, KIT, MAPK1(ERK2), MAPK2 (ERK1), MET, MYC, PIK3CA, RB1, TP53, STK11. Underlined genes have complete exon coverage by Foundation Medicine FoundationACT.

†Both Guardant and Foundation Medicine FoundationACT report four common fusions: ALK, ROS1, RET, and FGFR3 fusions. Guardant360 also reports additional NTRK1 and FGFR2 fusions, and Foundation Medicine FoundationACT reports additional EGFR and PDGFR-alpha fusions.

‡ROS1 rearrangement is the only ROS1 alteration detected by the current version of Foundation Medicine FoundationACT.

Abbreviations: PPV, positive predictive value; NA, not available; USD, US dollars.

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