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Characterization of anticancer drug resistance by Reverse Phase Protein Array: new targets and strategies

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Abstract

Introduction: Drug resistance is the main barrier to achieving cancer cures with medical therapy. Cancer drug resistance occurs, in part, due to adaptation of the tumor and microenvironment to therapeutic stress at a proteomic level. Reverse-phase protein arrays (RPPA) are well-suited to proteomic analysis of drug resistance due to high sample throughput, sensitive detection of phosphoproteins, and validation for a large number of critical cellular pathways.

Areas covered: This review summarizes contributions of RPPA to understanding and combating drug resistance. In particular, contributions of RPPA to understanding resistance to PARP inhibitors, BRAF inhibitors, immune checkpoint inhibitors, and breast cancer investigational therapies are discussed. Articles reviewed were identified by MEDLINE, Scopus, and Cochrane search for keywords “proteomics,” “reverse-phase protein array,” “drug resistance,” “PARP inhibitor,” “BRAF inhibitor,” “immune checkpoint inhibitor,” and “I-SPY” spanning October 1 1960 – October 1 2021.

Expert opinion: Precision oncology has thus far failed to convert the armament of targeted therapies into durable responses for most patients, highlighting that genetic sequencing alone is insufficient to guide therapy selection and overcome drug resistance. Combined genomic and proteomic analyses paired with creative drug combinations and dosing strategies hold promise for maturing precision oncology into an era of improved patient outcomes.

Keywords

Proteomics; reverse-phase protein array; RPPA; drug resistance; adaptive resistance; precision oncology; PARP inhibitor; BRAF inhibitor; immune checkpoint inhibitor

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1.0 Introduction

Drug resistance is the primary barrier to successful medical treatment of cancer in the modern era [1]. Over the past ten years, the United States Food and Drug Administration (FDA) has approved an average of twelve new oncology drugs each year, accounting for a quarter of all new drug approvals. With the exception of a few outstanding examples, however, these drugs do not cure patients of cancer, and unfortunately rarely extend survival by more than a year. Analyses of all new cancer drugs approved between 2002 and 2014 have found that these drugs increase overall survival on average between 2.1 and 3.4 months [2,3]. Despite initial response to anti-cancer drugs in many patients, the onset of drug resistance is nearly universal and often rapid, limiting the ability of both existing and newly-approved drugs to prolong length of life or improve quality of life for patients with cancer. While immune checkpoint blockade has resulted in prolonged overall survival in a subset of patients, less than half of patients across all diseases respond, and in many cases, the responses are of relatively short duration.

Anti-cancer drug resistance can be broadly characterized as innate, acquired, or adaptive. Innate, or primary, resistance to therapy exists before a tumor is exposed to a drug and can be due to mechanisms intrinsic to tumor cells and extrinsic contributions from the tumor environment [4]. Patients with high innate resistance to cancer therapy are “non-responders” to that therapy and ideally would be identified prior to treatment and either not receive a particular treatment, or receive that treatment only in combination with a sensitizing therapy. With acquired resistance, small, pre-existing subclonal populations are selected for and expand during and after drug treatment, leading to a stable state of drug resistance [5,6]. In some cases, mutations—such as estrogen receptor mutations in breast cancers treated with hormone manipulation—do not pre-exist, but rather are acquired while the patient is on therapy [7–10]. In contrast to patients with high innate resistance, patients with acquired resistance often have initial tumor response followed by tumor progression. Adaptive resistance is characterized by rapid signaling network alterations in response to therapeutic stress. Adaptive responses allow cells to withstand therapeutic stress until that stress has passed or compensatory genetic alterations accrue, and are increasingly being appreciated as stable and heritable independent of genetic mutation [11–13].

Importantly, for most patients, the interplay between multiple resistance mechanisms contributes to overall clinical response to a given therapy [14]. Rapid adaptive responses may allow cell populations, which are expected to be genetically sensitive to a treatment, time to accumulate heritable resistance-conferring mutations [15,16]. Conversely, cell populations expected to be genetically resistant to a treatment may revert to a sensitive phenotype under non-genetic influences. While acquired resistance is increasingly well-described by genetic analyses due to improving sequencing sensitivity and advances in single-cell technology, adaptive resistance is primarily mediated by the levels, locations, and functions of proteins. DNA and RNA analyses poorly predict protein abundance and activity, and as such genetic analyses have been less informative into adaptive mechanisms of resistance to cancer drugs [17]. Thus, proteomic analyses are a critical companion to genetic analyses in describing the landscape of tumor evasion of treatment.

Here, we describe unique contributions of proteomic studies to our understanding of mechanisms of resistance, candidate combination therapies, and when available, the clinical outcomes of these strategies. We focus specifically on contributions of reverse-phase protein arrays (RPPA), and four therapeutic areas of particular interest which have been studied extensively at the protein level: poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi), BRAF inhibitors (BRAFi), immune checkpoint inhibitors (ICI), and investigational therapies in early breast cancer adaptive trials. It is important to note that while this review focuses on the unique contributions of RPPA, other proteomic techniques – namely mass spectrometry, Nanostring digital spatial profiling, and single cell spatial proteomics including co-detection by indexing (CODEX), multiplex immunohistochemistry (mIHC), and cyclic immunofluorescence (Cyc-IF) – are poised to provide new insights (see section 7, "Frontiers in antibody-based proteomics for drug resistance") [18–28]. Finally, we speculate on the challenges that drug resistance has posed to the clinical successes of targeted therapies and the opportunities that exist for applying creative drug combination and dosing strategies to realize the promise of "precision oncology."

2.0 Methods

A database search including MEDLINE (PubMed), Scopus, and Cochrane (Wiley) was performed using combinations of the keywords "proteomics," "reverse-phase protein array," "drug resistance," "PARP inhibitor," "BRAF inhibitor," "immune checkpoint inhibitor," and "I-SPY" to identify articles for review. The search period spanned publication dates October 1 1960 – October 1 2021. Articles not available in English or without available English translation were excluded.

3.0 RPPA: A high-throughput antibody-based proteomic technology

RPPA is a high-throughput antibody-based proteomic technology that has enabled the semi-quantitative measurement of expression and post-translational modification of up to 500 proteins in parallel across a large number of samples. Using the RPPA approach, protein-containing samples are arrayed in serial dilution on nitrocellulose-coated glass slides. Example test specimens suitable for RPPA include serum, plasma, and lysate from cultured cells, fine need aspirates, fresh tissue, frozen tissue, formalin-fixed paraffin-embedded tissue, or laser capture microdissected specimens [29]. A variety of printers, including contact pin printers and non-contact microarrayers, are available for the production of sample microarrays [30]. Each glass slide can be printed with thousands of sample dots representing serial dilutions from hundreds of distinct protein samples. Printed protein microarrays are then incubated with validated high-affinity primary antibodies, spot signals are amplified with labeled secondary antibodies, spot intensities are quantified using a high-quality image acquisition device, and protein levels are determined using curve fitting software [31–38]. Because each printed array is probed with a single primary antibody, multiple identical arrays are typically printed and probed with a diversity of antibodies, which is enabled by the small amount of sample needed for each array [39]. Since its original implementation with protein lysates procured by laser capture microdissection, RPPA has been validated for a variety of protein substrates extracted through various methods; can be used with fluorescent, colorimetric, or chemiluminescent

signal amplification strategies; has been demonstrated with novel array substrata for various specific applications, including functionalized glass and hydrogels; and has evolved to incorporate refined spot analysis algorithms that improve data reliability [40–46]. Primary antibodies against more than 500 targets have been validated for RPPA to date, encompassing proteins critical to intracellular and extracellular signaling pathways including apoptosis, metabolism, cell cycle progression, replication stress, DNA damage repair, autophagy, invasion, and immune function. Notably, 20% of these antibodies are specific for protein post-translational modifications, and predominant among these post-translational modifications is protein phosphorylation [47].

3.1 Advantages and disadvantages of RPPA in comparison to other proteomic methods

RPPA offers distinct advantages and disadvantages as compared to other proteomic methods, including other antibody-based technologies and mass spectrometry. In comparison to low-throughput antibody-based methods such as Western blot, enzyme-linked immunosorbent assays, and immunohistochemistry (IHC), RPPA is resource and time efficient, requiring only picograms to fentograms of protein and allowing parallel preparation and interrogation of tens to thousands of protein samples with hundreds of antibody probes [36]. RPPA has demonstrated proficiency at characterizing proteins that appear at low abundance, which is particularly of benefit to the study of regulatory post-translational modifications of proteins that are present at low levels [36]. As compared to low-throughput methods, RPPA has been demonstrated to offer extremely high intra- and inter-array reproducibility, with inter-array variation shown at less than 15% [36]. Current standard practice for validation of antibodies used in RPPA involves demonstrating the specificity, selectivity, and reproducibility of the antibody by comparison to Western blot results, RNA levels generated by transcriptional profiling, and mass spectrometry protein levels in validation samples. Antibody quality can vary between antibody batches, necessitating time- and cost-intensive revalidation. Quality control processes with standard samples run in each experiment can identify antibodies that did not perform well in a single experiment, as well as where antibody quality has decreased in different batches. The need to thoroughly validate, and at times re-validate, RPPA antibodies represents a key intrinsic limitation of the technology [46].

In comparison to forward-phase protein arrays and bead-based arrays – which utilize printed arrays of antibodies or antibody-coated beads, respectively, probed with a single protein-containing sample – RPPA includes many samples on the same slide in serial dilution, which allows more direct comparison between samples and higher throughput of many protein samples. On the converse, because each array is probed with a single antibody, RPPA is relatively lower throughput with regard to different antibodies than forward-phase protein arrays [48]. The optimal choice of reverse- versus forward-phase array depends on the number and abundance of protein samples under investigation as well as the particular antibodies of interest to a given study.

As compared to mass spectrometry, RPPA is limited in its proteome coverage, and high quality antibody availability remains the major bottleneck to expansion of proteome coverage [33]. Mass spectrometry has the ability to unambiguously identify proteins including mutants, splice variants, and post-translational modified forms. Mass spectrometry

is better able to assess the full proteome (albeit current approaches do not cover the full proteome but only a subset of proteins, with particular limitations with regard to modified proteins and splice variants), while RPPA interrogates a limited and highly selected portion of the proteome for which high quality antibodies have been validated [49]. Nevertheless, the antibodies currently used for RPPA have been specifically chosen to cover pathways of high biological and clinical significance. Previous observations regarding the convergence of oncogenic changes on a limited number of biological pathways suggest that the critical pathways interrogated by RPPA would be expected to explain a significant proportion of tumor response and resistance to therapy [24,50]. Furthermore, the ability to sensitively and reliably characterize key sites of protein phosphorylation is central to RPPA's unique usefulness in interrogating signal network activity and in fact, RPPA has been demonstrated to be more sensitive than quantitative mass spectrometry in detecting a subset of regulatory phosphosites in tumor samples when directly compared [51]. The targeted approach offered by RPPA facilitates ease of data interpretation in comparison to the relatively greater quantity of data produced by mass spectrometry, which requires more extensive filtering for interpretability. On the contrary, the untargeted approach offers by mass spectrometry allows identification of unexpected proteins and pathways, which is a major advantage of mass spectrometry for new discovery. Because of the limited scope of RPPA with regards to available antibodies, RPPA may be more affected by pre-test hypotheses, while mass spectrometry may be seen as a better hypothesis-generating approach.

With regard to sample procurement, because RPPA analyzes protein contained in a single lysate prepared from a sample, cell-type heterogeneity within biopsy specimens poses a limitation as most biopsies contain a mix of tumor, stromal, immune, and normal organ cells, and the ratio of each can vary widely between biopsies. To overcome this limitation, an RPPA tumor content signature has been developed to help assess the proportion of tumor cells within each sample. Laser-aided microdissection of cell populations of interest can also be applied to overcome this limitation, although this comes with associated challenges with protein quantification and potential for tissue damage [52–54]. Warm and cold ischemia and tissue trauma during sample collection remain challenges to the analysis of clinical samples with all proteomic approaches; nevertheless, the low sample volume required and ability to capture sufficient tissue through microdissection is a major advantage of RPPA, increasing its usefulness to the clinical setting [50,55].

4.0 PARPi resistance

PARP proteins are nuclear enzymes that play a key role in DNA damage response. PARP detects and binds single-strand and double-strand DNA breaks and becomes activated upon DNA binding. PARP subsequently promotes repair of DNA lesions by recruitment and post-translational modification of repair machinery with poly(ADP-ribose) chains (PARylation) [56]. PARP also stabilizes replication forks during base excision repair [57]. PARP activity relies on cleavage of NAD+ as a source of ADP-ribose – a feature that has been exploited for the development of PARPi, the majority of which contain nicotinamide pharmacophores [58]. Monotherapy with PARPi has proven clinically useful in homologous recombination deficient (HRD) tumors, where a synthetic lethal combination of tumor cell genetic defects in DNA repair and PARP inhibition creates a therapeutic window; furthermore, activity in

tumors without HRD has led to approval of PARPi in maintenance therapy of all ovarian cancers [59,60]. PARPi both induce accumulation of single-strand breaks by inhibiting PARP activity and additionally have been shown to induce toxic double-strand breaks by trapping PARP on DNA [61].

As of now, four PARPi – olaparib, rucaparib, niraparib, and talazoparib – have been approved by the FDA. PARPi have been approved as first-line treatment for *BRCA*-mutant HER2-negative advanced or metastatic breast cancer, second-line treatment for HRD metastatic castration-resistant prostate cancer, and third-line treatment for *BRCA*-mutant advanced ovarian cancer. PARPi have additionally been approved as maintenance therapy for ovarian cancer independent of HRD status and for *BRCA*-mutant metastatic pancreatic cancer. Unfortunately, tumor response to PARPi is almost always transient, with median progression-free survival (PFS) in the clinical trials leading to these approvals ranging from 5 to 56 months [60,62–69]. Advances in sequencing at the single-cell and circulating cell-free DNA level have provided a new degree of detail about acquired resistance mechanisms in preclinical models and in patients [70–72], but genetic analyses have been less informative into adaptive resistance mechanisms to PARPi. RPPA has provided critical insight into adaptive PARPi resistance mechanisms, including activation of DNA damage checkpoints and activation of pro-survival pathways, leading to multiple completed and ongoing clinical trials (Table 1) [73–77].

4.1 DNA damage checkpoint activation in PARPi resistance

Cell cycle control checkpoints regulate progression through the eukaryotic cell cycle and allow DNA defects to be repaired before they are propagated to daughter cells. The S phase checkpoint slows replication, allowing time for nucleotide biosynthesis and replication fork stabilization, and the G2/M checkpoint allows time for repair of DNA double strand breaks. RPPA data from cancer cell lines demonstrate PARPi increases expression or phosphorylation of proteins involved in both the S and G2/M checkpoints, an adaptive resistance mechanism that could be targeted with combination therapy [74,75,78]. In particular, the well-described ATR/CHK1/WEE1 kinase cascade is active at both checkpoints and regulates cyclin-dependent cell cycle progression. Thus inhibitors of the ATR/CHK1/WEE1 kinase cascade promote mitotic entry despite unresolved DNA damage. RPPA data have demonstrated opposite effects of PARPi and WEE1 inhibitors (WEE1i) on mitotic gatekeepers including cdc2pY15 and FOXM1. Whereas PARPi increased cdc2pY15 and FOXM1 and induced G2/M arrest, WEE1i downregulated cdc2pY15, decreased FOXM1 and abrogated G2/M arrest. Addition of WEE1i to PARPi correspondingly bypassed G2/M arrest and induced mitotic catastrophe [75]. Concurrent therapy with PARPi and WEE1i is synergistic but poorly tolerated in mice, resulting in weight loss and anemia requiring cessation of therapy. Toxicity in human trials has limited dose and schedule and applicability. RPPA in animal model systems has demonstrated that proteomic response to PARPi persists over seven days post-treatment *in vitro* and *in vivo*, which suggested sequential therapy of the inhibitors may retain efficacy while limiting toxicity. Indeed, sequential treatment was less toxic to normal cells *in vitro* and retained efficacy in ovarian cancer patient-derived xenograft (PDX) models without substantial toxicity [75]. As a result of these preclinical studies, the Phase 1 Sequential Trial of Agents Against DNA Repair

(STAR) trial (NCT04197713) is now investigating the safety and tolerability of olaparib and the WEE1i adavosertib administered on a sequential schedule (5 days olaparib, 2 days off, 5 days adavosertib, 2 days off) in patients with HRD-deficient solid tumors. STAR has completed the dose expansion phase and is proceeding to an extended trial in PARPi-resistant ovarian cancer.

4.2 Pro-survival pathway signaling in PARPi resistance

Another adaptive resistance mechanism to PARPi identified by RPPA involves activation of pro-survival signaling pathways. PARPi has been demonstrated to activate the RAS/RAF/MAPK pathway, including an increase in pMEK, pMAPK, pPKC, pYB1, pBAD, and pS6, and decrease in FOXO3a, P27, and BIM, overall disfavoring apoptosis. Inverse changes were observed after treatment with a MEK inhibitor (MEKi), and combination PARPi plus MEKi demonstrated synergy in a subset of ovarian and other cancer cell lines, particularly in those with activating *KRAS* mutations. Cell lines cultured to acquire resistance to PARPi that have increased RAS/MAPK signaling, and in one case acquisition of an activating *KRAS* mutation, were re-sensitized to PARPi by the addition of MEKi [74]. These findings have informed the opening of the SOLAR trial (NCT03162627) to evaluate the combination of olaparib and the MEKi selumetinib in solid tumors with PARPi resistance and RAS pathway alterations.

A second pro-survival pathway activated by PARPi highlighted by RPPA is the PI3K/AKT/mTOR pathway [73]. In both *BRCA1* mutant and *BRCA*-proficient preclinical breast cancer models, dual PARPi/PI3K inhibition has been found to be synergistic [79,80]. Notably, in contrast to the *BRCA*-mutant or otherwise HRD setting, intrinsic or acquired homologous recombination proficiency generally imparts PARPi resistance. PI3K inhibition has been shown to downregulate BRCA1/2 and RAD51, thus inducing HRD in an otherwise homologous-recombination proficient background and imparting PARPi sensitivity [79,80]. Strikingly, while prior trials of olaparib monotherapy in patients with *BRCA*-wildtype platinum-resistant ovarian cancer have shown an overall response rate (ORR) around 5%, a recent Phase 1b trial including 26 platinum-resistant or refractory ovarian cancer patients treated with combination olaparib and the PI3K inhibitor (PI3Ki) alpelisib showed an ORR of 33% in patients with *BRCA*-wildtype platinum-resistant ovarian cancer [81]. A second recent Phase 1b trial assessing combination olaparib and the AKT inhibitor (AKTi) capivasertib in 38 patients with breast, endometrial, or ovarian cancer also showed response to combination therapy independent of both *BRCA* status and platinum resistance, including a 44% ORR in patients with endometrial cancer [82]. These results have led to more extensive multicenter trials to evaluate efficacy in greater depth.

As demonstrated in these cases, the application of RPPA in preclinical cancer models to define adaptive resistance mechanisms to PARPi has subsequently both been validated in and informed clinical studies. Other potential strategies to overcome PARPi resistance investigated with RPPA in the pre-clinical setting include combination therapy with BRD4 inhibitors in the homologous recombination proficient setting [76], dacarbazine in uveal melanoma [83], radiation therapy in hepatocellular carcinoma [84], and cisplatin and etoposide or irinotecan in small cell lung cancer [85].

4.3. PARPi window of opportunity trials

Proteomic studies have shed light on PARPi resistance mechanisms directly in the clinical setting in ovarian cancer window of opportunity trials [86]. In window trials, treatment-naïve patients are consented to receive an investigative drug in the period between their diagnosis and initiation of standard treatment. This allows new agents to be investigated directly in human patients whose tumor biology has not been altered by previous rounds of treatment. While the duration of therapy (typically a few days to a few weeks) is generally too short to confer therapeutic benefit, comparison of pre- and post-treatment biopsies can provide invaluable information about drug pharmacodynamics and early mechanisms of resistance that can inform subsequent new treatment strategies and trial design [87].

Among patients with ovarian cancer, laparoscopic staging of patients with presumed advanced-stage disease, in order to assess likelihood of tumor resection to no gross residual disease, is becoming increasingly common [88]. A standardized score from 0 to 14 is assigned based on laparoscopic findings, and patients with a score of less than 8 are generally recommended for primary debulking surgery, often at a later date, while patients with a score of 8 or more are recommended for neoadjuvant chemotherapy [89]. The ability to collect biopsy samples during laparoscopic staging creates an opportunity to collect pre-treatment samples from patients with ovarian cancer without excess morbidity from an additional procedure. Furthermore, the time (often a few days to weeks) between laparoscopic staging and primary surgery or initiation of neoadjuvant chemotherapy is a natural period for a window trial to be conducted. Thus both the ability to collect pre-treatment biopsies and the standard delay between diagnosis and treatment make advanced-stage ovarian cancer a ready setting for window trials.

One such window of opportunity trial has been conducted assessing adaptive responses to PARPi monotherapy in patients with advanced-stage ovarian cancer [86]. In this trial, three patients were treated with 7 to 14 days of talazoparib and pre- and post-treatment biopsies were characterized by DNA sequencing and RPPA. Unsupervised clustering of RPPA data from each biopsy showed a tendency towards clustering by treatment status. When post-treatment samples from each patient were normalized against each patient's own pre-treatment samples, post-treatment samples largely clustered by patient, suggesting patient-specific adaptive protein responses to PARPi that were consistent across biopsies taken from different anatomic locations. The response in patient 1 was characterized by increases in BCL-2, FAK, and proteins associated with the G2/M checkpoint, immune, and PI3K pathways. Data from patient 2 demonstrated an overlapping response, with increase in FAK and the G2/M and immune pathways but not PI3K. The response in patient 3 was markedly different than that in patient 1 or 2, with RAS/RAK/MAPK and immune pathway activation as well as increase in p16, phospho-S6 and FAK. Using model systems, it was then demonstrated that the degree of G2/M checkpoint activation by PARPi monotherapy predicted the synergy of combination PARPi plus an ATR inhibitor, suggesting that RPPA pathways analysis of patient samples could be used to predict which patients would benefit from treatment with multiple specific targeted agents. Importantly, this small trial provided evidence that adaptive resistance mechanisms in patients recapitulate mechanisms observed in cells lines, are conserved across lesions from the same patient, are heterogenous between

patients, and most importantly, are targetable with combination therapies [86]. We are currently performing similar window of opportunity trials with WEE1i in ovarian cancer (NCT02659241) and PARPi and MEKi in pancreatic cancer (NCT04005690) with the goal of identifying adaptive responses that could be targeted in subsequent trials (Table 2).

5.0 BRAFi resistance

The highly conserved RAS/RAF/MEK/ERK pathway (also called the MAPK pathway) transduces extracellular growth signals into intracellular responses including proliferation and resistance to apoptosis. RAF serine/threonine protein kinases (ARAF, BRAF, and CRAF) are key signaling kinases that rely on activation by RAS to, in turn, phosphorylate and activate MEK. Approximately half of cutaneous melanomas, and 6–8% of all solid tumors, harbor *BRAF* mutations, most frequently the BRAF(V600E) gain-of-function mutation which confers constitutive BRAF kinase activity [90–92].

Between 1975 and 2010, only one new drug, a synthetic form of interleukin-2, was approved as monotherapy for metastatic melanoma. The year 2011 ushered a decade of dramatic increase in FDA approvals for metastatic melanoma in two drug classes: kinase inhibitors targeting RAF and MEK, and immunotherapy [93]. Two large phase 3 trials of single-agent BRAFi in BRAF(V600E) mutant melanoma demonstrated an ORR of 48–50%, an impressive improvement from the standard therapy dacarbazine, which had an ORR of 5–6% in the control arm of these trials [94,95]. As of now, three BRAFi (vemurafenib, dabrafenib, and encorafenib) are approved as single-agent therapy for BRAF(V600) unresectable or metastatic melanoma, or as combination therapy with MEKi for BRAF(V600) metastatic melanoma, non-small cell lung carcinoma, and anaplastic thyroid cancer [96–98]. While the ORR to BRAFi in BRAF(V600E) mutant tumors has been remarkable, median PFS for patients on BRAFi monotherapy is on the order of 5 months due to rapid development of resistance [94,95]. Understanding and overcoming resistance to BRAFi is thus a major area of interest for improving patient outcomes in metastatic melanoma and other BRAF(V600) mutant cancers.

Investigation into BRAFi resistance has suggested both MAPK-dependent and MAPK-independent mechanisms. RPPA has proved a useful tool to characterize both of these mechanisms, given its unique ability to interrogate changes in protein levels and particularly in phosphorylation patterns. RPPA has been applied to human melanoma cell lines that have developed BRAFi resistance through prolonged culture in BRAFi-containing media and to xenograft melanoma models derived from these cells [99,100]; PDX and mouse melanoma models with BRAFi resistance developed through continuous treatment with BRAFi chow [101]; PDX models derived from patients who had progressed on BRAFi [102,103]; and biopsies from patients pre-treatment, on-treatment, and resistant to treatment with BRAFi [99,101]. RPPA analyses of these samples have identified distinct reproducible patterns of resistance including MAPK hyperactivation, PI3K pathway activation, and p21-activated kinase-mediated resistance, which are detailed below along with candidate strategies to overcome these mechanisms of resistance. Where relevant, clinical outcomes of these strategies are reviewed (Table 1). Other identified protein-mediated resistance mechanisms

not specifically discussed include histone methyltransferases [100], extracellular matrix remodeling collegenases [104], and topoisomerase 1 [105].

5.1 MAPK hyperactivation in BRAFi resistance

In one study, RPPA was applied to characterize a cross-species set of pre-treatment, post-treatment, and BRAFi-resistant biopsies from both human patients and mice [101]. On-treatment samples from both species showed downregulated proliferation and upregulated apoptosis. Drug resistant samples separated into three stable cross-species clusters, two of which featured MAPK pathway reactivation and the third of which did not. Of the two MAPK-reactivated clusters, one cluster featured pMEK and pERK levels restored to levels similar to pretreatment samples, while the other cluster featured pMEK and pERK levels increased above pretreatment levels. It has been demonstrated that in BRAFi-resistant melanomas with supraphysiologic MAPK signaling, the fitness advantage of MAPK pathway hyperactivation as a response to BRAFi becomes a fitness disadvantage when the pathway-dampening pressure of BRAFi is removed, as unchecked MAPK hyperactivation leads to cell cycle arrest and apoptosis [106–108]. Consistent with these observations, an intermittent BRAFi dosing schedule has been shown to delay tumor progression in PDX melanoma models [106]. In a case report of a single patient with concurrent BRAF(V600K) melanoma and NRAS(G12R) chronic myelomonocytic leukemia, vemurafenib treatment induced melanoma shrinkage but promoted leukemia proliferation (due to paradoxical MAPK activation by BRAFi in the setting of activated RAS, a known phenomenon [109]), such that the patient required an intermittent dosing schedule to optimize outcomes for his comorbid malignancies [110,111]. After 49 weeks of intermittent dosing with single agent vemurafenib, the patient achieved a near complete melanoma response [111]. Despite this encouraging case, a recent phase 2 trial of continuous versus intermittent (3-week-off, 5-week-on) combined BRAFi plus MEKi in BRAF(V600) melanoma failed to show a benefit of intermittent dosing, instead demonstrating significantly increased PFS in patients treated with continuous as compared to intermittent dosing (9 vs 5.5 months), with comparable toxicity and overall survival [112]. Additional studies may be required to assess whether there exists a subset of patients for whom intermittent dosing is a beneficial strategy and if this subset can be discerned *a priori*.

5.2 PI3K pathway activation in BRAFi resistance

RPPA analysis of 12 PDX from BRAFi-progressed melanoma patients found MAPK reactivation in 9/12 PDXs despite ongoing exposure to BRAFi [102]. An increase in AKT phosphorylation was seen in all three PDX that did not feature MAPK reactivation as well as three of the nine PDX with MAPK reactivation, suggesting PI3K pathway activation as a compensatory resistance mechanism. A selected PDX with increased AKT phosphorylation was expanded to a cohort of mice treated with either MAPK pathway inhibitors (MAPKi) with encorafenib plus the MEKi binimetinib or with the ERK inhibitor VX-11e; the PI3Ki buparlisib; or combination MAPKi/PI3Ki. Combination MAPKi/PI3Ki resulted in significantly decreased tumor growth as compared to MAPKi alone [102]. The phase 2 LOGIC2 trial evaluating the benefit of adding a third agent to encorafenib/binimetinib at the time of progression included buparlisib as a third agent, as well as the CDK4/6 inhibitor (CDK4/6i) LEE011 and the c-Met inhibitor (c-METi) INC280. Assignment to a third agent

was based on genetic tumor evaluation. Ultimately, 2 patients received triple therapy with the addition of PI3Ki, 25 patients with the addition of CDK4/6i, and 3 patients with the addition of c-METi. Disappointingly, ORR with the addition of a third agent was low in all groups at 0%, 5.3%, and 0% respectively [113]. A phase 1b study of continuous-dosed binimetinib plus buparlisib in advanced solid tumor patients with RAS/RAF alterations found a 12% partial response rate in advanced ovarian cancer patients; however, dosing was significantly limited by toxicity [114]. While combination MAPKi/PI3Ki may still be a promising strategy to overcome MAPKi resistance, more work is needed to define optimal dosing and patient selection. Alternatively, the mTORC1 inhibitor rapamycin and the combination PI3Ki/mTOR inhibitor BEZ235 have been applied in BRAFi/MEKi resistant cells with resultant down-regulation in cell cycle and anti-apoptotic proteins and induction of cell death, suggesting a new strategy to restore sensitivity to BRAFi [115]. This approach was subsequently proven safe and well-tolerated in a phase 1 trial of twenty patients with advanced cancer (NCT01596140) [116].

5.3 p21-activated kinases (PAKs) in BRAFi resistance

A third RPPA study of BRAFi resistance in melanoma found distinct mechanisms of resistance to BRAFi versus to combination BRAFi/MEKi, both mediated by p21-activated kinases (PAKs) [99]. In BRAFi-resistant cells, PAKs directly phosphorylated CRAF and MEK to reactivate MAPK signaling, which was reversible by the PAK inhibitor (PAKi) PF-3758309. In combination therapy-resistant cells, PAKi did not effect ERK activation, but did inhibit ERK downstream targets and mTOR pathway activity. PAK activation in these cells promoted JNK activity and decreased expression of pro-apoptotic proteins. Broadly, these findings suggest different therapeutic strategies may be necessary to overcome drug resistance to BRAFi monotherapy and to BRAFi/MEKi combination therapy.

6.0 ICI resistance

The last decade has seen a revolution in cancer therapy through the approval of ICIs. The first ICI, approved in 2011, was ipilimumab, a monoclonal antibody that binds and blocks CTLA4 on cytotoxic T lymphocytes (CTLs). CTLA4 is upregulated on CTLs in early activation, binds CD80 and CD86 on antigen-presenting cells, and induces CTL cell cycle arrest, thereby limiting sustained T cell activation despite presentation of an antigenic stimulus. Ipilimumab amplifies T-cell mediated antitumor responses and has been approved as monotherapy in melanoma [117]. A second class of ICIs target the checkpoint regulator PD-1, expressed on multiple immune cell lineages including a high proportion of tumor infiltrating lymphocytes, or its ligand PD-L1, expressed on peripheral tissues. The PD-1/PD-L1 interaction dampens T cell activation through immune cell tyrosine phosphatase activation [118]. Three monoclonal antibodies targeting PD-1 (nivolumab, pembrolizumab, and cemiplimb) and three monoclonal antibodies targeting PD-L1 (avelumab, durvalumab, and atezolizumab) have now been approved across 19 cancer types [119,120]. Impressively, as of 2018, an estimated 44% of US cancer patients were eligible for treatment with an ICI [121]. However, both innate and acquired resistance are major challenges to the clinical success of ICIs. Among US cancer patients, only 12.5% are estimated to respond to ICIs [121]. Furthermore, while some durable responses have been demonstrated among

responders, including 20% of melanoma patients showing continued response to ipilimumab at 5–10 years and 33% demonstrating response to pembrolizumab at 3 years, the majority of patients who initially respond develop resistance [122]. In addition, ICIs can mediate significant short and long-term toxicity including patient death, resulting in the need to identify patients likely to demonstrate toxicity and patients likely to demonstrate efficacy to warrant the potential toxicity [123,124].

6.1 ARID1A in ICI resistance

Studies have demonstrated that mismatch repair (MMR)-deficient tumors face increased neoantigen loads and are rendered susceptible to ICIs [125,126]. The MMR protein MSH2 was identified in a proteomic screen designed to identify binding partners of the chromatin remodeling complex SWI/SNF subunit ARID1A, which features common loss of function mutations in cancer [127]. This study subsequently found that ARID1A loss, as demonstrated at a protein level by RPPA, led to MMR deficiency, increased neoantigen load, and ICI sensitivity, thus highlighting a new potential predictor of clinical ICI response. A follow-up study aimed at identifying rational combination therapy to potentiate the effect of ICI in the ARID1A-deficienct setting analyzed RPPA data for proteins with differential expression in ARID1A-proficient and -deficient tumors and found increased expression of the DNA damage checkpoint kinase Chk2 in ARID1A-deficient tumors [128]. Paired with previous observation that ATR/Chk1 signaling is dampened in *ARID1A*-mutant tumors, the authors hypothesized that ARID1A-deficient tumors rely on ATM/Chk2 to respond to DNA damage [129]. Correspondingly, they found inhibition of ATM/Chk2 axis in ARID1A-mutant tumors led to buildup of cytosolic DNA, STING DNA-sensing pathway activation, and increased immune response [128]. Consistent with this, they observed that tumors with loss of both ARIDA1 and ATM had increased numbers of tumor infiltrating lymphocytes (TILs), and that tumors with comutations of *ARID1A* and *ATM* or *ARID1A* and *CHK2* had an overall more favorable prognosis than those with *ARID1A* mutations alone. Co-treatment of ovarian tumor-bearing mice with ICI and an ATM inhibitor led to significantly decreased tumor burden and increased survival specifically in mice bearing ARID1A-deficient tumors, suggesting a new strategy for combination therapy in the ARID1A-deficient clinical setting.

6.2 Exosomal PD-L1 in ICI resistance

A novel mechanism of tumor resistance to ICI involving PD-L1 positive exosomes, described below, was identified in an RPPA analysis of exosomes shed from melanoma cells [130]. The sensitivity of RPPA combined with the low input allowed identification of high levels of PD-L1 in exosomes from metastatic melanoma cells lines, which was confirmed in exosomes collected from melanoma PDX models and human melanoma patients. PD-L1 positive exosomes inhibited CD8 T cells, decreased TILs, and promoted tumor growth. Furthermore, analysis of fold change in exosomal PD-L1 pre- and post-treatment with ICI stratified responders from non-responders, with responders showing greater increase in exosomal PD-L1 at 3–6 weeks after initial treatment than non-responders, perhaps reflecting T cell reinvigoration with ICI in responders. This study suggested exosomal analysis as a potential clinical predictor of response to ICI which, attractively, could be developed as a blood-based biomarker without the need for tumor biopsy.

7.0 RPPA in adaptive clinical trials: I-SPY2

Only one-third of phase III clinical trials in oncology resulted in drug approvals in 2000–2015, despite the immense resource-intensiveness of these trials [131]. As a response to the spiraling costs and low success rates of oncology clinical trials, adaptive trial designs have been developed in an effort to increase the informativeness of clinical trials [132]. Adaptive trials are defined as those which allow pre-specified modifications to trial protocols on the basis of accrued data [133]. An exemplary adaptive trial, I-SPY2 TRIAL (Investigation of Serial Studies to Predict Your Therapeutic Response with Imaging and Molecular Analysis 2, NCT01042379) is a multicenter phase 2 trial assessing a diversity of drugs from different pharmaceutical companies in the neoadjuvant setting in patients with high risk stage II or III breast cancer [134–136]. I-SPY2 compares the efficacy of the novel agents in combination with standard chemotherapy to the efficacy of standard chemotherapy alone using pathologic complete response (pCR) at the time of surgery as primary endpoint [136]. Randomization probabilities are weighted on the accrued results for a given therapy and biomarker subtype, with better-performing therapies in a particular subtype given a greater probability of randomization to promote faster data accumulation for promising therapies [132]. A drug regimen “graduates” from the phase 2 trial to a phase 3 if and when its predicted probability of success in a randomized phase 3 trial of 300 patients who carry that regimen’s corresponding biomarker signature is greater than or equal to 85% [133]. Biomarkers assessed in I-SPY 2 include established biomarkers (those that are used clinically or are FDA-approved and were used to generate the 10 biomarker signatures from which drugs could graduate), qualifying biomarkers (those with promising predictive capability but not yet FDA approved), and exploratory biomarkers (those with compelling preliminary data included for discovery) [136]. RPPA-based measurements have been extensively evaluated as qualifying biomarkers in I-SPY2 [137–143].

7.1 Established biomarkers versus RPPA analysis of HER2 status in I-SPY1 and I-SPY2

Interestingly, in the I-SPY1 precursor trial to I-SPY2, RPPA was compared to established biomarkers for HER2 status (IHC and fluoresce in situ hybridization, FISH) and was found to extend sensitivity in detection of patients who may benefit from HER2-targeted therapy [144]. Laser-capture microdissected samples from patient core and surgical biopsies were analyzed by RPPA alongside standard IHC and FISH. While RPPA-based measurement of HER2 protein was highly concordant with IHC and FISH measurements, surprisingly, RPPA additionally revealed a subset of $HER2^-$ tumors that displayed similar levels of phospho-HER2 and activated downstream signaling proteins (SHC, FAK, and STAT5) as $HER2^+$ tumors, apparently coincident with EGFR and HER3 activation, thus suggesting a subset of patients who might benefit from HER2-targeted therapies but would be excluded from treatment on the basis of IHC or FISH [144]. In I-SPY2, pre-treatment HER2 levels and HER2 signaling activation was strongly predictive of response to HER2-targeted combination therapies (ado-trastuzumab emtansine plus pertuzumab, and paclitaxel, trastuzumab and pertuzumab) within $HER2^+$ tumors [141].

7.2 Biomarkers of investigational drug response in I-SPY2

Within I-SPY2, RPPA has also provided insight into patient subsets who might benefit from the addition of an AKTi [138,139] or the pan-erythroblastic oncogene B (ERBB) inhibitor neratinib [140] to overcome resistance to standard chemotherapy (doxorubicin and cyclophosphamide) in the neoadjuvant setting. Not specifically discussed here, RPPA has been additionally applied in analysis of response to veliparib plus carboplatin [142] and the type I insulin-like growth factor receptor (IGF-1R) inhibitor ganitubmab [143].

As described in previous sections, the PI3K/AKT/mTOR pathway is an area of strong therapeutic interest given its pro-survival role in mediating resistance to standard chemotherapy. The AKTi MK2206 graduated in I-SPY2 in the HER2+, HR-, and HR-/HER2+ signatures but not in the triple-negative (TN) signature. Notably, AKT-mTOR-HER pathway phospho-protein signatures, as determined by RPPA, were more predictive of AKTi response than AKT-mTOR-HER gene expression signatures or total protein levels [138]. RPPA analysis of pretreatment laser-capture microdissected specimen showed that AKTi response was predicted by higher levels of AKT substrate phospho-proteins including phospho-mTOR and phospho-TSC2 in the HER2+ background, but conversely by lower levels of AKT substrate phospho-proteins in the TN background [138].

In the case of neratinib in I-SPY2, while all 12 highly-selected *HER* pathway DNA biomarkers were either low prevalence or nonpredictive of neratinib response and only one of 10 *HER* pathway gene expression biomarkers (*STMN1*) was associated with neratinib response, six of 18 RPPA markers (three EGFR phosphosites, total HER2, phospho-HER2 and phospho-SHC) were associated with neratinib response [140]. Furthermore, EGFR Y1173 phosphorylation remained a significant independent predictor of response after adjustment for HR/HER2 status [140]. The pCR rate in the phospho-EGFR/phosph-HER2-high TN background was an impressive 82% with the addition of neratinib, compared to 36% in the control arm – a noteworthy advance for a patient population with historically few targeted therapeutic options [140].

8. Frontiers in antibody-based proteomics for drug resistance: spatial-oriented single cell proteomics

The therapeutic settings described above exemplify the contributions of RPPA to understanding adaptive drug resistance. A new wave of advances in understanding adaptive resistance is expected to be ushered by novel spatial-oriented single cell proteomic technologies such as imaging mass cytometry (IMC), multiplexed ion beam imaging (MIBI), tissue-based Cyc-IF, mIHC, multiplex immunofluorescence (mIF), and CODEX. Spatial mass spectrometry techniques like IMC and MIBI previously have been applied to describe the spatial distribution of cell surface metabolites and lipids, and increasingly are being applied to spatial proteomics [26,27]. With Cyc-IF, traditional low-plex fluorescence IHC is iteratively preformed, quenched, and repeated on a single sample. Images are then assembled into a composite high-dimensional image representing the spatial distribution of up to 60 proteins in a single tissue [28]. CODEX also uses iterative antibody binding but employs DNA barcodes and fluorescent dNTP analogs to visualize binding events

[25]. As with RPPA, these technologies offer the ability to analyze protein abundance and post-translational modifications with high sensitivity, with the added benefit of single cell sensitivity and retention of spatial information. The relative locations of tumor cells, immune cells, and the surrounding tumor microenvironment is preserved, and proteomic observations can be correlated with these spatial relationships. Cyc-IF in particular has been demonstrated as a feasible platform to analyze pre- and on-treatment biopsies from a series of patients undergoing olaparib monotherapy for metastatic TN breast cancer [145,146] and has been applied to predict response to combination niraparib and pembrolizumab in ovarian cancer in samples from a phase 1/2 trial [147]. mIHC has been applied to formalin-fixed paraffin-embedded head and neck squamous cell carcinomas and pancreatic ductal adenocarcinomas to characterize the relationship between immune context, disease progression and therapy response [148,149]. mIF has been applied alongside RPPA in the I-SPY2 platform described above with the conclusion that MHC-II expression on at least 5% of tumor cells is predictive of ICI benefit when added to standard neoadjuvant chemotherapy in HER2-negative breast cancer [137]. The use of spatial-oriented proteomics along with genomic studies and traditional bulk proteomics is expected to further expand our understanding of adaptive resistance and our ability to predict effective combination therapies to overcome drug resistance in patients.

9. Conclusion

RPPA is a powerful tool uniquely suited to investigate signaling network states in tumor cells. Rapid, protein-mediated signaling network alterations allow tumor cells to withstand therapeutic stress and contribute to drug resistance in the short-term, allowing continued cell division and emergence of stable resistant tumor populations in the long-term. Previous studies have shown that tumor cell adaptations converge on a limited number of critical pathways. These pathways (1) are within the scope of interrogation by RPPA and (2) can often be targeted using existing or in-development drugs. RPPA has proven useful in identifying which pathways mediate adaptive responses to drugs including PARPi, BRAFi, and ICI and is informing which combination therapies could interdict these adaptive responses. Some of these observations have been translated to the clinical setting with impressive success – such as the combination of olaparib and alpelisib in patients with platinum-resistant ovarian cancer, and olaparib and capivasertib in patients with breast, ovarian, or endometrial cancer – while other strategies have found limited success due to toxicity or poor response, underscoring that more work is needed to define optimal dosing strategies and patient selection. The year 2020 saw the highest number of new cancer drug approvals ever in the US, with 18 new drugs reaching the market. As the pharmacologic toolkit to combat cancer expands, the landscape of drug resistance grows, but the possibilities for combination therapies and novel dosing strategies grow as well. RPPA, as well as newer spatial-oriented proteomic methods, will continue to be critical contributors to our ability to make use of available drugs to extinguish drug resistance.

10. Expert Opinion

In the past, precision oncology has been fueled by the prospect that identification of a driver mutation in a patient’s cancer would guide paring that patient with a molecular targeted

therapy specially matched to their mutation, and that attacking each tumor's particular Achilles' heel would provide a durable response, if not a cure. The early success of imatinib, a BCR-ABL tyrosine kinase inhibitor specifically paired to the constitutive activation of BCR-ABL in chronic myeloid leukemia which saw 98% complete response in its inaugural phase 1 trial [150], spurred hope for this notion of precision oncology. With a handful of additional exceptional examples, imatinib has been an outlier, and most targeted therapies, even when prescribed on the basis of a matched molecular marker, are not cures and indeed durable responses are rare. A meta-analysis of 346 phase 1 oncology trials has found that biomarker-based patient selection is associated with improved response rate (30.6% vs 4.9% of patients not selected on the basis of a biomarker) and improved PFS (5.7 vs 2.95 months); but these humble figures, and in particular the short PFS as a result of emergence of resistance—even with biomarker-based selection—underscore how much is left to be achieved in precision oncology [151].

The studies highlighted in this review show that (1) drug resistance responses have both genetic and non-genetic, protein-level components, (2) resistance responses are diverse across different patients, and encouragingly, (3) preliminary data suggest responses may be relatively consistent within tumor biopsies collected from diverse anatomic sites within the same patient and (4) targetable with available drugs [86]. The degree to which observations (3) and (4) hold true across a diversity of tumor types has not yet been fully described and represents a potential major limitation on the ability to achieve complete responses to medical therapy in patients with metastatic disease. Further, intratumoral heterogeneity of adaptive responses has not been adequately characterized at the single cell level and could limit the utility of targeting adaptive responses. Nevertheless, the remarkable responses in some of the combination trials mentioned above targeting adaptive responses suggests that intratumoral heterogeneity of adaptive responses may not be a rate-limiting hurdle.

Despite the challenges of tumor heterogeneity and complex resistance mechanisms, an immense opportunity to help patients live longer and with high quality of life lies within the enormous landscape of drug combinations – encompassing drug sequencing, dosing, and timing – that has yet to be explored. The efficacy of combined PARPi and WEE1i in ovarian cancer PDX models, for example, is an encouraging example of a proteomics-informed approach to eliminating the DNA damage checkpoint as a means by which tumor cells can withstand PARPi. The clinical outlook of this strategy is promising for patients with both HRD and homologous recombination competent tumors, with results of the STAR trial forthcoming. Where clinical translation of anti-resistance strategies has been less successful, dose toxicities and poor efficacy have been major pitfalls. The sequential dose strategy in the STAR trial, previously shown to retain efficacy and ameliorate toxicity in PDX models, holds promise that creative dosing strategies can be applied to overcome dose-limiting toxicities for a number of combination therapies, as the territory of possible dosing schedules is vast. With respect to poor efficacy, a number of exceptional patient responders to particular drugs or drug combinations – such as the described patient with near-complete melanoma response after 49 weeks of intermittent dosing with single-agent vemurafenib – give hope that increasingly nuanced pre-treatment analysis of patient tumors will provide ever-better guidance for patient selection for therapy.

While the disappointments of precision oncology have suggested that DNA sequencing of a set of target genes, often from a single biopsy, is not sufficient to turn the armament of targeted therapies into durable responses, we are hopeful that advances in our understanding of the complexity of drug resistance at the proteomic level and particularly how these evolve under therapeutic pressure will improve these efforts. Importantly, the unique insights provided by antibody-based proteomics described herein highlight that proteomic data are a critical addition to DNA and RNA-based studies in understanding resistance mechanisms in patients. As noted above, the emergence of single cell spatially resolved proteomics and their applications to understanding adaptive resistance, intratumoral heterogeneity and the interaction of the diverse cells in the tumor ecosystem has the potential to open a new era in precision oncology. It will be critical to deploy single cell proteomic approaches to fully understand the effects of therapy on the individual cells in the tumor and the tumor ecosystem as they evolve under therapeutic pressure. The convergence of oncogenic alterations and adaptive responses into a limited number of critical pathways, many of which are already druggable, suggests that though the dimensionality of the problem is high, it is finite. Continued work with combined genomic and proteomic approaches is expected to help mature precision oncology from a promise into a reality.

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Abbreviations

RPPA	Reverse-phase protein array
FDA	Food and Drug Administration
PARP	Poly (ADP-ribose) polymerase
PARPi	PARP inhibitors
BRAFi	BRAF inhibitors
ICI	Immune checkpoint inhibitors
IHC	immunohistochemistry
HRD	homologous recombination deficient
PFS	progression-free survival

WEE1i	WEE1 inhibitors
PDX	patient-derived xenograft
AKTi	AKT inhibitors
MEKi	MEK inhibitors
ORR	overall response rate
MAPKi	MAPK pathway inhibitors
PI3Ki	PI3K inhibitors
CDK4/6i	CDK4/6 inhibitors
c-METi	c-MET inhibitors
PAK	p21-activated kinase
PAKi	PAK inhibitors
CTL	cytotoxic T lymphocyte
MMR	mismatch repair
TIL	tumor infiltrating lymphocyte
pCR	pathologic complete response
FISH	fluoresce in situ hybridization
TN	triple-negative
IMC	imaging mass cytometry
MIBI	multiplexed ion beam imaging
Cyc-IF	cyclic immunofluorescence
mIHC	multiplex immunohistochemistry
mIF	multiplex immunofluorescence
CODEX	co-detection by indexing

References

1. Vasan N, Baselga J, Hyman DM. A view on drug resistance in cancer. Nature. 2019;575(7782):299–309. [PubMed: 31723286]
2. Salas-Vega S, Iliopoulos O, Mossialos E. Assessment of overall survival, quality of life, and safety benefits associated with new cancer medicines. JAMA Oncol. 2017;3(3).
3. Fojo T, Mailankody S, Lo A. Unintended consequences of expensive cancer therapeutics—the pursuit of marginal indications and a me-too mentality that stifles innovation and creativity. JAMA Otolaryngol Head Neck. 2014;140(12).

4. Sharma P, Hu-Lieskovan S, Wargo JA, et al. Primary, adaptive, and acquired resistance to cancer immunotherapy. Cell. 2017 Feb 9;168(4):707–723. [PubMed: 28187290] * This article is the first of two studies describing epigenetic variability as a means by which cells escape drug treatment
5. Diaz LA Jr., Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012 Jun 28;486(7404):537–40. [PubMed: 22722843]
6. Bozic I, Nowak MA. Timing and heterogeneity of mutations associated with drug resistance in metastatic cancers. Proc Natl Acad Sci U S A. 2014 Nov 11;111(45):15964–8. [PubMed: 25349424]
7. Jeselsohn R, Yelensky R, Buchwalter G, et al. Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. Clin Cancer Res. 2014 Apr 1;20(7):1757–1767. [PubMed: 24398047]
8. Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, et al. D538G mutation in estrogen receptor-alpha: A novel mechanism for acquired endocrine resistance in breast cancer. Cancer Res. 2013 Dec 1;73(23):6856–64. [PubMed: 24217577]
9. Robinson DR, Wu YM, Vats P, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet. 2013 Dec;45(12):1446–51. [PubMed: 24185510]
10. Toy W, Shen Y, Won H, et al. ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet. 2013 Dec;45(12):1439–45. [PubMed: 24185512]
11. Marine J-C, Dawson S-J, Dawson MA. Non-genetic mechanisms of therapeutic resistance in cancer. Nat Rev Cancer. 2020;20(12):743–756. [PubMed: 33033407]
12. Vander Velde R, Yoon N, Marusyk V, et al. Resistance to targeted therapies as a multifactorial, gradual adaptation to inhibitor specific selective pressures. Nat Comm. 2020;11(1).
13. Labrie M, Brugge JS, Mills GB, et al. Therapy resistance: opportunities created by adaptive responses to targeted therapies in cancer. Nat Rev Cancer. 2022 Mar 9.
14. Salgia R, Kulkarni P. The genetic/non-genetic duality of drug 'resistance' in cancer. Trends Cancer. 2018 Feb;4(2):110–118. [PubMed: 29458961]
15. Shaffer SM, Dunagin MC, Torborg SR, et al. Rare cell variability and drug-induced reprogramming as a mode of cancer drug resistance. Nature. 2017 Jun 15;546(7658):431–435. [PubMed: 28607484] * This article is the second of two studies describing epigenetic variability as a means by which cells escape drug treatment
16. Sharma SV, Lee DY, Li B, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell. 2010 Apr 2;141(1):69–80. [PubMed: 20371346]
17. Akbani R, Ng PK, Werner HM, et al. A pan-cancer proteomic perspective on The Cancer Genome Atlas. Nat Commun. 2014 May 29;5:3887. [PubMed: 24871328] ** This article describes the largest application of RPPA to date, covering 3,467 patient samples and 11 tumor types
18. Merritt CR, Ong GT, Church SE, et al. Multiplex digital spatial profiling of proteins and RNA in fixed tissue. Nat Biotechnol. 2020 May;38(5):586–599. [PubMed: 32393914]
19. Zawistowski JS, Graves LM, Johnson GL. Assessing adaptation of the cancer kinome in response to targeted therapies. Biochem Soc Trans. 2014 Aug;42(4):765–9. [PubMed: 25109955]
20. Wu P, Heins ZJ, Muller JT, et al. Integration and analysis of CPTAC proteomics data in the context of cancer genomics in the cBioPortal. Mol Cell Proteomics. 2019 Sep;18(9):1893–1898. [PubMed: 31308250]
21. Cooper MJ, Cox NJ, Zimmerman EI, et al. Application of multiplexed kinase inhibitor beads to study kinome adaptations in drug-resistant leukemia. PLoS One. 2013;8(6):e66755.
22. Duncan JS, Whittle MC, Nakamura K, et al. Dynamic reprogramming of the kinome in response to targeted MEK inhibition in triple-negative breast cancer. Cell. 2012 Apr 13;149(2):307–21. [PubMed: 22500798]
23. Mertins P, Mani DR, Ruggles KV, et al. Proteogenomics connects somatic mutations to signalling in breast cancer. Nature. 2016 Jun 2;534(7605):55–62. [PubMed: 27251275]
24. Zhang H, Liu T, Zhang Z, et al. Integrated proteogenomic characterization of human high-grade serous ovarian cancer. Cell. 2016 Jul 28;166(3):755–765. [PubMed: 27372738]
25. Goltsev Y, Samusik N, Kennedy-Darling J, et al. Deep profiling of mouse splenic architecture with CODEX multiplexed imaging. Cell. 2018 Aug 9;174(4):968–981 e15. [PubMed: 30078711]

26. Baharlou H, Canete NP, Cunningham AL, et al. Mass cytometry imaging for the study of human diseases—applications and data analysis strategies. Frontiers Immunol. 2019;10.
27. Piehowski PD, Zhu Y, Bramer LM, et al. Automated mass spectrometry imaging of over 2000 proteins from tissue sections at 100-μm spatial resolution. Nat Commun. 2020;11(1).
28. Lin JR, Izar B, Wang S, et al. Highly multiplexed immunofluorescence imaging of human tissues and tumors using t-CyCIF and conventional optical microscopes. Elife. 2018 Jul 11;7.
29. Becker KF. Lysate preparation for reverse phase protein arrays. Adv Exp Med Biol. 2019;1188:21–30. [PubMed: 31820381]
30. McWilliam I, Chong Kwan M, Hall D. Inkjet printing for the production of protein microarrays. Methods Mol Biol. 2011;785:345–61. [PubMed: 21901611]
31. Liotta LA, Espina V, Mehta AI, et al. Protein microarrays: Meeting analytical challenges for clinical applications. Cancer Cell. 2003;3(4):317–325. [PubMed: 12726858]
32. Nielsen UB, Cardone MH, Sinskey AJ, et al. Profiling receptor tyrosine kinase activation by using Ab microarrays. Proc Natl Acad Sci U S A. 2003;100(16):9330–9335. [PubMed: 12876202]
33. Sheehan KM, Calvert VS, Kay EW, et al. Use of reverse phase protein microarrays and reference standard development for molecular network analysis of metastatic ovarian carcinoma. Mol Cell Proteomics. 2005;4(4):346–355. [PubMed: 15671044]
34. Guo H, Liu W, Ju Z, et al. An efficient procedure for protein extraction from formalin-fixed, paraffin-embedded tissues for reverse phase protein arrays. Proteome Sci. 2012 Sep 24;10(1):56.
35. Paweletz CP, Charboneau L, Bichsel VE, et al. Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. Oncogene. 2001;20(16):1981–1989. [PubMed: 11360182]
36. Tibes R, Qiu Y, Lu Y, et al. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. Mol Cancer Ther. 2006 Oct;5(10):2512–21. [PubMed: 17041095]
37. Zhang L, Wei Q, Mao L, et al. Serial dilution curve: a new method for analysis of reverse phase protein array data. Bioinformatics. 2009 Mar 1;25(5):650–4. [PubMed: 19176552]
38. Petricoin E, Wulfkuhle J, Howard M, et al. RPPA: Origins, transition to a validated clinical research tool, and next generations of the technology. Adv Exp Med Biol. 2019;1188:1–19. [PubMed: 31820380] ** This article is the first chapter in an excellent reference book discussing RPPA technical consderations, analytical principles, and research applications
39. Neeley ES, Kornblau SM, Coombes KR, et al. Variable slope normalization of reverse phase protein arrays. Bioinformatics. 2009 Jun 1;25(11):1384–9. [PubMed: 19336447]
40. Gallagher RI, Silvestri A, Petricoin EF, 3rd, et al. Reverse phase protein microarrays: fluorometric and colorimetric detection. Methods Mol Biol. 2011;723:275–301. [PubMed: 21370072]
41. Chiechi A, Mueller C, Boehm KM, et al. Improved data normalization methods for reverse phase protein microarray analysis of complex biological samples. Biotechniques. 2012 Sep;0(0):1–7.
42. Chiechi A.Normalization of reverse phase protein microarray data: choosing the best normalization analyte. Methods Mol Biol. 2016;1362:77–89. [PubMed: 26519170]
43. Ju Z, Liu W, Roebuck PL, et al. Development of a robust classifier for quality control of reverse-phase protein arrays. Bioinformatics. 2015 Mar 15;31(6):912–8. [PubMed: 25380958]
44. Hu J, He X, Baggerly KA, et al. Non-parametric quantification of protein lysate arrays. Bioinformatics. 2007 Aug 1;23(15):1986–94. [PubMed: 17599930]
45. Paweletz CP, Charboneau L, Bichsel VE, et al. Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. Oncogene. 2001 Apr 12;20(16):1981–9. [PubMed: 11360182]
46. Akbani R, Becker KF, Carragher N, et al. Realizing the promise of reverse phase protein arrays for clinical, translational, and basic research: a workshop report: the RPPA (reverse phase protein array) society. Mol Cell Proteomics. 2014 Jul;13(7):1625–43. [PubMed: 24777629]
47. Petricoin EF, Bichsel VE, Calvert VS, et al. Mapping molecular networks using proteomics: A vision for patient-tailored combination therapy. J Clin Oncol. 2005;23(15):3614–3621. [PubMed: 15908672]

48. Haab BB. Antibody arrays in cancer research. Mol Cell Proteomics. 2005;4(4):377–383. [PubMed: 15671041]
49. Lu Y, Ling S, Hegde AM, et al. Using reverse-phase protein arrays as pharmacodynamic assays for functional proteomics, biomarker discovery, and drug development in cancer. Semin Oncol. 2016 Aug;43(4):476–83. [PubMed: 27663479]
50. Labrie M, Fang Y, Kendsersky ND, et al. Using reverse phase protein array (RPPA) to identify and target adaptive resistance. Adv Exp Med Biol. 2019;1188:251–266. [PubMed: 31820393]
51. Mertins P, Yang F, Liu T, et al. Ischemia in tumors induces early and sustained phosphorylation changes in stress kinase pathways but does not affect global protein levels. Mol Cell Proteomics. 2014 Jul;13(7):1690–704. [PubMed: 24719451]
52. Mueller C, Davis JB, Liotta LA. Combining the “sibling technologies” of laser capture microdissection and reverse phase protein microarrays. Adv Exp Med Biol. 2019;1188:95–111. [PubMed: 31820385]
53. Wulfkuhle JD, Aquino JA, Calvert VS, et al. Signal pathway profiling of ovarian cancer from human tissue specimens using reverse-phase protein microarrays. Proteomics. 2003;3(11):2085–2090. [PubMed: 14595806]
54. Mueller C, deCarvalho AC, Mikkelsen T, et al. Glioblastoma cell enrichment is critical for analysis of phosphorylated drug targets and proteomic-genomic correlations. Cancer Res. 2014 Feb 1;74(3):818–28. [PubMed: 24346432]
55. Zhao W, Li J, Akbani R, et al. Credentialing individual samples for proteogenomic analysis. Mol Cell Proteomics. 2018 Aug;17(8):1515–1530. [PubMed: 29716986]
56. Pilie PG, Tang C, Mills GB, et al. State-of-the-art strategies for targeting the DNA damage response in cancer. Nat Rev Clin Oncol. 2019 Feb;16(2):81–104. [PubMed: 30356138]
57. Ronson GE, Piberger AL, Higgs MR, et al. PARP1 and PARP2 stabilise replication forks at base excision repair intermediates through Fbh1-dependent Rad51 regulation. Nat Commun. 2018 Feb 21;9(1):746. [PubMed: 29467415]
58. Curtin NJ. PARP inhibitors for cancer therapy. Expert Rev Mol Med. 2005;7(4):1–20.
59. Lord CJ, Ashworth A. PARP inhibitors: Synthetic lethality in the clinic. Science. 2017 Mar 17;355(6330):1152–1158. [PubMed: 28302823]
60. Del Campo JM, Matulonis UA, Malander S, et al. Niraparib maintenance therapy in patients with recurrent ovarian cancer after a partial response to the last platinum-based chemotherapy in the ENGOT-OV16/NOVA trial. J Clin Oncol. 2019 Nov 10;37(32):2968–2973. [PubMed: 31173551]
61. Pommier Y, O’Connor MJ, de Bono J. Laying a trap to kill cancer cells: PARP inhibitors and their mechanisms of action. Sci Transl Med. 2016 Oct 26;8(362):362ps17.
62. Pujade-Lauraine E, Ledermann JA, Selle F, et al. Olaparib tablets as maintenance therapy in patients with platinum-sensitive, relapsed ovarian cancer and a BRCA1/2 mutation (SOLO2/ENGOT-Ov21): a double-blind, randomised, placebo-controlled, phase 3 trial. Lancet Oncol. 2017 Sep;18(9):1274–1284. [PubMed: 28754483]
63. Litton J, Rugo H, Ettl J, et al. EMBRACA: A phase 3 trial comparing talazoparib, an oral PARP inhibitor, to physician’s choice of therapy in patients with advanced breast cancer and a germline BRCA mutation. Cancer Res. 2018;78.
64. Banerjee S, Moore KN, Colombo N, et al. Maintenance olaparib for patients with newly diagnosed, advanced ovarian cancer and a BRCA mutation: 5-year follow-up from SOLO1. Ann Oncol. 2020;31:S613.
65. Golan T, Hammel P, Reni M, et al. Maintenance olaparib for germline BRCA-mutated metastatic pancreatic cancer. N Engl J Med. 2019 Jul 25;381(4):317–327. [PubMed: 31157963]
66. Ledermann J, Harter P, Gourley C, et al. Olaparib maintenance therapy in platinum-sensitive relapsed ovarian cancer. N Engl J Med. 2012 Apr 12;366(15):1382–92. [PubMed: 22452356]
67. Coleman RL, Oza AM, Lorusso D, et al. Rucaparib maintenance treatment for recurrent ovarian carcinoma after response to platinum therapy (ARIEL3): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet. 2017 Oct 28;390(10106):1949–1961. [PubMed: 28916367]
68. Robson M, Im SA, Senkus E, et al. Olaparib for metastatic breast cancer in patients with a germline BRCA mutation. N Engl J Med. 2017 Aug 10;377(6):523–533. [PubMed: 28578601]

69. Swisher EM, Lin KK, Oza AM, et al. Rucaparib in relapsed, platinum-sensitive high-grade ovarian carcinoma (ARIEL2 Part 1): an international, multicentre, open-label, phase 2 trial. Lancet Oncol. 2017 Jan;18(1):75–87. [PubMed: 27908594]
70. Farkkila A, Rodriguez A, Oikkonen J, et al. Heterogeneity and clonal evolution of acquired PARP inhibitor resistance in TP53- and BRCA1-deficient Cells. Cancer Res. 2021 May 15;81(10):2774–2787. [PubMed: 33514515]
71. Lin KK, Harrell MI, Oza AM, et al. BRCA reversion mutations in circulating tumor DNA predict primary and acquired resistance to the PARP inhibitor rucaparib in high-grade ovarian carcinoma. Cancer Discov. 2019 Feb;9(2):210–219. [PubMed: 30425037]
72. Mehta AK, Cheney EM, Hartl CA, et al. Targeting immunosuppressive macrophages overcomes PARP inhibitor resistance in BRCA1-associated triple-negative breast cancer. Nat Cancer. 2021 Jan;2(1):66–82. [PubMed: 33738458]
73. Sun C, Fang Y, Labrie M, et al. Systems approach to rational combination therapy: PARP inhibitors. Biochem Soc Trans. 2020 Jun 30;48(3):1101–1108. [PubMed: 32379297]
74. Sun C, Fang Y, Yin J, et al. Rational combination therapy with PARP and MEK inhibitors capitalizes on therapeutic liabilities in RAS mutant cancers. Sci Transl Med. 2017 May 31;9(392).
75. Fang Y, McGrail DJ, Sun C, et al. Sequential therapy with PARP and WEE1 inhibitors minimizes toxicity while maintaining efficacy. Cancer Cell. 2019 Jun 10;35(6):851–867 e7. [PubMed: 31185210]
76. Sun C, Yin J, Fang Y, et al. BRD4 inhibition is synthetic lethal with PARP inhibitors through the induction of homologous recombination deficiency. Cancer Cell. 2018 Mar 12;33(3):401–416 e8. [PubMed: 29533782]
77. Labrie M, Kendsersky ND, Ma H, et al. Proteomics advances for precision therapy in ovarian cancer. Expert Rev Proteomics. 2019 Oct;16(10):841–850. [PubMed: 31512530]
78. Kim H, Xu H, George E, et al. Combining PARP with ATR inhibition overcomes PARP inhibitor and platinum resistance in ovarian cancer models. Nat Commun. 2020 Jul 24;11(1):3726. [PubMed: 32709856]
79. Juvekar A, Burga LN, Hu H, et al. Combining a PI3K inhibitor with a PARP inhibitor provides an effective therapy for BRCA1-related breast cancer. Cancer Discov. 2012 Nov;2(11):1048–63. [PubMed: 22915751]
80. Ibrahim YH, Garcia-Garcia C, Serra V, et al. PI3K inhibition impairs BRCA1/2 expression and sensitizes BRCA-proficient triple-negative breast cancer to PARP inhibition. Cancer Discov. 2012 Nov;2(11):1036–47. [PubMed: 22915752]
81. Konstantinopoulos PA, Barry WT, Birrer M, et al. Olaparib and alpha-specific PI3K inhibitor alpelisib for patients with epithelial ovarian cancer: a dose-escalation and dose-expansion phase 1b trial. Lancet Oncol. 2019 Apr;20(4):570–580. [PubMed: 30880072]
82. Westin SN, Labrie M, Litton JK, et al. Phase Ib dose expansion and translational analyses of olaparib in combination with capivasertib in recurrent endometrial, triple-negative breast, and ovarian cancer. Clin Cancer Res. 2021 Sep 13.
83. de Koning L, Decaudin D, El Botty R, et al. PARP inhibition increases the response to chemotherapy in uveal melanoma. Cancers. 2019 May 29;11(6).
84. Gerossier L, Dubois A, Paturel A, et al. PARP inhibitors and radiation potentiate liver cell death in vitro. Do hepatocellular carcinomas have an achilles' heel? Clin Res Hepatol Gastroenterol. 2020 Nov 9:101553.
85. Byers LA, Wang J, Nilsson MB, et al. Proteomic profiling identifies dysregulated pathways in small cell lung cancer and novel therapeutic targets including PARP1. Cancer Discov. 2012 Sep;2(9):798–811. [PubMed: 22961666]
86. Labrie M, Kim TB, Ju Z, et al. Adaptive responses in a PARP inhibitor window of opportunity trial illustrate limited functional interlesional heterogeneity and potential combination therapy options. Oncotarget. 2019 May 28;10(37):3533–3546. [PubMed: 31191824]
87. Schmitz S, Duhoux F, Machiels JP. Window of opportunity studies: Do they fulfil our expectations? Cancer Treat Rev. 2016 Feb;43:50–7. [PubMed: 26827692]

88. Fleming ND, Nick AM, Coleman RL, et al. Laparoscopic surgical algorithm to triage the timing of tumor reductive surgery in advanced ovarian cancer. Obstet Gynecol. 2018 Sep;132(3):545–554. [PubMed: 30095787]

89. Fagotti A, Ferrandina G, Fanfani F, et al. A laparoscopy-based score to predict surgical outcome in patients with advanced ovarian carcinoma: a pilot study. Ann Surg Oncol. 2006 Aug;13(8):1156–61. [PubMed: 16791447]

90. Cancer Genome Atlas N.Genomic classification of cutaneous melanoma. Cell. 2015 Jun 18;161(7):1681–96. [PubMed: 26091043]

91. Bollag G, Tsai J, Zhang J, et al. Vemurafenib: the first drug approved for BRAF-mutant cancer. Nat Rev Drug Discov. 2012 Nov;11(11):873–86. [PubMed: 23060265]

92. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature. 2002 Jun 27;417(6892):949–54. [PubMed: 12068308]

93. Jenkins RW, Fisher DE. Treatment of advanced melanoma in 2020 and beyond. J Invest Dermatol. 2021 Jan;141(1):23–31. [PubMed: 32268150]

94. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med. 2011 Jun 30;364(26):2507–16. [PubMed: 21639808]

95. Hauschild A, Grob JJ, Demidov LV, et al. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. Lancet. 2012 Jul 28;380(9839):358–65. [PubMed: 22735384]

96. Dummer R, Ascierto PA, Gogas HJ, et al. Encorafenib plus binimetinib versus vemurafenib or encorafenib in patients with BRAF-mutant melanoma (COLUMBUS): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol. 2018 May;19(5):603–615. [PubMed: 29573941]

97. Long GV, Stroyakovskiy D, Gogas H, et al. Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. N Engl J Med. 2014 Nov 13;371(20):1877–88. [PubMed: 25265492]

98. Robert C, Karaszewska B, Schachter J, et al. Improved overall survival in melanoma with combined dabrafenib and trametinib. N Engl J Med. 2015 Jan 1;372(1):30–9. [PubMed: 25399551]

99. Lu H, Liu S, Zhang G, et al. PAK signalling drives acquired drug resistance to MAPK inhibitors in BRAF-mutant melanomas. Nature. 2017 Oct 5;550(7674):133–136. [PubMed: 28953887]

100. Emran AA, Marzese DM, Menon DR, et al. Distinct histone modifications denote early stress-induced drug tolerance in cancer. Oncotarget. 2018 Feb 2;9(9):8206–8222. [PubMed: 29492189]

101. Kwong LN, Boland GM, Frederick DT, et al. Co-clinical assessment identifies patterns of BRAF inhibitor resistance in melanoma. J Clin Invest. 2015 Apr;125(4):1459–70. [PubMed: 25705882]

102. Krepler C, Xiao M, Sproesser K, et al. Personalized preclinical trials in BRAF inhibitor-resistant patient-derived xenograft models identify second-line combination therapies. Clin Cancer Res. 2016 Apr 1;22(7):1592–602. [PubMed: 26673799]

103. Krepler C, Sproesser K, Brafford P, et al. A comprehensive patient-derived xenograft collection representing the heterogeneity of melanoma. Cell Rep. 2017 Nov 14;21(7):1953–1967. [PubMed: 29141225]

104. Marusak C, Thakur V, Li Y, et al. Targeting extracellular matrix remodeling restores BRAF inhibitor sensitivity in BRAFi-resistant melanoma. Clin Cancer Res. 2020 Nov 15;26(22):6039–6050. [PubMed: 32820016]

105. Oliveira EA, Chauhan J, Silva JRD, et al. TOP1 modulation during melanoma progression and in adaptative resistance to BRAF and MEK inhibitors. Pharmacol Res. 2021 Nov;173:105911. [PubMed: 34560251]

106. Das Thakur M, Salangsang F, Landman AS, et al. Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance. Nature. 2013 Feb 14;494(7436):251–5. [PubMed: 23302800]

107. Petti C, Molla A, Vegetti C, et al. Coexpression of NRASQ61R and BRAFV600E in human melanoma cells activates senescence and increases susceptibility to cell-mediated cytotoxicity. Cancer Res. 2006 Jul 1;66(13):6503–11. [PubMed: 16818621]

108. Moriceau G, Hugo W, Hong A, et al. Tunable-combinatorial mechanisms of acquired resistance limit the efficacy of BRAF/MEK cotargeting but result in melanoma drug addiction. Cancer Cell. 2015 Feb 9;27(2):240–56. [PubMed: 25600339]
109. Heidorn SJ, Milagre C, Whittaker S, et al. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell. 2010 Jan 22;140(2):209–21. [PubMed: 20141835]
110. Callahan MK, Rampal R, Harding JJ, et al. Progression of RAS-mutant leukemia during RAF inhibitor treatment. N Engl J Med. 2012 Dec 13;367(24):2316–21. [PubMed: 23134356]
111. Abdel-Wahab O, Klimek VM, Gaskell AA, et al. Efficacy of intermittent combined RAF and MEK inhibition in a patient with concurrent BRAF- and NRAS-mutant malignancies. Cancer Discov. 2014 May;4(5):538–45. [PubMed: 24589925]
112. Algazi AP, Othus M, Daud AI, et al. Continuous versus intermittent BRAF and MEK inhibition in patients with BRAF-mutated melanoma: a randomized phase 2 trial. Nat Med. 2020 Oct;26(10):1564–1568. [PubMed: 33020646]
113. Dummer R, Sandhu SK, Miller WH, et al. A phase II, multicenter study of encorafenib/binimetinib followed by a rational triple-combination after progression in patients with advanced BRAF V600-mutated melanoma (LOGIC2). J Clin Oncol. 2020;38(15_suppl):10022–10022.
114. Bardia A, Gounder M, Rodon J, et al. Phase Ib study of combination therapy with MEK inhibitor binimetinib and phosphatidylinositol 3-kinase inhibitor buparlisib in patients with advanced solid tumors with RAS/RAF alterations. Oncologist. 2020 Jan;25(1):e160–e169. [PubMed: 31395751]
115. Wang B, Zhang W, Zhang G, et al. Targeting mTOR signaling overcomes acquired resistance to combined BRAF and MEK inhibition in BRAF-mutant melanoma. Oncogene. 2021;40(37):5590–5599. [PubMed: 34304249]
116. Subbiah V, Sen S, Hess KR, et al. Phase I study of the BRAF inhibitor vemurafenib in combination with the mammalian target of rapamycin inhibitor everolimus in patients with BRAF-mutated malignancies. JCO Precision Oncology. 2018 (2):1–12.
117. 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):711–723. [PubMed: 20525992]
118. Kubli SP, Berger T, Araujo DV, et al. Beyond immune checkpoint blockade: emerging immunological strategies. Nat Rev Drug Discov. 2021.
119. Twomey JD, Zhang B. Cancer immunotherapy update: FDA-approved checkpoint inhibitors and companion diagnostics. AAPS J. 2021 Mar 7;23(2):39. [PubMed: 33677681]
120. Vaddepally RK, Kharel P, Pandey R, et al. Review of indications of FDA-approved immune checkpoint inhibitors per NCCN guidelines with the level of evidence. Cancers. 2020;12(3).
121. Haslam A, Prasad V. Estimation of the percentage of US patients with cancer who are eligible for and respond to checkpoint inhibitor immunotherapy drugs. JAMA Netw Open. 2019;2(5).
122. Jenkins RW, Barbie DA, Flaherty KT. Mechanisms of resistance to immune checkpoint inhibitors. Br J Cancer. 2018;118(1):9–16. [PubMed: 29319049]
123. Jing Y, Liu J, Ye Y, et al. Multi-omics prediction of immune-related adverse events during checkpoint immunotherapy. Nat Commun. 2020 Oct 2;11(1):4946. [PubMed: 33009409]
124. Berti A, Bortolotti R, Dipasquale M, et al. Meta-analysis of immune-related adverse events in phase 3 clinical trials assessing immune checkpoint inhibitors for lung cancer. Crit Rev Oncol Hematol. 2021 Jun;162:103351.
125. Le DT, Uram JN, Wang H, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med. 2015;372(26):2509–2520. [PubMed: 26028255]
126. Le DT, Durham JN, Smith KN, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science. 2017;357(6349):409–413. [PubMed: 28596308]
127. Shen J, Ju Z, Zhao W, et al. ARID1A deficiency promotes mutability and potentiates therapeutic antitumor immunity unleashed by immune checkpoint blockade. Nat Med. 2018 May;24(5):556–562. [PubMed: 29736026]
128. Wang L, Yang L, Wang C, et al. Inhibition of the ATM/Chk2 axis promotes cGAS/STING signaling in ARID1A-deficient tumors. J Clin Invest. 2020 Nov 2;130(11):5951–5966. [PubMed: 33016929]
129. Shen J, Peng Y, Wei L, et al. ARID1A deficiency impairs the DNA damage checkpoint and sensitizes cells to PARP inhibitors. Cancer Discov. 2015;5(7):752–767. [PubMed: 26069190]

130. Chen G, Huang AC, Zhang W, et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. Nature. 2018 Aug;560(7718):382–386. [PubMed: 30089911]
131. Wong CH, Siah KW, Lo AW. Estimation of clinical trial success rates and related parameters. Biostatistics. 2019 Apr 1;20(2):273–286.
132. Berry DA. Adaptive clinical trials in oncology. Nat Rev Clin Oncol. 2011;9(4):199–207. [PubMed: 22064459]
133. Berry DA. The Brave New World of clinical cancer research: Adaptive biomarker-driven trials integrating clinical practice with clinical research. Mol Oncol. 2015;9(5):951–959. [PubMed: 25888066]
134. Park JW, Liu MC, Yee D, et al. Adaptive randomization of neratinib in early breast cancer. N Engl J Med. 2016;375(1):11–22. [PubMed: 27406346]
135. Rugo HS, Olopade OI, DeMichele A, et al. Adaptive randomization of veliparib–carboplatin treatment in breast cancer. N Engl J Med. 2016;375(1):23–34. [PubMed: 27406347]
136. Barker AD, Sigman CC, Kelloff GJ, et al. I-SPY 2: An adaptive breast cancer trial design in the setting of neoadjuvant chemotherapy. Clin Pharmacol Ther. 2009;86(1):97–100. [PubMed: 19440188]
137. Gonzalez-Ericsson PI, Wulfkhule JD, Gallagher RI, et al. Tumor-specific Major Histocompatibility-II expression predicts benefit to anti-PD-1/L1 therapy in patients with HER2-negative primary breast cancer. Clin Cancer Res. 2021 Jul 27.
138. Wolf DM, Yau C, Wulfkuhle J, et al. Mechanism of action biomarkers predicting response to AKT inhibition in the I-SPY 2 breast cancer trial. NPJ Breast Cancer. 2020;6:48. [PubMed: 33083527]
139. Wulfkuhle JD, Wolf DM, Yau C, et al. Phosphorylation of AKT kinase substrates to predict response to the AKT inhibitor MK2206 in the I-SPY 2 trial in both HER2- and HER2+ patients. J Clin Oncol. 2018;36(15_suppl):12099–12099.
140. Wulfkuhle JD, Yau C, Wolf DM, et al. Evaluation of the HER/PI3K/AKT family signaling network as a predictive biomarker of pathologic complete response for patients with breast cancer treated with neratinib in the I-SPY 2 TRIAL. JCO Precision Oncol. 2018 (2):1–20.
141. Clark AS, Yau C, Wolf DM, et al. Neoadjuvant T-DM1/pertuzumab and paclitaxel/trastuzumab/pertuzumab for HER2+ breast cancer in the adaptively randomized I-SPY2 trial. Nat Comm. 2021;12(1).
142. Wolf DM, Yau C, Sanil A, et al. DNA repair deficiency biomarkers and the 70-gene ultra-high risk signature as predictors of veliparib/carboplatin response in the I-SPY 2 breast cancer trial. NPJ Breast Cancer. 2017;3(1).
143. Yee D, Isaacs C, Wolf DM, et al. Ganitumab and metformin plus standard neoadjuvant therapy in stage 2/3 breast cancer. NPJ Breast Cancer. 2021;7(1).
144. Wulfkuhle JD, Berg D, Wolff C, et al. Molecular analysis of HER2 signaling in human breast cancer by functional protein pathway activation mapping. Clin Cancer Res. 2012;18(23):6426–6435. [PubMed: 23045247]
145. Li A, Labrie M, Vuky J, et al. Feasibility of real-time serial comprehensive tumor analytics: Pilot study of olaparib and durvalumab in metastatic triple negative breast cancer (mTNBC). J Clin Oncol. 2020;38(15_suppl):e13092–e13092.
146. Labrie M, Li A, Creason A, et al. Multi-omics analysis of serial samples from metastatic TNBC patients on PARP inhibitor monotherapy provide insight into rational PARP inhibitor therapy combinations. medRxiv. 2020.
147. Farkkila A, Gulhan DC, Casado J, et al. Immunogenomic profiling determines responses to combined PARP and PD-1 inhibition in ovarian cancer. Nat Commun. 2020 Mar 19;11(1):1459. [PubMed: 32193378]
148. Banik G, Betts CB, Liudahl SM, et al. High-dimensional multiplexed immunohistochemical characterization of immune contexture in human cancers. Methods Enzymol. 2020;635:1–20. [PubMed: 32122539]
149. Tsujikawa T, Kumar S, Borkar RN, et al. Quantitative multiplex immunohistochemistry reveals myeloid-inflamed tumor-immune complexity associated with poor prognosis. Cell Rep. 2017 Apr 4;19(1):203–217. [PubMed: 28380359]

150. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001;344(14):1031–1037. [PubMed: 11287972]

151. Schwaederle M, Zhao M, Lee JJ, et al. Association of biomarker-based treatment strategies with response rates and progression-free survival in refractory malignant neoplasms. JAMA Oncol. 2016;2(11).

Article Highlights

- Reverse-phase protein arrays (RPPA) have enabled the high-throughput measurement of expression and post-translational modification of up to 500 proteins of high biological and clinical significance in low-volume samples including serum, plasma, and lysates from cultured cells, fine need aspirates, fresh tissue, frozen tissue, formalin-fixed paraffin-embedded tissue, and laser capture microdissected specimens.
- RPPA has been applied in the pre-clinical setting to generate hypotheses regarding mechanisms of drug resistance and strategies to overcome drug resistance that have subsequently been validated in clinical trials. Notable examples include combination PARP inhibition and PI3K pathway inhibition to impart sensitivity to PARP inhibitors in the *BRCA*-wildtype setting, and combination BRAF inhibition and mTOR pathway inhibition to restore sensitivity to BRAF inhibitors in heavily-pretreated patients with *BRAF*-mutant tumors. Newer areas of RPPA investigation include resistance to targeted breast cancer therapies and immune checkpoint inhibitors, with clinical trials designed to overcome drug resistance in these settings both underway and expected soon.
- RPPA has been applied directly in the clinical setting to identify potential biomarkers in completed and ongoing window of opportunity clinical trials and adaptive clinical trials. In this setting RPPA is used to analyze pre- and on-treatment patient biopsy specimens to characterize the molecular underpinnings of differential patient response to treatment.
- Genomic analyses alone have thus far been limited in their ability to describe and predict patient responses to targeted therapy. Genomic analyses have been demonstrated to poorly characterize intracellular protein levels and activities, making proteomics a critical companion to genomics in the goal to understand and devise new strategies to overcome cancer drug resistance.

Table 1.

Example mechanisms of anticancer drug resistance and strategies to overcome resistance that have been proposed based on data generated by RPPA and translated to the clinical setting

Drug class	Mechanism of resistance identified in RPPA analysis	Therapeutic strategy to overcome resistance	Clinical study	Clinical study outcome
PARPi				
PARPi	Increased expression and phosphorylation of S and G2/M checkpoint proteins, allowing time for repair of DNA double strand breaks	Combination treatment with PARPi and WEE1i to bypass PARPi-induced G2/M arrest	Phase 2 trial EFFORT (NCT03579316), investigating adavosertib monotherapy and combination therapy with olaparib and adavosertib in PARPi-resistant ovarian cancer	Completed: ORR of 23% and CBR of 63% with adavosertib monotherapy; ORR of 29% and CBR of 89% with combination olaparib and adavosertib
		Sequential treatment with PARPi and WEE1i to bypass PARPi-induced G2/M arrest and decrease toxicity of PARPi and WEE1i combination therapy	Phase 1 trial STAR (NCT04197713) investigating sequentially-dosed olaparib and adavosertib in patients with HRD-deficient solid tumors	Ongoing: Has completed does escalation phase
PARPi	Activation of the pro-survival RAS/RAF/MAPK pathway	Combination therapy with PARPi and MEKi to overcome MAPK pathway activation	Phase 1–2 trial SOLAR (NCT03162627) evaluating combination olaparib and selumetinib in solid tumors with PARPi resistance and RAS pathway alterations	Ongoing: Nearing completion
PARPi	Activation of the pro-survival PI3K/AKT pathway	Combination therapy with PARPi and PI3K/AKT/mTOR pathway inhibition	Phase 1 trial NCT01623349 investigating combination olaparib and alpelisib in recurrent TN breast cancer and high grade serous ovarian cancer	Completed: ORR of 33% for combination olaparib and alpelisib in patients with *BRCA*-wildtype platinum-resistant ovarian cancer
			Phase 1b trial OCTOPUS (NCT02208375) investigating combination olaparib and capivasertib in breast, endometrial, and ovarian cancer	Completed: ORR of 44% for combination olaparib and capivasertib, regardless of *BRCA* status and platinum resistance, in endometrial cancer, and 19% across all enrolled cancer types
BRAFi				
BRAFi	Compensatory MAPK pathway hyperactivation	Intermittent dosing of combination BRAFi and MEKi to periodically remove MAPK pathway dampening and allow unchecked MAPK hyperactivation to induce apoptosis	Phase 2 trial S1320 (NCT02196181) evaluating continuous versus intermittent dosing of combination dabrafenib and trametinib in metastatic and unresectable *BRAF*-mutant melanoma	Completed: Poorer progression-free survival with intermittent dosing as compared to continuous dosing (5.5 versus 9.0 months)
BRAFi	Activation of the PI3K/AKT/mTOR pathway	Combination therapy with BRAFi and PI3K/AKT/mTOR pathway inhibition	Phase 2 trial LOGIC2 (NCT02159066) evaluating benefit of addition of buparlisib to combination encorafenib/binimetinib at time of progression	Completed: ORR 0% with addition of buparlisib
			Phase 1 trial NCT01596140 investigating combination vemurafenib and everolimus in heavily-pretreated patients with *BRAF*-mutant solid tumors	Completed: ORR 22% across all enrolled patients

Table 2.

Window of opportunity clinical trials utilizing RPPA to identify adaptive responses in on-treatment patient samples

Clinical trial	Investigational agent(s)	Phase	Status	Conclusions
POSITION: Talazoparib in Determining Genetic Effects on Disease Response in Patients With Advanced Ovarian, Fallopian Tube, or Primary Peritoneal Cancer (NCT02316834)	Talazoparib	Early Phase 1	Completed	Patients treated with PARPi monotherapy display individual-specific adaptive responses, with limited interlesional heterogeneity within each patient. PARPi increased RAS/MAPK signaling, PI3K signaling, and activation of S phase and G2/M checkpoint, leading to PARPi trials described in Table 1.
Adavosertib Before Surgery in Treating Patients With Advanced High Grade Ovarian, Fallopian Tube, or Primary Peritoneal Cancer (NCT02659241)	Adavosertib	Early Phase 1	Recruiting	Ongoing
Targeted PARP or MEK/ERK Inhibition in Patients With Pancreatic Cancer (NCT04005690)	Olaparib and cobimetinib	Early Phase 1	Recruiting	Ongoing