Review

Clinical Cancer Research

Intratumoral Heterogeneity: From Diversity Comes Resistance

Ariel Pribluda, Cecile C. de la Cruz, and Erica L. Jackson

Abstract

Tumors consist of a heterogeneous mixture of functionally distinct cancer cells. These functional differences can be caused by varying levels of receptor activity, differentiation, and distinct metabolic and epigenetic states. Intratumoral heterogeneity can lead to interdependence among different subpopulations of cells for sustained tumor growth. In addition, subpopulations can vary widely in their responses to therapeutic agents. As such, it is believed that intratumoral heterogeneity may underlie incomplete treatment responses, acquired and innate resistance, and disease relapse observed in the clinic in response to conventional chemotherapy and targeted agents. *Clin Cancer Res; 21(13); 2916–23.* *©2015 AACR.*

Introduction

Cytotoxic chemotherapies, the traditional mainstay of cancer treatment, have led to significant improvements in patient survival. However, the toxicity of these compounds limits their therapeutic window, and complete responses to chemotherapy are rare. Partial responses and acquired resistance demonstrate that some, but not all the cells in a given tumor, are sensitive to treatment. The introduction of combination chemotherapy in the 1960s further improved patient response and survival (1) due to the eradication of a larger fraction of the tumor cells than with individual therapies alone. This could be due to the combined cytotoxic effect on all tumor cells, or could result from the specific effects of the individual drugs on distinct subpopulations in the tumor.

Tumors contain cancer cells and different types of stromal cells that contribute to their growth and progression. Within a tumor, the cancer cells themselves display heterogeneity in a variety of features that may contribute to therapeutic resistance, including gene expression, differentiation state, and proliferative capacity. Nonuniform expression of specific tumor markers has been appreciated for decades based on histologic evaluation. It is only with the recent advent of technologies that enable more in-depth, multiparameter analysis of single cells, such as mass cytometry and single-cell RNA-seq, that we are beginning to grasp the true extent of phenotypic variation among tumor cells.

Therapeutic strategies for cancer treatment have evolved significantly over the past two decades. Molecularly targeted cancer drugs have resulted in more dramatic but often short-lived responses, further highlighting the problem of acquired resistance (2). In some cases, secondary mutations are the clear underlying cause of this resistance. For example, mutation of a critical threonine residue of EGFR renders this protein insensitive to ATP-competitive inhibitors, and thus the oncogene remains active in the presence of drug (3). Moreover, intratumoral genetic heterogeneity is a well-established phenomenon, and there is increasing evidence that tumors harbor numerous genetically distinct subclones that differ in their sensitivities to chemotherapy and molecularly targeted agents (4). Moreover, therapeutic intervention may enhance tumor evolution by providing a selective pressure that promotes the expansion of minor, resistant subclones (5, 6). As there have been several recent reviews on this topic, we will not provide a detailed discussion of genetic heterogeneity.

Many studies have shown that acquired resistance occurs in the absence of secondary genetic mutations (2). Responses to rechallenge therapy with both conventional and targeted therapies provide ample clinical evidence for transient drug-resistance mechanisms. There are many examples of patients who progressed on therapy and then showed objective responses when retreated with the same therapy after a drug holiday (7). In some cases, rechallenge therapy has proven more efficacious than approved second-line therapies, resulting in prolonged overall survival (8). Here, we discuss some of the forms of intratumoral heterogeneity that have been linked to nongenetic drug resistance. The stromal and immune components also contribute to the heterogeneity of the tumor and to therapeutic resistance (9), but a discussion of this topic is beyond the scope of this review.

Cancer Stem Cells

The link between heterogeneity and drug resistance was initially explored in the context of cancer stem cells (CSC) by John Dick's laboratory in 1994 (10). Several years later, the first solid tumor CSCs were reported by Al-Hajj and colleagues (11) and since then, hundreds of reports have described CSCs in nearly every type of cancer. The consensus definition of a CSC is "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor" (12). However, this unidirectional, hierarchical definition of CSCs has been controversial, as substantial evidence exists that the tumorigenic capacity of an individual cell reflects a reversible or bidirectional state (13, 14). In addition, specific markers of CSCs have proven to be irreproducible across different

Discovery Oncology, Genentech, Inc., South San Francisco, California.

Corresponding Author: Erica Jackson, Genetech, Inc., 1 DNA Way, MS42, South San Francisco, CA 94080. Phone: 650-467-7460; Fax: 650-467-5454; E-mail: jackson.erica@gene.com

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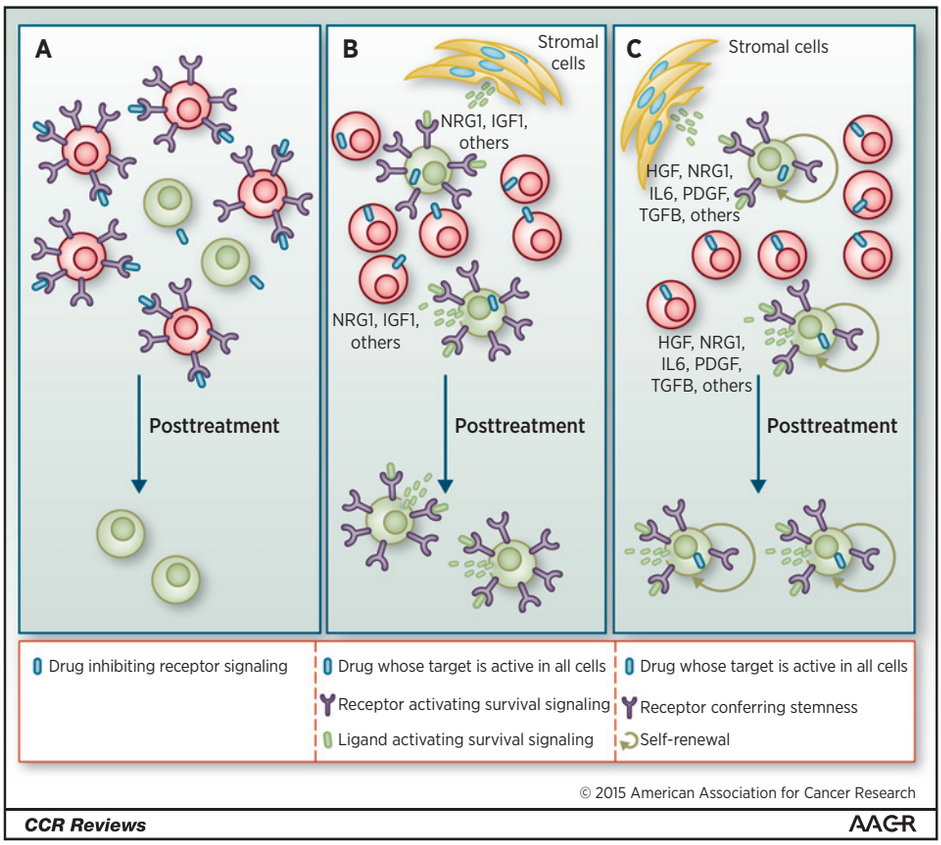

Figure 1.
Heterogeneity in ligand/receptor expression contributes to resistance. A, a subpopulation of cells does not express the drug target. Cells depicted in red represent a subpopulation that expresses and depends on the targeted receptor for survival. Cells depicted in green represent a subpopulation that does not express the targeted receptor, rendering them resistant to the drug. B, receptor-mediated survival signaling. A subpopulation of cells tolerates drug exposure due to survival signaling from receptors absent in the rest of the population. C, stemness-mediated resistance. A subpopulation of cells expresses ligands/receptors that confer stemness, making them resistant to drug treatment.

cancer types, or even between individual tumors of a given subtype. Regardless of the strict definition of CSCs, it is clear that at any given time, a tumor contains cells with varying degrees of "stemness," meaning that only a subset of the cells in a tumor is capable of sustaining tumor growth over time.

There are many reviews on CSCs and plasticity, and we will not describe their characterization in depth (15, 16). However, it is important to note that numerous reports have documented that CSCs have innate tolerance to cellular stresses, and resistance to radiation, chemotherapy, and molecularly targeted agents (17). Moreover, many properties of stem cells and drivers of stemness also confer therapeutic resistance. For example, induction of epithelial-to-mesenchymal transition (EMT) converts immortalized breast epithelial cells into breast CSCs, rendering them more chemoresistant (18). EMT is recognized as a resistance mechanism to both kinase inhibitors and conventional chemotherapies (19, 20). Additional signaling pathways have been implicated in conferring both stemness and resistance, including IL6, JAK/STAT, HGF, WNT, NOTCH, TGFB, PDGF, and others (16).

Heterogeneity in Ligand/Receptor Expression

Many targeted cancer therapies act by inhibiting signaling from either mutated or wild-type cell surface receptors that are established drivers of tumor growth. EGFR mutation or overexpression is a common driver of non–small cell lung cancer (NSCLC), glioblastoma, and colorectal cancer, and several inhibitors of EGFR have been approved for the treatment of cancer (21). Similarly, *ERBB2* (HER2) mutation or amplification drives certain breast and gastric cancers and it is the target of several approved therapies (22). Luminal breast cancers are mostly driven by wild-type estrogen receptor signaling, making endocrine therapy the mainstay in this disease.

Within a given tumor, cancerous cells are often heterogeneous in their expression of ligands and cell surface receptors, even when these receptors are important drivers of tumor growth. This heterogeneity has been linked to therapeutic resistance, not only to the therapies targeting that particular receptor, but also to therapies targeting other receptors and downstream effectors, and to cytotoxic chemotherapies as well (Fig. 1).

Patel and colleagues recently reported an in depth analysis of intratumoral heterogeneity in primary glioblastoma using single-cell RNA-seq (23). Within the cancer cells of an individual tumor, they found mosaic expression of numerous ligands and cell surface receptors. Interestingly, even when activating mutations in *EGFR* are present in a tumor, only a fraction of the tumor cells express the mutant receptor. EGFR-negative cells express other receptor tyrosine kinases (RTK), perhaps to compensate for their lack of *EGFR* activity, and *EGFR* expression is anticorrelated with *PDGFRA* and *PDGFC* expression. Although they did not explore the functional consequences of this heterogeneity, their findings suggest that inhibition of a single RTK would not impact all of the cells in the tumor.

Heterogeneous receptor expression also occurs in luminal breast cancers, which are defined by being estrogen receptor positive (ER^+) or progesterone receptor positive (PR^+) and/or PR^+. Endocrine therapy provides the basis for treatment of this disease.

However, hormone receptor expression levels are not uniform across all of the cells within these tumors. IHC analysis of a cohort of ER^+ breast cancers revealed that in most cases, all of the tumor cells were ER^+, but in approximately 22% of cases, the proportion of ER^+ cells within the tumor ranged from 20% to 90% (24). In another study, an endocrine therapy-resistant $ER^-PR^-CK5^+$ subpopulation was found in luminal breast cancer cell lines (25, 26). This population expresses markers of breast CSCs, which have been linked to drug resistance and are thought to give rise to circulating tumor cells (CTC). CTCs are associated with poor prognosis and poor response to therapy in breast cancer and were recently shown to be heterogeneous in ER expression (27). Single-cell analysis of CTCs in ER^+ metastatic breast cancer patients showed that in nearly half of evaluable patients, all CTCs were ER^-, and the remaining patients harbored CTCs with mixed ER expression. Trials are currently under way to assess whether the endocrine therapy index of CTCs is correlated with patient outcome (28).

Heterogeneous expression of the ERBB3 (HER3) and its ligand NRG1 have been reported in a variety of human cancers, including breast cancer, lung cancer, and glioblastoma (23, 29, 30). HER3 signals as heterodimer with other receptors of the HER family leading to potent activation of the PI3K/AKT pathway (22). Activation of HER3 by NRG1 has been widely implicated in resistance to kinase inhibitors. Using a panel of cancer cell lines, Wilson and colleagues demonstrated that treatment with exogenous NRG1 rescued numerous cell lines from growth inhibition by several kinase inhibitors (31). Inhibition of RAF or MEK signaling results in increased HER3 expression through multiple mechanisms, causing cancer cells to become refractory to inhibitor treatment if NRG1 is present (32, 33). HER3 activation also confers resistance to EGFR inhibitors, radiotherapy, and several chemotherapeutic agents (34). Our laboratory found that primary NSCLCs contain a small subpopulation of $NRG1^+$ cells and that NRG1 and its receptors HER3 and HER4 are enriched in residual tumor cells that survive chemotherapy treatment. Moreover, inhibition of NRG1 signaling enhanced the magnitude and duration of the response to chemotherapy in several *in vivo* models (29).

Recent studies highlight the impact of intratumoral heterogeneity in NRG1 and HER3 expression in breast cancer. Although HER2 targeted therapies are currently only approved to treat patients with HER2-positive disease, analysis of results from two adjuvant trastuzumab (anti-HER2) trials revealed that patients with Her2-low tumors also benefit from adjuvant treatment (35, 36). On the basis of these findings, Lee and colleagues explored the role of HER2 signaling in CSCs from Her2-low tumors and found that activation of HER2/HER3 by NRG1 autocrine signaling supports their proliferation and self-renewal (30). NRG1 alone is sufficient to support mammosphere formation by breast CSCs (37), and NRG1 also stimulates the growth of primary lung CSCs in sphere cultures (38).

Metabolic Heterogeneity

Metabolic reprogramming is recognized as a new hallmark of cancer (39). The fact that cancer cells display altered glucose metabolism and become dependent on oxidative glycolysis rather than oxidative phosphorylation for energy generation was first observed by Otto Warburg in the 1930s. Because glycolysis is less efficient than oxidative phosphorylation (OXPHOS) at generating ATP, cancer cells must compensate by increasing their glucose uptake (39). This increased glucose uptake provides the basis for the commonly used imaging modality, ^{18}F-deoxyglucose positron emission tomography (FDG-PET). Heterogeneous 18F-FDG uptake provides ample clinical evidence for intratumoral metabolic heterogeneity, which has been associated with poor treatment response (40, 41).

Metabolic reprogramming in cancer is much more complex than just a switch from OXPHOS to glycolysis. After being converted to glucose-6-phosphate, some of the increased glucose uptake is shunted into the pentose phosphate pathway, to generate ribose and nicotinamide adenine dinucleotide phosphate (NADPH). Both ribose and NADPH are integral in the biosynthesis of nucleotides and fatty acids, which are in high demand in rapidly dividing cancer cells (42). NADPH also serves as the antioxidant substrate for the glutathione (GSH) and thioredoxin antioxidant systems, helping to reduce levels of reactive oxygen species (ROS) in the cell (Fig. 2). Low levels of ROS are beneficial to the cancer cell, stimulating proliferation and survival pathways. However, oncogene activation induces high levels of ROS, resulting in oxidative stress that can lead to apoptosis. Therefore, certain cancer cells rely on antioxidant systems to balance ROS levels (43, 44). Additional metabolic hallmarks of cancer cells include increased rates of glutamine metabolism and dysregulated autophagy.

Several studies have linked distinct ROS levels in tumor cell subpopulations with their resistance to therapy. Diehn and colleagues found that CSCs in both mouse and human tumors have higher expression of antioxidant genes and low ROS levels compared with non-CSCs. Moreover, these cells show less DNA damage and increased survival following exposure to ionizing radiation, and GSH depletion in these cells sensitizes them to radiation (45). Similarly, ROS^{low} cells in head and neck cancers express CSC markers and have enhanced tumor-initiating capacity. ROS^{low} cells are enriched after cisplatin treatment and inhibition of antioxidant systems in these cells sensitizes them to the drug (46). Aldehyde dehydrogenase (ALDH) proteins can function as antioxidants by controlling the oxidation of a variety of aldehydes, including toxic byproducts generated by ROS (47). Interestingly, Raha and colleagues found that drug-tolerant persisters (DTP), a small population of cells that survive kinase inhibitor treatment, are $ALDH^{high}$ and that $ALDH^{high}$ cells in the naïve culture are innately drug tolerant (48). ALDH has been used to identify CSCs in a variety of cancer types (16). Raha and colleagues found that treatment-naïve $ALDH^{high}$ cells have increased ROS levels, and increased ALDH activity is required to protect the DTPs from high ROS levels that arise following drug exposure. Inhibition of ALDH activity results in the depletion of DTPs upon kinase inhibitor treatment (48). Together, these findings indicate that metabolic defenses against ROS are an important drug resistance mechanism.

Dysregulated autophagy is another metabolic hallmark of cancer cells. Autophagy is a process in which cellular organelles, proteins, and other cytoplasmic components are sequestered in specialized vacuoles called autophagosomes, where they undergo catabolic breakdown following fusion with the lysosome. The energy and molecular building blocks generated through this breakdown process can then be "recycled" for the synthesis of new macromolecules. Autophagy serves as an adaptive metabolic response to stresses commonly encountered by cancer cells, such as hypoxia, endoplasmic reticulum stress, and nutrient

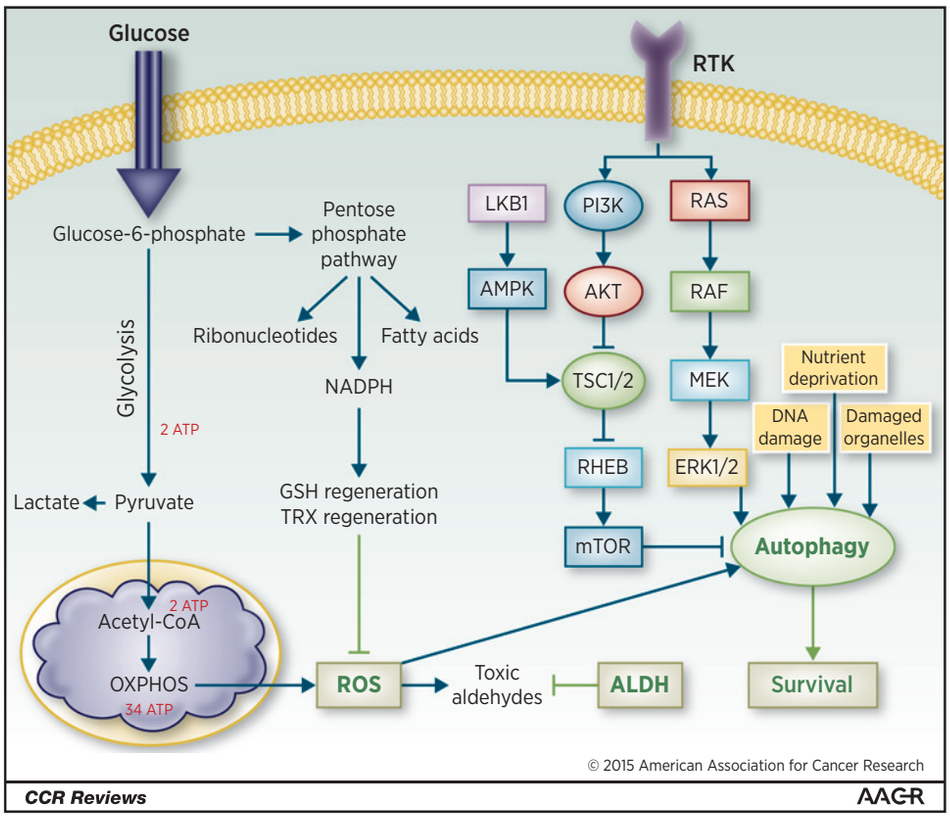

Figure 2.
Metabolic pathways contributing to drug resistance in cancer cells. Cancer cells depend on increased glucose uptake that is used in glycolysis for the generation of energy and shunted into the pentose phosphate pathway for the production of ribonucleotides, fatty acids, and antioxidant regeneration. Certain metabolic properties have been linked to intratumoral heterogeneity and drug resistance, including ROS levels, the ability to counteract toxic byproducts of ROS through the activity of ALDH and activation of autophagy (green). Autophagy is also regulated by several signaling pathways that are frequently activated in cancer.

deprivation (49, 50) and several critical oncogenic pathways feed into the regulation of autophagy in the cell (Fig. 2).

Increased autophagy can contribute to chemoresistance (51). Treatment of cancer cell lines with platinum chemotherapies induces autophagy and inhibition of autophagy sensitizes cell lines and xenograft tumors to treatment with these drugs (52, 53). Likewise, irradiation also induces autophagy in cancer cell lines and siRNA-mediated inhibition of autophagy sensitizes resistant cells to irradiation (52–55). Autophagy also contributes to kinase inhibitor resistance. Inhibition of this process can overcome resistance to BRAF inhibitors in BRAF-mutant melanoma models, and high autophagy levels are also associated with poor prognosis and therapeutic resistance in patients with advanced melanoma (56, 57).

Variation in basal autophagy levels has also been linked to the therapeutic resistance of cancer cell subpopulations. In BCR/ABL-driven chronic myelogenous leukemia (CML), CSCs are known to survive treatment with kinase inhibitors against BCR/ABL, and the persistence of these cells in patients may be why these therapies are not curative (58). $CD34^+$ CML stem cells have increased basal autophagy levels, and the $CD34^+$ cells in imatinib nonresponders have higher baseline autophagy than those of responders (59). Autophagy can serve as a resistance mechanism to these inhibitors, and cotreatment with an autophagy inhibitor sensitizes CML CSCs to imatinib (59, 60).

Viale and colleagues studied cells that are resistant to oncogene ablation in a mouse model of *Kras*-induced pancreatic adenocarcinoma driven by an inducible *Kras* allele. They found that the surviving cells that remain when *Kras* expression is turned off have reduced glycolysis, and show increased dependence on OXPHOS and autophagy. Pharmacologic inhibition of OXPHOS or autophagy significantly reduces the viability of surviving cells. Moreover, they showed that inhibition of OXPHOS with oligomycin synergizes with *Kras* ablation *in vivo*, resulting in a dramatic increase in animal survival upon reintroduction of *Kras* expression (61).

Epigenetic Heterogeneity

The term "epigenetics" is often used to refer to alterations in chromatin structure that result in changes in gene expression (62). The epigenome consists of different layers of regulation, which control the accessibility of transcriptional regulators to genomic DNA (63, 64). These include chemical modifications or "marks" on DNA and histone proteins. These marks affect nucleosomal occupancy and positioning and thus define boundaries between transcriptionally permissive and repressive chromatin states (64). There are several classes of epigenetic regulators, those that write the marks, DNA methyltransferases, histone methyltransferases, and histone acetyltransferases; those that read the marks, the bromodomain, chromodomain, and tudor proteins; those that erase the marks, histone deacetylases (HDAC) and histone demethylases (HDM); and those that remodel the chromatin, such as components of the SWI/SNF complex. The frequent, recurrent mutation of specific epigenetic modifiers in a variety of cancers demonstrates that altered epigenetic regulation plays an important role in driving tumorigenesis (65).

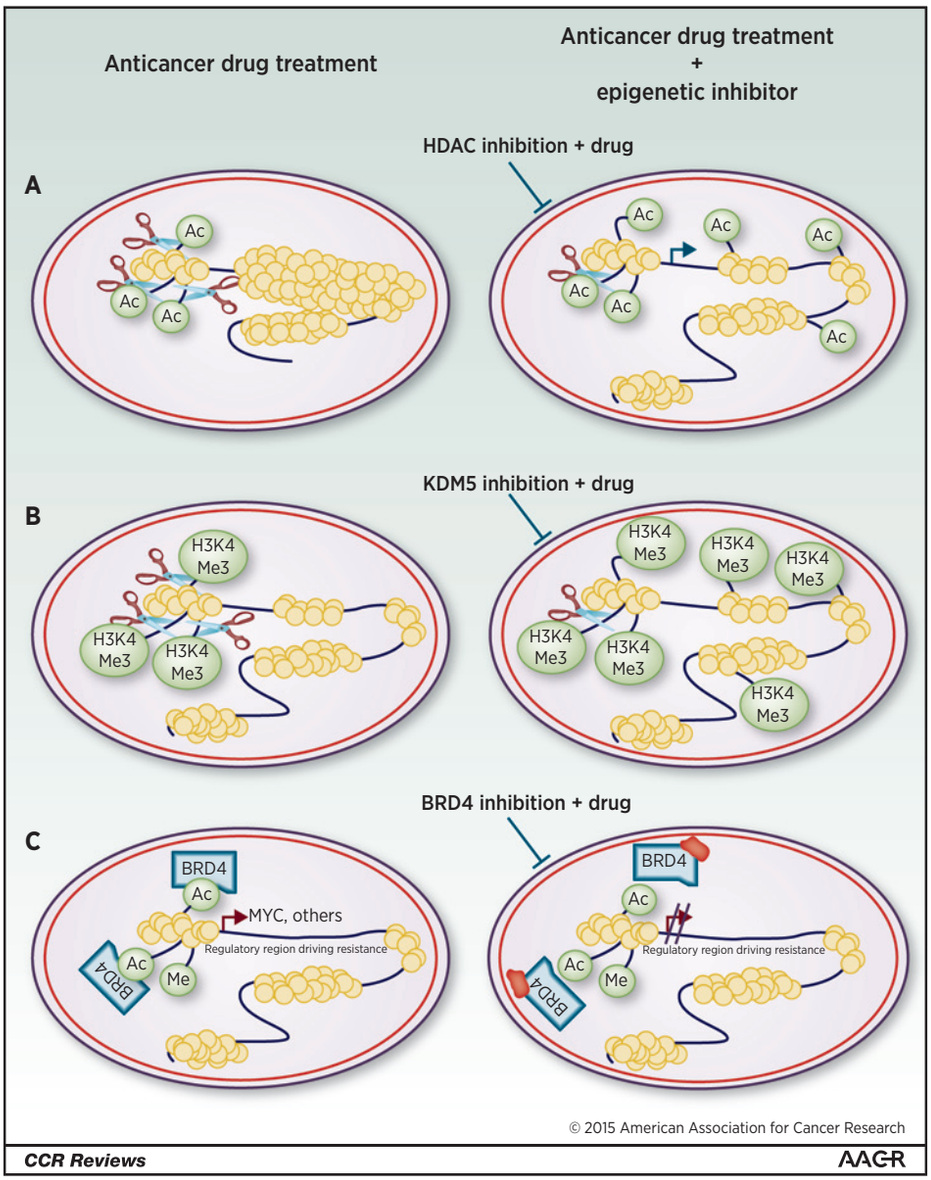

Figure 3.
Epigenetic drivers of resistance. A and B, resistance mechanisms conferred by HDAC and KDM5 HDM erasers found in residual cells that survive drug treatment (left) or on-treatment (right), respectively. Erasers are represented by scissors removing the relevant marks. HDAC or KDM5 inhibition under drug eliminates the persister population. C, BRD4 confers resistance by facilitating MYC and/or other transcriptional programs that drive this resistance state (left). BRD4 inhibition under relevant drug treatment eliminates resistant cells by suppressing a BRD4-dependent transcriptional program.

The epigenome is a crucial mediator of cell fate determination, allowing cells with the same genome to differentiate into distinct cell types. Several classes of epigenetic regulators have been implicated in drug resistance and intratumoral heterogeneity (Fig. 3). Histone acetylation appears to play an important role in driving tumorigenesis and drug resistance. Pan-HDAC inhibitors are successfully used for the treatment of cancer, but their use has been limited by toxicity and more selective HDAC inhibitors are currently being evaluated in the clinic (66). HDAC inhibitor treatment can sensitize a patient's tumors to radiation and chemotherapy, and combination regimens are currently in clinical trials (67). It is likely that HDACs contribute to the innate resistance of CSCs or slow-cycling tumor subpopulations. The combination of HDAC inhibitor plus imatinib, but neither agent alone, can induce apoptosis in quiescent CML stem cells *in vitro* and *in vivo* (68). HDACs also contribute to therapeutic resistance in a small subpopulation (DTPs) identified by Sharma and colleagues (69). DTPs in cell lines from several cancer types were found to be multidrug resistant and epigenetic modifiers play a key role in regulating the drug-tolerant state of these cells. Notably, HDAC inhibitor cotreatment could deplete DTPs and prevent the outgrowth of drug tolerant cells.

Efforts are also underway to develop drugs targeting the readers of histone acetylation, specifically the bromodomains and extra terminal (BET) family of bromodomain proteins. Among the BET family, BRD4 has been most widely studied and may be unique in its ability to bind to "superenhancers" that drive the expression of several developmental genes linked to oncogenesis (70). Several BET inhibitors have shown impressive single-agent activity in *in vivo* cancer models and are currently in clinical trials (71). Hematologic malignancies appear to be particularly sensitive to BET inhibition, perhaps due to their dependence on MYC whose expression is driven by

the activity of BRD4 on its superenhancer. In normal and malignant pre-T cells, NOTCH1 drives MYC expression and it is a common driver in T-cell acute lymphoblastic leukemia (T-ALL). Interestingly, in T-ALL persister cells, *MYC* expression is BRD4 dependent and NOTCH independent, making them resistant to NOTCH inhibition by γ-secreatase inhibitor and dependent on BRD4 for survival (72).

Histone demethylases also appear to drive drug resistance in a subpopulation of tumor cells. Recent studies identified drug resistance mechanisms involving HDMTs in NSCLC and melanoma models. Sharma and colleagues found the H3K4-specific HDM, KDM5A, is enriched in DTPs, contributing to their innate drug tolerance (69). Roesch and colleagues found that the activity of another H3K4-specific KDM, KDM5B (JARID1B) defines a population of slow cycling cells that is required for sustaining tumor growth (13). Like the DTPs, these JARID1B$^+$ melanoma cells also display multidrug resistance (73). Although Sharma and colleagues found that IGFIR, acting through KDM5A, is required for the reversible drug-tolerant state, Roesch and colleagues linked KDM5B to a metabolic resistance. Interestingly, like the surviving cells in pancreatic cancer models described by Viale and colleagues (61), the JARID1B$^+$ melanoma cells displayed increased dependence on oxidative phosphorylation relative the to the bulk population, and could be depleted by OXPHOS inhibitors.

Summary

Patients with cancer present with an established tumor and receive the therapeutic intervention that is appropriate for their stage and type of disease. Unfortunately, in many cases, some of their cancer cells survive treatment. It was long believed that these residual cells were no different than the bulk of the tumor and their survival was simply stochastic. We now know that intratumoral heterogeneity can significantly affect treatment response, and that certain subpopulations of tumor cells have unique properties that render them insensitive to drugs. Here, we have touched on some of these properties, including stemness, ligand/receptor expression, epigenetic regulation, and metabolic state. There are additional factors that contribute to non-uniform treatment response across tumor subpopulations, such as stromal interactions, genetic variation, and others that are not yet understood. Ideal treatment regimens would target all the different subpopulations of cancer cells present at the time of treatment, thus avoiding resistance and delaying relapse. This will likely require combination therapies. Combining targeted therapies directed against different subpopulations is one such approach, but may require novel, more sensitive diagnostic strategies capable of detecting small subpopulations of cells. Although not necessarily effective on their own, therapies targeting resistance mechanisms might also drive uniform responses when used in combination. Although many challenges lie ahead, it is clear that intratumoral heterogeneity will have to be addressed therapeutically in order to achieve durable responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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