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Review

Cytokines in cancer drug resistance: Cues to new therapeutic strategies

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ABSTRACT

The development of oncoprotein-targeted anticancer drugs is an invaluable weapon in the war against cancer. However, cancers do not give up without a fight. They may develop multiple mechanisms of drug resistance, including apoptosis inhibition, drug expulsion, and increased proliferation that reduce the effectiveness of the drug. The collective work of researchers has highlighted the role of cytokines in the mechanisms of cancer drug resistance, as well as in cancer cell progression. Furthermore, recent studies have described how specific cytokines secreted by cancer stromal cells confer resistance to chemotherapeutic treatments. In order to gain a better understanding of mechanism of cancer drug resistance and a prediction of treatment outcome, it is imperative that correlations are established between global cytokine profiles and cancer drug resistance. Here we discuss the recent discoveries in this field of research and discuss their implications for the future development of effective anti-cancer medicines.

Contents

Abbreviations: AM, adrenomedullin; AMF, autocrine motility factor; AML, acute myeloid leukemia; BCL, B-cell lymphoma; BCR, breakpoint cluster region; BM, bone marrow; CCL, chemokine (C-C motif) ligand; CML, chronic myeloid leukemia; ECM, extracellular matrix; EGF, epidermal growth factor; EMT, epithelial–mesenchymal transition; FGF, fibroblast growth factor; G-CSF, granulocyte colony stimulating factor; GEM, gemcitabine monotherapy; HER2, human epidermal growth factor receptor 2; HGF, hepatocyte growth factor; IL, interleukin; Mcl-1, myeloid cell leukemia-1; MDR, multidrug resistance; MMP, matrix metalloproteinase; MRCC, metastatic renal cell carcinoma; NGAL, neutrophil gelatinase-associated lipocalin; PARP, poly ADP-ribose polymerase; PFS, progression-free survival; PI3, phosphoinositide 3; PSCs, pancreatic stellate cells; RANTES, regulated on activation, normal T cell expressed and secreted; SDF-1, stromal cell-derived factor-1; STAT3, signal transducer and activator of transcription 3; TGF, transforming growth factor; TKI, tyrosine kinase inhibitor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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1. Introduction

The development of effective treatments against cancer has been an ongoing biomedical endeavor over the past 50 years [1,2]. Many treatments involve the use of cytotoxic chemotherapy agents that have powerful anti-cancer activities. Despite the effectiveness of these agents against tumors, most chemotherapy protocols do not effectively increase patient survival. However, decades of research have brought us a deeper understanding of the molecular basis of many cancers, enabling newer, more advanced strategies for tumor killing. The recent development of oncoprotein-targeted drugs, a breakthrough weapon in the war on cancer, uses precise inhibitors and antibodies to block pathways or targets that are specific to the tumor. Tremendous progress in cancer treatment has emerged from this latest generation of drugs, most notably the kinase inhibitors targeting the V600E-B-RAF mutation in late stage melanoma, the BCR-ABL fusion protein in chronic myeloid leukemia (CML), and the EML4-ALK translocation in non-small cell lung cancer (NSCLC) [3]. However, even after a profound initial response to the targeted therapy, the majority of cancers acquire resistance to these agents and begin to grow again. Thus, despite the improvement in patient survival rates and reduction of side effects, relapse remains our primary challenge [4].

The resistance to cytotoxic or targeted chemotherapy drugs may be present before cancer therapy, or may arise as a result of cancer therapy. In some cases, there may be only subpopulations of cells within the tumor that possess resistance mechanisms, thus resulting in their competitive expansion following drug administration. As the tumor develops, mutations may then occur in some cells, resulting in those possessing the most efficient improvements to proliferate, conferring a selective advantage. Moreover, as cells within the tumor acquire somatic mutations, the genetic profile of the tumor becomes heterogeneous, wherein different populations of cells acquire dissimilar genetic fingerprints. [5]. Intensifying this problem are observations of multidrug resistance (MDR), meaning that more complex strategies of chemotherapy must be developed to overcome resistance. Thus, the development of the next generation of cancer therapies must occur with resistance mechanisms as hindsight, and in this review, we attempt to summarize the key drug resistance pathways which represent putative therapeutic strategies.

Chemotherapy drug resistance may originate from various mechanisms including dysregulated apoptosis, somatic mutations within drug targets, or modification of drug metabolism and transport. Resistance may be a pre-existing, innate feature of the cancer cell (intrinsic drug resistance), or may be acquired during the course of treatment (acquired resistance) as tumors adapt to and counter drug exposure. Both scenarios typically lead to a relapse following therapy. There are two major mechanisms, both not mutually exclusive, that may account for intrinsic or acquired resistance of tumors against chemotherapeutic drugs. The first is simply a failure of plasma membrane receptors on tumor cells to actively take up anti-cancer agents. The second mechanism involves drug elimination through the ejection of cytostatic substances across the cell membrane. This phenomenon is responsible for many multidrug resistance (MDR) phenotypes, and is a major influence in the failure of chemotherapy strategies [5,6]. On the other hand, acquired resistance develops following continuous exposure to drugs that induce genetic and/or epigenetic changes eventuating in proapoptotic pathway blockade, and/or constitutive expression of anti-apoptotic proteins [7,8] as well as increased efficiencies in cellular DNA damage repair mechanisms [9].

However, the combination of all the mechanisms described above does not fully explain chemotherapy drug resistance, indicating that unknown cancer drug resistance mechanisms remain to be discovered. With the expanding arsenal of anticancer agents and the introduction of comprehensive high-throughput screening, there are now prospects of overcoming drug resistance through the clinical assessment and strategic implementation of drug combinations, and through the identification of predictive biomarkers.

Recent studies have implicated dysregulated cytokine expression as a crucial aspect of many drug resistance mechanisms [4,5,10]. Cytokines, which we broadly define here as a diverse category of secreted molecules which includes chemokines, growth factors, pro- and antiangiogenic factors, adipokines, soluble receptors and extracellular proteases, play pivotal roles in both normal and pathologic cellular events. The proliferation of tumor cells and the formation of stromal blood vessel networks, both of which support progressive tumor growth, are directed by aberrant cytokine signaling. Cytokines are responsible for a number of physiologic processes that are strongly correlated with tumorigenesis, tumor development and metastasis; these processes include inflammation [11], cell migration [12], angiogenesis [13], and apoptosis [14]. Additionally, evidence is emerging that the cytokines secreted by cancer cells and their associated stroma play a key role in a number of drug resistance mechanisms [15,16]. Although our understanding of how cytokines influence drug resistance is currently sparse, it has become clear that cytokines represent legitimate therapeutic targets and biomarkers, and further exploration of their function in these contexts is urgently needed.

It is likely that early diagnosis of cancer drug resistance may eventually be accomplished by monitoring changes in circulating cytokine levels during chemotherapy. This would allow investigators to evaluate tumor response to a particular drug, and glean guidance in the application of alternative chemotherapeutic strategies. Fortunately, cytokine levels can be easily measured in serum or plasma, fluids that are collected by minimally invasive methods. However, an obstacle to this approach is the realization that alteration in the levels of a single cytokine rarely constitutes an accurate biomarker, and that the true pathology of the disease state is understood fully by measuring multiple cytokines in parallel [17]. This broader perspective of cytokine expression requires high content, high-throughput methodologies for measuring protein levels in blood. To address this need, a number of platforms have been introduced within the last 20 years that employ the simultaneous use of multiple antibodies to expand the efficiency of protein detection in small sample volumes. These platforms have a promising track record of being successfully utilized in the discovery of biomarkers and key drug resistance-related molecules. For example, the multiplex immunoassay, an ELISA-based technique utilizing many antibodies regularly arranged on a solid support, enables efficient cytokine expression profiling from low volume biospecimens. This approach has contributed significantly to our understanding of cytokine dynamics in the tumor microenvironment [18] and has greatly accelerated biomarker discovery [19,20].

Multiplex immunoassays are proteomic techniques that are used for the detection of panels of key proteins involved in disease states. They exist on two major platforms: 1) antibody arrays (antibodies spotted onto planar solid supports such as glass slides, membranes, or microtiter plates) and 2) bead-based assays (antibodies coupled to fluorescent-labeled microbeads). Both techniques are technically straightforward and may be used to detect protein expression profiles from diverse biological fluids. Moreover, multiplex immunoassays have proven crucial in instances of biomarker detection for many diseases including immunologic disorders, asthma, neurological dysfunction, renal disease, and others [17].

Herein, we review recent advancements in the identification of novel drug resistance mechanisms, in which drug resistance is conferred by specific cytokines secreted by the cancer cells. In addition, we highlight the contribution of stromal cells within the tumor, and how their secretion profiles contribute to cancer drug resistance. We also discuss the utility of multiplex immunoassay platforms as predictive methods in determining cancer drug efficacy, and present examples of how researchers employed antibody-based screens to assess the extent to which cytokines function as effector molecules in cancer drug resistance mechanisms.

2. Cytokine perturbations in cancer development

Cytokines that directly influence cancer progression include a variety of angiogenic growth factors that regulate cancer cell proliferation and the formation of vessel networks within tumors. For example, vascular endothelial growth factor (VEGF), a highly potent proangiogenic factor, is highly expressed within most tumors, where it stimulates the migration and proliferation of endothelial cells and formation of blood vessels [21,22]. In addition, malignant transformation is often associated with aberrant expression of fibroblast growth factor (FGF), epidermal growth factor (EGF) and hepatocyte growth factor (HGF), all of which stimulate proliferation of tumor and stromal cells and manifest potent angiogenic effects [23–26]. Transforming growth factor beta (TGF-beta), a highly pleiotropic growth factor with complex and paradoxical implications for cancer, has been found to exhibit both pro and anti-tumorigenic effects, depending on the context. In some instances TGF-beta suppresses growth or activates apoptosis, inhibiting cancer progression. In other instances, it promotes cancer through the induction of epithelial–mesenchymal cell transition (EMT), which can induce cancer cells to de-differentiate and acquire cancer stem-cell-like properties [27]. Tumor cells are known to acquire aberrant responses to TGF-beta and matrix metalloproteinases (MMPs), which can induce specific EMT programs [28,29].

Tumor cells also produce cytokines and chemokines such as interleukin-6 (IL-6), IL-8, IL-10, monocyte chemoattractant protein 1 (MCP-1), and regulated on activation, normal T cell expressed and secreted (RANTES) all of which have complex autocrine and paracrine effects. IL-6 is a pleiotropic pro-inflammatory cytokine that promotes B- and T-cell differentiation, induces acute phase reactant production, and stimulates hematopoiesis. IL-6 has been demonstrated to directly stimulate proliferation of tumor cells and promote angiogenesis [30–32]. Several reports have found that IL-6 levels correlate with disease progression and inversely correlate with response to treatment and survival [33]. IL-8 belongs to the superfamily of CXC chemokines and has a wide range of pro-inflammatory effects. It stimulates migration of neutrophils, monocytes, and lymphocytes, and promotes tumor cell proliferation and metastasis [34–36]. In addition, IL-8 exhibits strong angiogenic activity [34,35,37]. Chemokines of the CC superfamily such as RANTES and MCP-1 also are able to stimulate migration of normal and malignant cells, as well as promote tumor angiogenesis [38–40]. IL-10, a potent immunosuppressive cytokine which is frequently overexpressed in tumors, plays an important role in protecting cancer cells from immune-mediated destruction [41]. Collectively, this nexus of secreted signals serves to drive tumor progression and subvert cell function by potentiating the key hallmarks of cancer, such as chronic proliferation, perturbation of immunosurveillance, recruitment of blood vessels, and avoidance of apoptosis.

In addition, these aberrant cytokine signals eventuate in other effects which are now widely recognized to be crucial for cancer progression, i.e., reprogramming the tumor microenvironment (TME) to facilitate tumor growth. Specifically, tumor cells hijack local non-malignant cell types (tumor-associated stromal cells), directing them to actively serve the tumor's agenda. These non-malignant stromal cells, which include endothelial cells, fibroblasts, lymphocytes, and macrophages, can be re-educated to supply a specific cocktail of cytokines, chemokines, growth and angiogenic factors to the tumor, influencing the survival and proliferation of tumor cells. Thus, there exists a continuous cross-talk between tumor and stromal cells, where generated soluble factors represent a tumor cytokine network that plays an essential role in tumor growth and protection from endogenous (hypoxia, oxygen free radicals) and exogenous (chemotherapy drugs, ionizing radiation) damage. Overproduction of these factors by growing tumors has been shown to lead to the increases in circulating levels of cytokines, chemokines, angiogenic and growth factors which are often associated with resistance to therapy and overall poor prognosis [33,34,42–44]. We will now describe specific examples of seminal discoveries in the field that significantly advanced our understanding of the function of cytokines in cancer drug resistance.

3. Cancer cells secrete cytokines to evade drug-induced death

One of the more extensively characterized mechanisms of drug efflux from cancer cells is attributable to dysregulation of the *mdr1* gene, which results in abnormally high expression of the protein MDR1, also known as P-glycoprotein (P-gp). Belonging to the ATP-binding cassette (ABC) family of transporter proteins, P-gp is a transmembrane pump which serves to eliminate a variety of toxic compounds, including major cancer chemotherapeutics [5]. Expression of P-gp can in some instances be upregulated by cytokines [45,46]. P-gp-mediated MDR involves signaling through several signal pathways and transcription factors, including the ERK, JNK, p38, PI3-kinase and protein kinase C signaling pathways. Thus, drugs targeted against these pathways may provide new therapies for treatment of ABCB1/Pgp-mediated MDR [47].

Since the 1990s, it has been repeatedly observed that breast cancer patients with elevated serum levels of IL-6 exhibited poorer prognosis [48,49]. Studies into the mechanistic role of IL-6 in breast cancer progression revealed that the drug-sensitive breast cancer cell line MCF-7 does not express IL-6, whereas high levels of IL-6 are produced by multidrug-resistant sublines. It was further observed that both pretreatment with exogenous IL-6 and constitutive expression of IL-6 rendered drug-sensitive breast cancer cells resistant to several chemotherapy agents (doxorubicin, vincristine, and taxol). This protection was accompanied by the activation of the CCAAT enhancer-binding protein (C/EBP) family of transcription factors, and *mdr1* gene expression. This study revealed that breast cancer cells could acquire the ability to express IL-6, which confers autocrine cellular multi-drug resistance, and thus represents a self-protective mechanism [50]. This finding is especially significant in light of previous Phase I/II clinical trials in which IL-6 had been administered to breast and lung cancer patients in combination with chemotherapy for its ability to stimulate platelet growth [51].

Several groups have observed that an autocrine IL-6 loop contributes substantially to drug resistance of prostate cancer. LNCaP-IL6 + cells, which are a model system for therapy-resistant prostate cancer, were found to express increased levels of Mcl-1 protein, an anti-apoptotic member of the Bcl-2 family. Treatment of cells with a chimeric anti-IL-6 antibody led to the induction of apoptosis and the down regulation of Mcl-1 protein levels [52]. Moreover, some prostate cancer cell lines are resistant to enzalutamide (an androgen antagonist) by a mechanism thought to be due to autocrine IL-6-induced constitutive activation of signal transducer and activator of transcription 3 (STAT3) and its target genes. Indeed, inhibition of STAT3 expression or addition of the STAT3 inhibitor AG490, led to increased sensitivity of prostate cancer cells to enzalutamide. This study implies that targeting the IL-6-STAT3 axis along with enzalutamide treatment may be a viable therapeutic strategy for patients with enzalutamide resistant prostate cancer. [53].

To identify molecular changes involved in MDR, the cytokine profile of a multidrug-resistant human breast cancer cell line MCF-7/R was assessed using a cytokine antibody array that simultaneously detects 120 target proteins [54]. From this screen, expression of both IL-6 and IL-8 were found to be significantly increased compared to the sensitive parent cell line (MCF-7/S) and neutralizing antibody assays indicated that MDR was dependent on the activities of IL-6 and IL-8. An independent study also observed high levels of IL-6 and IL-8 in an antibody array experiment investigating drug resistance to the gamma-secretase inhibitor RO4929097 (an inhibitor of the Notch signaling axis). Here, xenograft models indicated that resistance to RO4929097 was associated with increased tumor cell-derived IL-6 and IL-8 expression levels [55]. Collectively, these findings suggest that IL-6 and IL-8 are key markers for the development of resistance to several drug types.

The Nuclear Factor (NF)-kappa B transcription factor activates an array of cellular defense responses such as the induction of anti-apoptotic factors and the production of pro-inflammatory and pro-angiogenic cytokines such as IL-6 and IL-8. NF-kappa B signaling occurs in response to a broad range of external stimuli, including bacteria, viruses, pro-inflammatory cytokines, ionizing radiation, and genotoxic substances. Of importance to cytokines and cancer drug resistance is the fact that NF-kappa B is activated by a number of cytotoxic chemotherapy agents including cisplatin, paclitaxel, docetaxel, and doxorubicin [56,57]. Unfortunately, this response commonly eventuates in drug-resistant tumors due to the upregulation of pro-survival cell signaling pathways. This, combined with the fact that many cancers are inherently characterized by dysregulation or constitutive activation of NF-kappa B, has stimulated a great deal of interest in therapeutics targeting various aspects of NF-kappa B signaling [58,59]. For example, one particularly effective compound, bortezomib (trade name Velcade), can suppress NF-kappa B-dependent gene expression, including cytokine production, through inhibition of the 26S proteasome. Inhibition of the 26S subunit has the effect of blocking the degradation of the Inhibitor of kappa B (I-kappa B), which keeps NF-kappa B in check, preventing its translocation to the nucleus. The net effect of bortezomib is blockade of NF-kappa B-dependent gene transcription. [60,61]. While bortezomib has proven effective against multiple myeloma, it is less effective for certain solid tumors and can in some instances, paradoxically activate NF-kappa B [62,63]. Furthermore, in ovarian and prostate cancer cells, bortezomib treatment has been shown to stimulate the secretion of IL-8. This upregulation proceeds through the direct phosphorylation and accumulation of the p65 subunit of NF-kappa B within the nucleus. Here, phospho-p65 is recruited to the IL-8 promoter along with EGR-1 (early growth response-1), and the inhibitor of kappa B kinase beta (IKK-beta) [64]. Given that ovarian cancer progression has been associated with elevated expression of IL-8 and other proinflammatory cytokines, this finding may explain the failure of bortezomib as an effective treatment for ovarian cancer.

Other cytokines contributing to anti-cancer drug resistance were discovered in the human fibrosarcoma cells. The HT 1080 cell line secreted high levels of AMF (autocrine motility factor) and were also resistant to mitomycine C (MMC)-induced apoptosis. Analysis indicated that AMF abrogated expression of Apaf-1 and caspase-9, which are essential for the progression of intrinsic apoptosis. Corroborating these findings, treatment with antibodies against AMF induced apoptosis in vitro and effectively aided MMC-induced apoptosis in vivo [65], altogether showing that AMF expression represents an important protective mechanism in the in vitro cultured human fibrosarcoma HT-1080 line.

Another secreted molecule which has recently emerged as a potential marker of drug resistance is adrenomedullin (AM), a 52-amino acid vasodilatory peptide. AM is expressed and secreted in both normal and malignant prostate cells, where it acts in an autocrine fashion. Under the anti-cancer drug etoposide, AM inhibited apoptosis in PC-3 and LNCaP prostate carcinoma cells. Constitutive expression of AM in PC-3 cells lowered basal levels of phosphorylated ERK1/2, which was unaffected following etoposide treatment. In addition, etoposide-induced apoptosis in PC-3 cells was significantly inhibited following constitutive AM expression, demonstrating that AM plays a critical role in preventing etoposide-induced apoptosis of prostate cancer cells. [66,67].

Based on observations that T-helper lymphocyte-derived cytokines could mediate autoimmune thyroid destruction, one group focused investigations on the autocrine activities of the Th2/Th3 cytokines IL-4 and IL-10 in modulating thyroid cancer resistance to cytotoxic chemotherapy agents. This group observed upregulation of the anti-apoptotic factors Bcl-2 and Bcl-xL in thyroid carcinoma cells which were refractory to cisplatin, doxorubicin or taxol. IL-4 and IL-10 secretion was associated with increased levels of Bcl-2 and Bcl-xL, thus protecting thyroid cells from chemotherapeutic agents. Furthermore, neutralization of IL-4 and IL-10 promoted thyroid cancer cell apoptosis and dramatically increased the effect of chemotherapy drugs. These data suggest that both chemokines have potent modulating influences on tumor cell survival [68].

The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) erlotinib causes feedback activation of STAT3 signaling in PC-9 cells, a non-small-cell lung carcinoma (NSCLC) line harboring *EGFR*-activating mutations. Intriguingly, the conditioned medium from erlotinib-treated PC-9 cells significantly increased erlotinib resistance when applied to naïve PC-9 cells. To identify the secreted factors that contribute to this induced drug resistance, a large antibody array-based screen of the culture medium was conducted. The array revealed that IL-6, IL-1 alpha and galectin were highly expressed, resulting in activation of STAT3 and its target IRF-1. Importantly, STAT3 knockdown efficiently prevented PC-9 from developing erlotinib resistance, which was thought to be due to STAT3 activation through IL-6/Janus kinase 1 (JAK1) signaling. Given that cytokine-induced STAT3 feedback activation is associated with poor prognosis in lung adenocarcinoma, this finding provides rationale for combination therapies that disrupt this feedback mechanism [69]. In agreement with this finding, the importance of STAT3 signaling in chemotherapeutic drug resistance was also demonstrated in a tamoxifen-resistant MCF-7 (TRM-7) cell model. TRM-7 culture medium was shown to strongly induce STAT3 phosphorylation in parental MCF-7 cells. Subsequently, a cytokine antibody array analysis revealed increased levels of RANTES in TRM-7 culture medium. This finding was validated by the addition of a neutralizing antibody to RANTES, which blocked STAT3 activation and lowered the resistance of TRM-7 cells to tamoxifen (Fig. 1). Thus STAT3-RANTES autocrine signaling is essential for tamoxifen resistance in human breast cancer cells [70].

There is evidence to suggest that, within a population of tumor cells, minority clones of intrinsically drug-resistant cells actually benefit from targeted drug therapy, allowing the tumor to progress faster. A recent study examined the mechanisms by which heterogeneous tumor cell populations evolve and adapt to therapeutic stress [71]. Rather than focusing on a few pre-selected proteins or pathways, a broader approach was taken. Transcriptomic and antibody array analysis of melanoma cells revealed that drug-sensitive cells stressed by vemurafenib (a B-RAF inhibitor) produce a complex secretome which not only promotes their survival, but hastens the growth and dissemination of neighboring vemurafenib-resistant cells. This therapy-induced secretome included many upregulated cytokines which resulted in hyperactivated PI3-kinase/Akt signaling. Since this finding predicted a possible route of combination therapy, cells were co-treated with vemurafenib and either MK2206 (Akt inhibitor) or BEZ235 (PI3-kinase/mTOR inhibitor). Indeed, the outgrowth of resistant cells was blunted under both of these conditions, diminishing the benefits of the therapy-induced secretome [71]. Thus, even as a tumor regresses in response to therapy, its microenvironment becomes infused with a variety of tumor-promoting signaling cues. Fortunately, the emerging population of resistant cells appears to be vulnerable to inhibition of the alternate pathways it activates.

Intrinsic or acquired resistance to targeted therapies may proceed through expression of growth factors that bind to alternate receptors and activate downstream survival pathways (thus bypassing the drug target entirely). Evidence of this type of adaptive signaling was demonstrated when HGF, a ligand of the hepatocyte growth factor receptor (HGFR, otherwise known as MET), was shown to induce gefitinib resistance in lung cancer cells with *EGFR*-activating mutations. Resistant cells were demonstrated to have restored PI3-kinase/Akt signaling via phosphorylation of the receptor MET, and that this activation occurred independently of EGFR. Reversal of gefitinib resistance by an anti-HGF antibody in vitro suggested that specific inhibition of the HGF/MET axis may be a viable therapeutic strategy to overcome TKI resistance [72]. In agreement with this rationale, later studies found that, in vitro and in vivo, small preexisting populations of lung adenocarcinoma cells exhibit *MET* amplification (and thus drug resistance) prior to TKI treatment. Furthermore, treatment with the EGFR kinase inhibitor

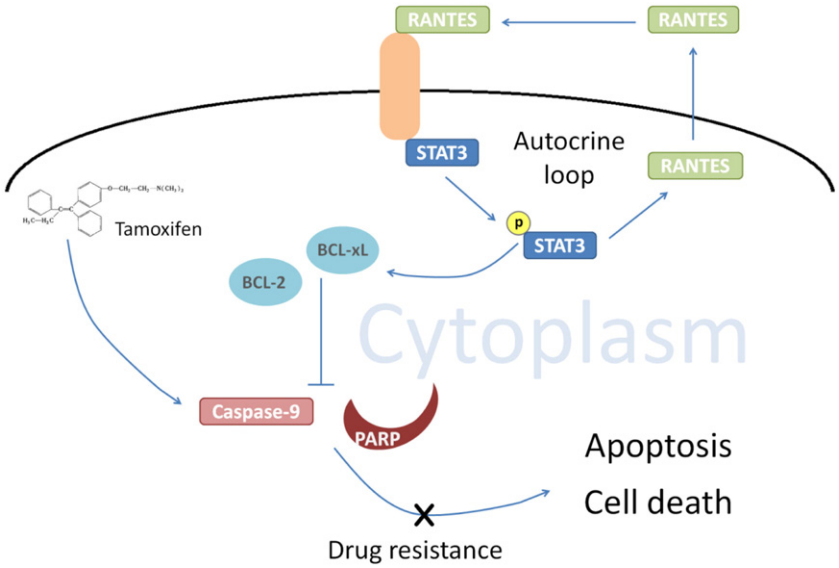

Fig. 1. Acquisition of tamoxifen resistance in MCF-7. The RANTES autocrine loop activates STAT3 pathway signaling which in turn, promotes RANTES secretion and inhibits caspase-9/PARP activity via the modulation of BCL-2/BCL-xL activity. Tamoxifen suppresses tumor cells by inducing apoptosis and caspase-9/PARP inactivation, thus sensitizing cells to chemotherapy drugs.

PF00299804 in the presence of HGF was shown to significantly accelerate the development of *MET* amplification, resulting in clonal expansion of these rare, TKI-resistant cells. Thus, specific mechanisms of acquired drug resistance may be predetermined. Importantly, the combination of MET inhibitor and TKI effectively reversed drug resistance. These findings suggest that treatment-naïve lung cancer patients harboring *EGFR* and *MET* mutations may benefit from initial combination therapy to block development of drug resistance [73].

In breast carcinomas, human epidermal growth factor receptor 2 (HER2) is highly expressed in 15–20% of cases. Trastuzumab (also known as Herclon or Herceptin), a monoclonal antibody that targets the HER2 receptor, is widely used to improve the prognosis of patients with HER2-positive breast cancer. However, a significant portion of HER2-positive breast cancer patients eventually develops trastuzumab resistance. Putative ligands of HER2 have been previously investigated [74] but no ligand binding directly to HER2 has been identified. HER2 shares common signaling pathways and forms a heterodimer with EGFR [75]. Amphiregulin is one of the ligands of EGFR and plays a prominent role in mammary gland development as well as tumorigenesis. Investigations into the clinical relevance of circulating amphiregulin on trastuzumab therapy in HER2-positive breast cancer patients revealed that high serum amphiregulin levels were associated with early disease progression, possibly due to Akt and ERK signaling activation by amphiregulin [76]. This observation represents a mechanism of cytokine-mediated drug resistance which involves activation of downstream signaling pathways common to the cognate receptor and the

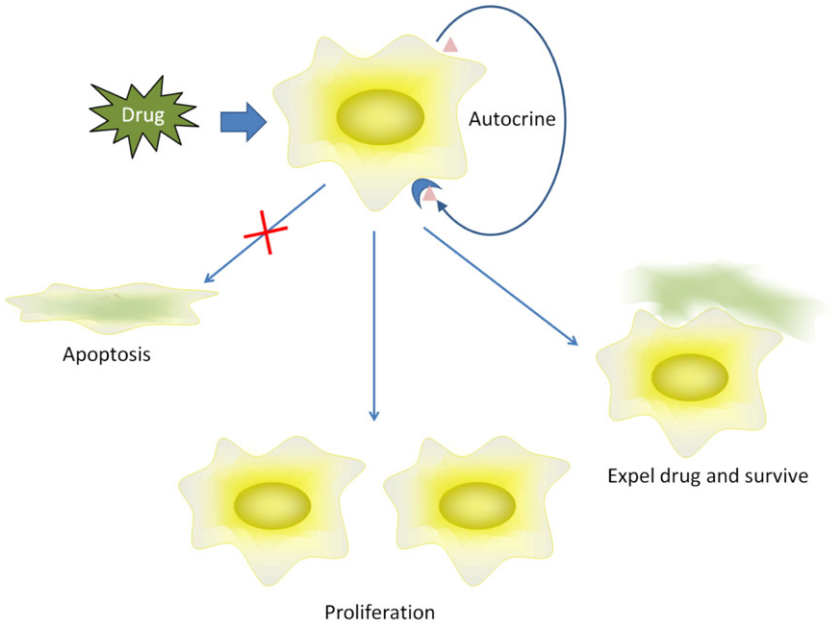

Fig. 2. Tumor cells evade drug inhibition by autocrine regulation. Cytokines secreted by tumor cells activate a variety of signal pathways that are involved in cell survival and proliferation, thus countering the chemotherapy drug effect. Some activated pathways upregulate expression of membrane proteins that function to expel drugs from the cytoplasm, thus reducing cytoplasmic drug concentration and contributing to the development of drug resistance.

therapeutic target (in this case, EGFR and HER2, respectively). It also implicates amphiregulin as a putative biomarker for resistance to first-line trastuzumab-based therapy in HER2-positive breast cancer patients.

4. Cytokines secreted from stromal cells contribute to cancer drug resistance

The TME plays an integral and increasingly appreciated role in tumorigenesis and cancer progression [77–79]. The resident cell types within the TME are varied and include numerous bone marrow-derived cell types (neutrophils, macrophages, mast cells, and others), endothelial cells of blood or lymphatic origin, pericytes, cancer-associated fibroblasts (CAFs), and mesenchymal stem cells. These non-malignant cells and the soluble factors they secrete can influence cancer cell growth and metastasis in diverse ways, for example by increasing cell survival, enabling invasiveness, and modulating drug response (Fig. 2). Immune cells may promote cancer initiation by secreting cytokines and growth factors which stimulate epithelial proliferation and generate reactive oxygen species, which can culminate in DNA damage [80,81]. In response to overproduction of VEGF, endothelial cells proliferate and form pathological vasculatures. Pericytes, which are recruited to the tumor by platelet-derived growth factor-beta (PDGF beta) gradients [82], take up residence on the exterior of blood vessels, where they are thought to play a role in immunosuppression. Fibroblasts, the predominant non-immune stromal cell type in the TME, not only synthesize and remodel the extracellular matrix (ECM) of the tumor stroma, but also support cancer cell growth by paracrine secretion of growth factors (Fig. 3).

While the role of the TME in cancer initiation, growth, and metastasis has been extensively studied, its role in chemotherapy drug resistance has only been partially described. Our sparse understanding of stroma-mediated drug resistance mechanisms is a major impediment to successful cancer treatment due to a lack of target molecules upon which to focus therapies. The contributions of the TME and its constituent stromal cell populations to targeted drug resistance are beginning to be appreciated; in fact, one study employed antibody arrays to probe stromal secretomes in order to begin to answer this question. Therein, it was discovered that tumor stroma can directly elicit innate resistance to RAF inhibitors through HGF secretion [83]. Using a co-culture system, 23 different stromal cell types were surveyed for their capacity to generate innate resistance in 45 cancer cell lines to 35 different anticancer drugs (both cytotoxic and oncoprotein-targeted agents). From this multitude of data, it was found that stroma-mediated resistance occurred frequently, particularly with the targeted drugs. Furthermore, this resistance was recapitulated by stromal cell medium alone, indicating a soluble secreted factor was responsible. Antibody array analysis of the medium revealed that HGF was expressed highly, and this resulted in downstream activation of MAPK and PI3-kinase/Akt signaling through MET, which conferred immediate resistance to the RAF inhibitor PLX4720 [83]. As with other studies that employ a dual inhibition strategy, the disruption of both HGF and MET function resulted in reversal of drug resistance, again showing that combination targeted therapy is a potential therapeutic strategy for melanoma, and exemplifying a powerful method of uncovering mechanisms underlying drug resistance.

Pancreatic cancer has the poorest prognosis of all the cancers and exhibits a high degree of resistance to available chemotherapeutic agents. The contribution of pancreatic stellate cells (PSCs) has been shown to be critical for pancreatic cancer progression [84] and potentially critical for chemoresistance [85,86]. Recently, a study probed the mechanism of PSC-mediated pancreatic cancer chemoresistance, specifically querying the contribution of SDF-1 alpha/CXCR4 signaling based on its critical role in a variety of other epithelial cancers [87–90]. It was demonstrated that primary PSCs expressed SDF-1 alpha, while its receptor CXCR4 was highly expressed in pancreatic cancer cells (PCCs). The culture media from PSCs repressed gemcitabine-induced cytotoxicity and apoptosis in PCCs; this effect was antagonized by an SDF-1 alpha neutralizing antibody and recapitulated with recombinant SDF-1 alpha treatment. Additionally, recombinant SDF-1 alpha increased IL-6 expression in PCCs in a CXCR4-dependent fashion, and increased focal adhesion kinase (FAK), ERK1/2, Akt and p38 phosphorylation. Taken together, these data demonstrated that PSCs can confer chemoresistance to PCCs via paracrine SDF-1 alpha/CXCR4-mediated activation of intracellular FAK-Akt and ERK1/2, with a subsequent IL-6 autocrine loop [91].

The bone marrow (BM) is composed of both hematopoietic cells and nonhematopoietic cells which include BM stromal cells, endothelial cells, osteoclasts, and osteoblasts. These cell types and the soluble factors which they secrete including cytokines, growth factors, and chemokines, along with the ECM, constitute the entire BM microenvironment [92]. The BM microenvironment plays an important role in differentiation, migration, proliferation, survival, and drug resistance of

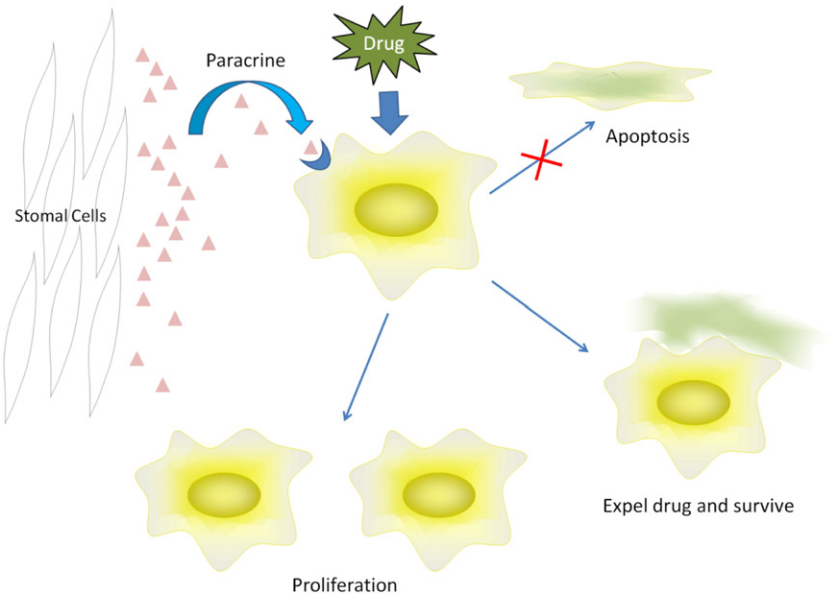

Fig. 3. The paracrine model of cancer drug resistance. The tumor microenvironment plays a crucial role in drug resistance. Stromal cells and tumor-associated cells secrete cytokines which act in a paracrine fashion, activating signaling pathways within tumor cells that contribute to drug resistance. Mechanisms include apoptosis inhibition, proliferation, and active transport drug elimination.

leukemia cells. For example, the c-KIT inhibitor imatinib, a highly effective therapy for treatment of chronic myeloid leukemia (CML), inhibits the *BCR-ABL* kinase oncogene in CML cells. However, the BM is known to confer imatinib resistance in K562 cells by secreting BM stroma-derived soluble factors that increase STAT3 phosphorylation (tyrosine 705) and subsequently increase the expression of anti-apoptotic proteins. [93]. Additionally, treatment with ABL TKIs cures only a minority of CML patients due to the activation of survival signals in CML stem/progenitor cells (SPCs) through pathways such as JAK2/STAT5 [94]. However, it has been reported that combination treatment of the JAK1/2 inhibitor ruxolitinib with the tyrosine kinase inhibitor nilotinib, reduced the activity of the JAK2/STAT5 pathway and contributed to the elimination of CML $CD34^+$ cells in vitro and in vivo. Thus, the JAK2/STAT5 pathway is a relevant therapeutic target for eradication of persistent disease in CML patients [95].

Acute myeloid leukemia (AML) is a malignant and aggressive disease which is insensitive to chemotherapy. The dynamic interaction between AML cells and BM microenvironment plays a critical role in the response of this disease to chemotherapy [96]. For example, imatinib and nilotinib (both inhibitors of c-KIT/ABL) have been shown to block proliferation of two c-KIT mutant AML cell lines, an effect which was significantly diminished when the cells were cultured in conditioned medium from BM stromal cells (either HS-5 or primary BM stromal cells). After testing the ability of several cytokines to rescue AML cell lines from c-KIT induced apoptosis, it was found that granulocyte colony stimulating factor (G-CSF) treatment could mimic the effects observed with HS-5 conditioned medium [97]. Thus, a clear role was demonstrated for G-CSF in modulating the response of AML cells to c-KIT inhibition (Fig. 4).

Bone is the main site of metastasis for prostate cancer cells, which depend on bone-derived factors for their drug resistance. Indeed, the culture medium from either primary osteoblast-like cells or from the MC3T3 cell line can stimulate proliferation of prostate cancer cell lines in a sphingosine 1-phosphate (S1P)-dependent manner [98]. S1P, a bioactive lipid mediator, is an important component of various processes in cancer biology, including cell proliferation, differentiation and survival. These effects are transmitted mainly through its five high-affinity surface G-protein-coupled receptors $S1P_{1-5}$. One study noted that the culture medium from MC3T3 cells enhanced the survival of CaP cells treated with docetaxel (a cytotoxic chemotherapy drug of the taxane family) or gamma-irradiation. These proliferative and survival effects were abolished when S1P secretion or activity from osteoblastic cells was blocked. This established that osteoblast-derived S1P can act as a paracrine survival factor and confer resistance to therapeutics against bone metastasis-derived prostate cancer [98].

5. Cytokines can be predictive biomarkers in cancer drug treatment

Sunitinib (known as Sutent by Pfizer), a broad spectrum TKI, is FDA-approved for the treatment of metastatic renal-cell carcinoma (MRCC). Research endeavors have focused on identifying predictive markers of sunitinib activity in MRCC. To this end, a cytokine antibody array detecting 174 human cytokines was employed to screen serum samples from 31 MRCC patients treated with sunitinib. In MRCC patients that did not respond to sunitinib, TNF-alpha and MMP-9 baseline levels were found to be significantly increased, and significantly associated with reduced overall survival and time-to-progression, concluding that levels of these two cytokines may be predictive markers of sunitinib activity in MRCC [99]. Because many patients develop sunitinib resistance and progressive disease after about 1 year of treatment, other studies have focused on the mechanisms of resistance. To this end, xenografts were developed to model clinical presentation. In this system, sunitinib-resistant tumors were found to exhibit increased secretion of IL-8 and higher microvessel density, while neutralization of IL-8 resensitized the tumor to sunitinib. Thus, IL-8 is both an important marker of sunitinib resistance and a candidate therapeutic target to reverse it [100]. In further attempts to identify factors that might predict response to sunitinib in MRCC, serum VEGF and neutrophil gelatinase-associated lipocalin (NGAL) levels were determined in 85 patients. Both VEGF and NGAL levels in serum proved accurate predictors of progression-free survival, and thus represent candidate biomarkers of the efficacy of sunitinib in MRCC patients [101].

In a study of clinical patients with pancreatic cancer treated with gemcitabine (a nucleoside analog), patients with both high serum IL-6 and IL-1 beta levels exhibited shortened overall and progression-free survival and a reduction in the tumor control rate. Together, serum levels of IL-6 and IL-1 beta can predict the efficacy of gemcitabine in patients with advanced pancreatic cancer [102].

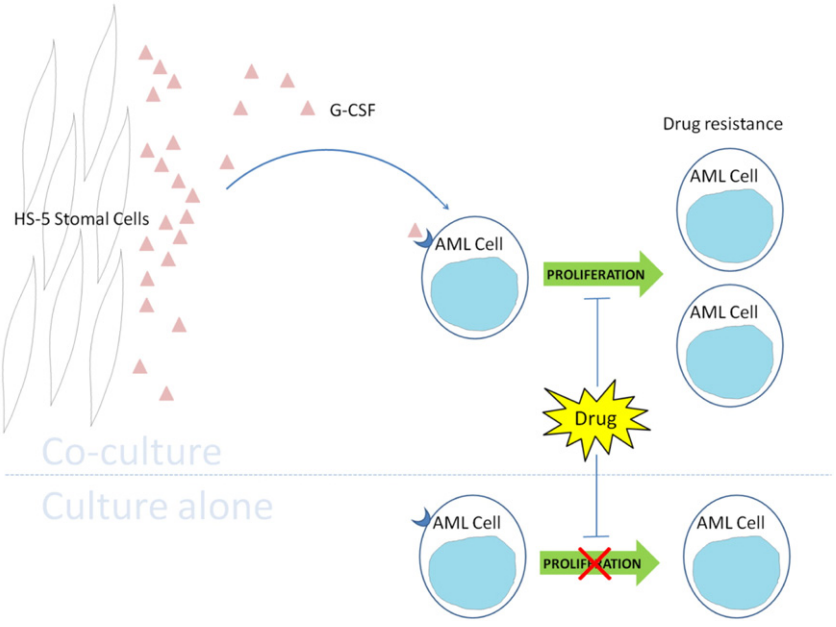

Fig. 4. Stromal cell-derived G-CSF contributes to acute myeloid leukemia drug resistance. When in co-culture with HS-5 stromal cells or grown in conditioned medium from HS-5 cells, AML cells continue to proliferate in the presence a c-KIT/ABL inhibitor. This protection is conferred by high levels of G-CSF secreted from stromal cells.

6. Conclusion

Resistance to chemotherapy and oncoprotein-targeted drugs remains the biggest impediment in oncology, disrupting the long term success of treatment of cancer patients. In this review, we described and assessed examples of the mechanisms by which cells and tissues develop resistance against chemotherapy drugs, particularly those involving altered cytokine expression and their downstream signaling networks. As mentioned earlier, our understanding of the molecular mechanisms of chemoresistance is woefully incomplete. The studies described here however, have contributed foundational insights, identifying several key pathways which cancer cells commonly hyperactivate or block in order to evade targeted therapeutic agents. These pathway perturbations are caused by (or result in) a telltale cytokine signature. Individually, these cytokines can stimulate tumor-promoting pathways through autocrine loops and/or paracrine mechanisms emanating from re-educated stromal cells. Collectively, these cytokines contribute to a TME which is permissive to tumor progression and drug resistance (Table 1).

Perhaps the most habitual mediator of resistance mechanisms is IL-6. IL-6 is in fact, one of the most universally dysregrulated cytokines in cancer patients [103]. The relevance of this cytokine as a prognostic biomarker was initially noted in breast and prostate cancers, where elevated serum levels correlated with advanced stage disease and poor prognosis. A pleiotropic cytokine with a variety of biological activities in inflammation, hematopoiesis, and immune regulation, IL-6 is expressed by many cell types. The pleiotropy and redundant functions of IL-6 are attributable to its bifunctional receptor system, which consists of the IL-6 receptor (the IL-6-specific component) and the common signaling receptor gp130. The dimerization of these 2 components results in activation of the JAK–STAT, Akt, and Ras–MAPK pathways [104]. At present, the involvement of the IL-6/IL-6R signaling axis in oncogenic transformations is well established.

The IL-6 gene has a promoter region containing an NF-kappa B-binding site [105]. NF-kappa B, a master orchestrator of inflammation and innate immunity, has recently emerged as a crucial component of oncogenesis and tumor progression [106]. NF-kappa B regulates many of the cellular processes which, when dysregulated, become the hallmarks of cancer; these include proliferation, cell adhesion, pro-survival programs, and secretion of a plethora of cytokines which modulate immune response and influence the microenvironment. The same can be said of STAT3, a rapidly inducible transcription factor which controls a broad array of cellular defense responses, and which is often inappropriately activated in tumor cells. It is now apparent that constitutive activation of either NF-kappa B or STAT3 (or both) typifies many cancer types. The dysregulation of these pathways may arise from sustained exposure of the cell to paracrine cytokines or from mutations in upstream signaling molecules that result in pathway hyperactivation within the cell [106–108]. The end result is the release of key cytokines which create a microenvironment that is hypervascularized and replete with immunosuppressive stromal cells.

HGF and its cognate receptor MET have emerged as lynchpin markers in certain melanomas and NSCLCs. Oncogenesis has been documented as a result of either overexpression of HGF (which activates MET in an autocrine fashion) or overexpression of MET itself [83,109]. This then leads to dysregulated signaling through the RAS/RAF/MEK/ERK axis, which stimulates abnormal proliferation.

There is mounting evidence for the emergence of compensatory pathway activation by tumor cells to escape the effects of oncoprotein-targeted drugs. For example, lung adenocarcinoma cells can bypass gefitinib-mediated ErbB3 blockade by autocrine upregulation of HGF, which in turn stimulates the PI3-kinase/Akt pathway downstream of MET [72,109]. HGF was also identified as a stroma-derived paracrine mediator of RAF inhibitor resistance in B-RAF mutant melanoma, and this protection proceeds through ERK and Akt pathway activation [83]. These alternate pathways may represent an important Achilles heel in resistant cancer cells, as suggested by the observation that STAT3 knockdown resensitizes *EGFR* mutant PC-9 cells to erlotinib [69]. It should be noted that in the studies mentioned above, the identification of alternate cancer-sustaining pathways was enabled by the screening of secreted

Table 1
Cytokines and their proposed roles in drug resistance.

Cancer type	Drug	Drug target	Cytokine	Cytokine function(s)	Ref #
Prostate	Enzalutamide	Androgen receptor	IL-6	Blocks enzalutamide apoptosis via STAT3	[53]
Prostate	Etoposide	Topoisomerase II	Adrenomedullin	Reduces ERK1/2 phosphorylation and PARP cleavage	[66]
Breast	Doxorubicin	Topoisomerase II	IL-6	Induces upregulation of *mdr-1*	[50]
Breast	Paclitaxel, Doxorubicin	Microtubule function; topoisomerase II	IL-6, IL-8	Associated with P-gp expression; required for MDR phenotype	[54]
Breast	Tamoxifen	Estrogen receptor	RANTES	Induces phosphorylation of STAT3 (Tyr705)	[70]
Breast	Trastuzumab	HER2/neu	Amphiregulin	Associated with poor response to trastuzumab, possibly through increased AKT and ERK signaling	[76]
Thyroid	Cisplatinum, Doxorubicin, Taxol	DNA replication & repair; microtubule function; topoisomerase II	IL-4, IL-10	Upregulates Bcl-2 and Bcl-xL; blocks apoptosis	[68]
Fibrosarcoma	Mitomycine C	DNA replication & repair	Autocrine motility factor	Blocks expression of Apaf-1 and caspase-9	[65]
Lung	Gefitinib	EGFR	HGF	Restores P13-kinase signaling through MET activation	[72]
Lung	Erlotinib	EGFR	IL-6, IL-1 alpha	Feedback activation of STAT3 and IRF1 through IL-6/JAK1	[69]
Lung	Dacomitinib (PF-00299804)	EGFR	HGF	Accelerates development of *MET* amplification, which then restores Akt signaling	[73]
Melanoma	PLX4720	B-RAF	HGF	Stromally-derived HGF activates MAPK and Akt signaling through MET	[83]
Melanoma	Vemurafenib	B-RAF	IGF-1, EGF, PDGFD, others	Hyperactivation of PI3 kinase/Akt signaling	[71]
Pancreatic	Gemcitabine	DNA replication & repair	SDF-1 alpha	Stromally-derived SDF-1 alpha activates FAK/Akt & ERK1/2 signaling in a CXCR4-dependent manner	[91]
Chronic myeloid leukemia	Imatinib, Nilotinib, Dasatinib	BCR-ABL kinase/c-KIT	IL-6, G-CSF, GM-CSF	Stromally-derived cytokines putatively activate JAK2 signaling	[94]
Acute myeloid leukemia	Imatinib, Nilotinib	BCR-ABL kinase/c-KIT	G-CSF	Attenuates apoptosis	[97]
Renal cell carcinoma	Sunitinib	Tyrosine kinase	VEGF, NGAL	Predicts PFS	[101]
Renal cell carcinoma	Sunitinib	Tyrosine kinase	TNF alpha, MMP-9	Associated with reduced overall survival and time-to-progression	[99]
Renal cell carcinoma	Sunitinib	Tyrosine kinase	IL-8	Associated with resistance	[100]
Other	RO4929097	Gamma-secretase	IL-6, IL-8	Restores angiogenesis	[55]

factors or receptor activation using antibody array technology. It is thus expected that the approach of collecting broad protein signatures of both cancerous and stromal cells will reveal a wealth of information about the biology of the TME and its enigmatic role in chemoresistance. In fact, Straussman et al. noted that the HGF-mediated resistance to RAF inhibition which they reported was but one of the scores of drug resistance interactions identified in their screen [83].

The combination of many recent insights into the signaling networks of chemoresistance are repeatedly converging on the idea that drug resistance may be circumvented by using treatment regimens employing combinations of precisely targeted inhibitors, provided the tumor can be sufficiently characterized first. In fact, a number of combination targeted therapies have been investigated both clinically and preclinically, with the idea that coinhibition of 2 or more important compensatory signaling pathways can block the tumor's potential escape route. However, targeted combination therapies are still in very early stages of development and only a few clinical successes have been achieved (notably, dual inhibition of HER2 and mTOR pathways) [110]. But how should we approach characterizing tumor cells and prescreening patients to prescribe the optimal combination of drugs? Recent advances have equipped scientists with many investigative tools to identify the prominent adaptive and microenvironmental changes that underlie resistance against therapies. Identifying the cobweb of cellular modifications even in the context of one drug and one cancer type is an arduous task, as is evident by the multitude of research manuscripts devoted to this topic. For example, open ended screens that employ short guide RNAs that activate CRISPR/Cas9-based gene knockouts, identified constitutively expressed genes that drive resistance [111,112]. Other cell culture-based screening approaches in combination with antibody array-based expression profiling identified proteins that function in microenvironmental induced drug resistance [83]. Extensive drug-based screening discovered mechanisms of resistance using pharmacologic probes that block resistance [113]. Indeed, specific mechanisms of resistance were discovered in individual tumors by taking biopsy samples from EGFR mutation-positive lung adenocarcinoma patients. Cell lines established from these biopsies were subjected to drug screens, whereupon potential chemotherapeutic drugs that increased the sensitivity of patient-derived cancer cells were discovered [95]. Together, it is expected that approaches like these will progress and become more sophisticated in their approach to study drug resistance.

Using these reports as rationale, we have shown that multiplex immunoassays which can quickly collect inflammatory, angiogenic, apoptotic, or growth factor profiles, identify prognostic biomarkers and reveal critical but unexpected pathway activation in multiple cell types. Due to the heterogeneity of tumor cell populations and of different cancer types, the idea of common mechanisms seems to now be obsolete. Likewise, the idea of a single protein as a cancer biomarker is of little use, as it is now apparent that panels of multiple cytokines are more informative as prognostic measures of cancer [114].

The last decade has brought an explosive growth in our understanding of how tumors evade and usurp the immune system, and with it, the much anticipated realization of cancer immunotherapy. This novel treatment modality refers to agents which exploit and augment the immune system's intrinsic ability to target specific mutations expressed by cancers. Employing the strategy of immune checkpoint therapy has given us three breakthrough agents: a monoclonal antibody against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (ipilimumab) and two monoclonal antibodies against programmed death-1 (PD-1) (pembrolizumab and nivolumab) for the treatment of advanced melanoma. Another immunotherapy strategy which is currently on an upward trajectory is chimeric antigen receptors (CAR; composed of a single chain antibody fused to intracellular signaling chains) T cell adoptive immunotherapy. In the wake of the successes of anti-CTLA-1 and anti-PD-1, the biggest obstacle to further advancement of cancer immunotherapy is the identification of more target molecules which are cancer cell-specific.

Enhanced knowledge of oncogene signaling pathways and networks will undoubtedly advance chemotherapeutic procedures by determining the best consolidations of drugs to employ. This knowledge is gathered from measuring both the intrinsic protein signature within tumors, and the protein signature of circulating blood. With the increased knowledge of the cytokine profiles that defines cancer resistance, we expect to see longer-term remissions in the future.

Statement of conflict of interest

All of the authors of this paper are employees of RayBiotech, Inc., a company producing commercial antibody arrays which were used in some cited references.

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