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Mechanisms of Drug Resistance in Cancer Therapy

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Mechanisms of Drug Resistance in Cancer Therapy

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Preface

The remarkable advances in precision medicine and the development of targeted cancer treatments have generated significant optimism based on the assumption that a better knowledge of genetic or molecular features sustaining cancer growth could determine long-term control of many types of cancer and potentially cure the majority of patients. After several years of basic and translational research, the oncology community has tempered this optimism by the recognition that in only a minority of patients cure can be achieved by innovative strategies such as targeted therapies, immunotherapy and cytotoxic agents. In the vast majority of cancer patients, cancer wins the battle, and the reason for this failure is drug resistance. Ultimately, advanced cancer patients die because some or all of their tumour cells exhibit or develop resistance to available therapeutic strategies. The challenge of drug resistance therefore represents an important barrier that hinders the ultimate goal of cure or at least long-term control of cancer.

As a corollary of this, response rates in the setting of tumour progression/relapse are dismal. Despite the considerable importance of drug resistance to cancer therapies, our understanding of their biological mechanisms – and plausible therapeutic avenues to intercept them – remains highly incomplete.

Knowledge of specific resistance mechanisms can inform novel therapeutic approaches to counter this phenomenon, and improvement of our understanding of key driver genes could guide new therapeutics approaches capable of eliciting meaningful tumour responses in patients with advanced malignancies.

As previously reported with chemotherapy, it is reasonable to suppose that a combination of multiple targeted therapies and immunotherapy strategies as well as planned sequence of treatments will be necessary to effectively prevent and/or treat drug-resistant cancers. The potential number of therapeutic combinations is immense; thus, new preclinical paradigms are needed to prioritize high-yield combinations and define the genetic or molecular contexts in which they would most likely be efficacious. To improve outcome of patients a strict collaboration between academia and industry will be needed so that the appropriate resources and innovation may be brought to bear on this challenge. The goal to achieve durable control of many cancer subtypes will likely require dedicated, multidisciplinary teams of preclinical and clinical experts that work together guided by rigorous translational and analytical science.

With the increasing armamentarium of anticancer agents and the advent of powerful high-throughput screening techniques, there are now unprecedented opportunities to understand and overcome drug resistance through the clinical assessment of rational therapeutic drug combinations and the use of predictive and prognostic biomarkers to enable patient stratification and tailor treatments.

The main aim of this book is to offer to the readers an updated overview on the possible reasons of failure of new and promising therapeutic opportunities.

The first part of the book covers the basic mechanisms of such hot topics. The other chapters cover specific pathways of resistance that from the knowledge of basic mechanisms translate this information in clinical routine or in translational clinical research.

Readers will discover diverse perspectives of the contributing authors (such as basic scientists, clinical pharmacologists and clinicians) and extensive discussions of issues including pharmacodynamic and pharmacokinetic mechanisms of resistance, alterations in the drug target, activation of prosurvival pathways, ineffective induction of cell death and plasticity of microenvironment.

This book will be of interest to scholars and researchers, biologists, pharmacologists, medical oncologists, haematologists and immunologists.

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Molecular and Pharmacological Mechanisms of Drug Resistance: An Evolving Paradigm

Benedetta Colmegna, Lavinia Morosi, and Maurizio D'Incalci

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Abstract

The high heterogeneity and genomic instability of malignant tumors explains why even responsive tumors contain cell clones that are resistant for many possible mechanisms involving intracellular drug inactivation, low uptake or high efflux of anticancer drugs from cancer cells, qualitative or quantitative changes in the drug target. Many tumors, however, are resistant because of insufficient exposure to anticancer drugs, due to pharmacokinetic reasons and inefficient and heterogeneous tumor drug distribution, related to a deficient vascularization and high interstitial pressure. Finally, resistance can be related to the activation of anti-apoptotic and cell survival pathways by cancer cells and often enhanced by tumor microenvironment.

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Apoptosis • Drug distribution • Mass spectrometry imaging • Multidrug resistance • Pharmacokinetics

1 Introduction

Although significant improvement of the therapy of several human malignancies have been achieved in the last two decades with the use of small molecules or antibodies designed to hit cancer-specific molecular targets, for the majority of solid human tumors at advanced stage pharmacological treatments have only a transient antitumor effect with small or no impact on patients' survival. The major reason for this disappointing result is the phenomenon of drug resistance.

Although most recent research has been focused on molecular and cellular mechanism of resistance, it seems likely that in many cases the lack of antitumor activity is related to pharmacokinetic reasons, essentially associated with an insufficient drug concentration in the cancer cells.

The pharmacokinetic factors that can be involved in the failure of treatment can be dependent on the route of administration. For example, in the case of orally administered drugs the variable absorption as well as the first pass effect can be responsible for an insufficient bioavailability. For many anticancer drugs the variable metabolism can be a clinically relevant problem, also in consideration of potential interactions with other drugs interfering with the activity of cytochromes involved in drug biotransformation. These pharmacokinetic factors can be investigated by monitoring plasma drug levels, thus allowing, at least in theory, a dose adjustment or a change in the route of administration.

However, the drug plasma levels that for other classes of drugs predict the drug concentration at the pharmacological target, for anticancer drug are often meaningless. The circulating drug concentrations in fact do not necessarily mirror those present in the neoplastic tissue and its metastases because of tumor architecture and microenvironment (Fuso Nerini et al. 2014).

It is known that the vascularization of solid tumors is often inefficient, as the angiogenic process occurring in growing tumors leads to unorganized capillary network with fenestrated vessels and significant leakage of proteins responsible for an increase in the interstitial pressure. Probably further factors contributing to the high interstitial pressure are the inefficient lymphatic drainage as well as a significant infiltration of inflammatory and mesenchymal cells that represent common features of many solid tumors. This peculiar abnormal vascularization and tissue architecture is responsible for a low and heterogeneous tumor drug distribution (Minchinton and Tannock 2006). The evidence of this heterogeneous distribution of anticancer drugs was already available several years ago by examining different parts of tumors. It was demonstrated that in necrotic or hypovascularized and hypoxic parts of the tumor the drug concentrations were lower than those that were normally perfused by blood.

Recently new powerful technologies have been developed to investigate drug distribution in different parts of the tumor by simultaneous visualization and quantification of the drug levels. Among these new approaches, mass spectrometry imaging (MSI) is an interesting technique to obtain specific information on the spatial distribution of a drug linking qualitative molecular information of compounds to their spatial coordinates and distribution within the investigated tissue (Morosi et al. 2013).

MSI can be applied to visualize potentially every tissue component localizing the molecule of interest on the base of its molecular mass and fragmentation pattern, without the need to label the analyte, a great advantage over other imaging techniques such as fluorescence microscopy, positron emission tomography, magnetic resonance spectroscopy, and autoradiography (Sugiura and Setou 2010).

MSI was applied to localize drug distribution and penetration inside solid tumors, thanks to its superior spatial resolution (20–100 μm) and specificity allowing the detection of parent compound and metabolites simultaneously (Buck et al. 2015; Connell et al. 2015).

MSI can also provide the quantitative amounts of target compounds in well-defined region of interest of the examined tissue, ideally in a single pixel. This goal is extremely challenging depending on the technical possibilities and limitations of the MSI instrument hardware, but equally on the chosen calibration/standardization strategy (Giordano et al. 2016a; Rzagalinski and Volmer 2016).

MSI has also some limitation: it is only applicable to molecules that are ionizable, the selection of optimal matrix is arduous and driven by empirical results, moreover the interpretation of quantitative data is often complex especially because of ion suppression effect and the chemical noise from the matrix ions covering the drug ion signal and finally, the sensitivity is quite limited (Prideaux and Stoeckli 2012).

By applying this methodology it has become clear that drug distribution in tumors is very heterogeneous comparing different tumor models having different histopathological characteristics (Giordano et al. 2016b). Figure 1 shows the intratumor distribution of the anticancer drug paclitaxel by MSI. The drug distribution is homogeneus only in the ovarian cancer model while it is highly irregular in the other exemples of tumor types. As you can see in other models such as sarcoma or breast cancer, areas of the tumor where the drug is highly concentrated and areas where it is almost absent can be observed at the same time. Broad parts of the tumor where the drug is not present at all are present especially in mesothelioma xenograft. This aspect surely contributes to explain tumor resistance. Torok et al. recently correlate the antitumor activity of several antiangiogenic drugs with their intratumoral distribution data obtained by MSI (Torok et al. 2017). Moreover, Cesca et al. observed an enhancement in paclitaxel activity combined with bevacizumab associated with the improvement of its intratumor distribution (Cesca et al. 2016).

The possibility to superimpose MSI molecular images with histological and immune-histological evaluation could help understanding the factors that impair drug penetration. Finally, this technology could be used in future to evaluate the

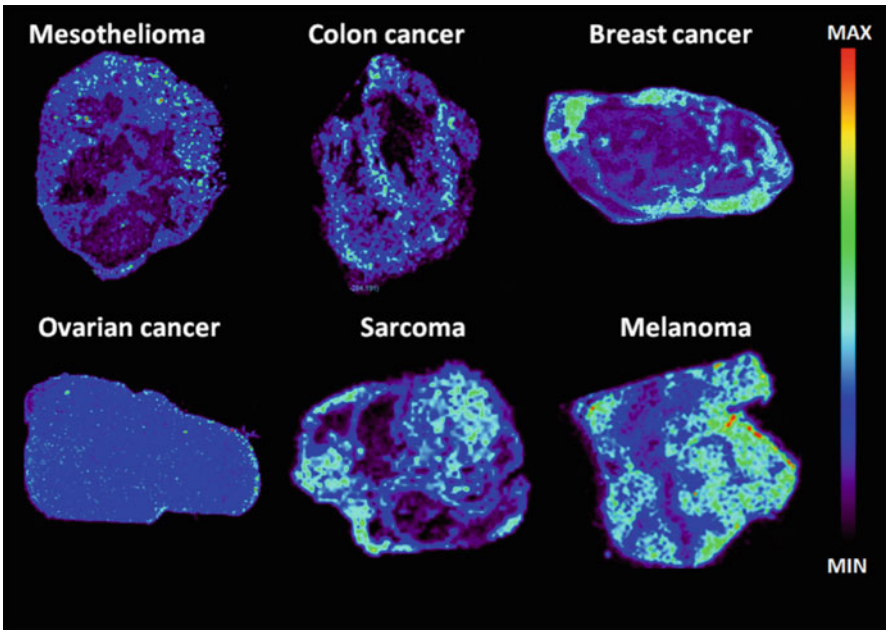

Fig. 1 Paclitaxel distribution in different cancer models analyzed by MALDI mass spectrometry imaging. *Light blue* and *green* mark the drug presence on the basis of the color scale on the right

effect of therapeutic strategies aimed at modifying tumor microenvironment or altering drug properties to facilitate drug uptake and intratumor distribution.

2 Cellular Mechanism of Multidrug Resistance

The hallmarks of cellular anticancer drug resistance fall into distinct categories:

2.1 Drug Activation and Inactivation

Altered local drug metabolism and detoxification are key resistance mechanisms highly specific for each class of drugs. Lack of prodrug activation or epigenetic silencing – by promoter methylation – of genes involved in drug processing are examples of potential cause of resistance and are frequently tumor-specific. Classical examples could be the inactivation of platinum drugs by the thiol glutathione (Meijer et al. 1992) or metallothioneins (Amable 2016), or by epigenetic mechanisms, e.g., silencing – by methylation – of gene encoding for thymidine phosphorylase, an enzyme involved in the conversion of capecitabine (prodrug) to 5-fluorouracil (active drug) (Longley et al. 2003). For many DNA interacting agents, e.g., alkylating agents, anthracyclines, and platinum drugs, an important

mechanism of inactivation is related to the overexpression of Glutathione–S–Transferases (GSTs) that metabolizes the drugs into inactive molecules (Kauvar et al. 1998).

2.2 Expression of Drug Efflux Pumps

Plasma membrane transport proteins are key players in the uptake of nutrients, such as sugars, amino acids, nucleosides, and inorganic ions and the efflux of xenobiotic toxins, including many anticancer drugs. Transport proteins can be classified into two major families, the solute carrier (SLC) and the ATP-binding cassette (ABC) transporters and can affect drug absorption, distribution, and excretion conferring sensitivity or resistance to anticancer drugs.

The importance of ABC transporters in cancer therapy has been well documented while the impact of the solute carriers (SLC) on cancer therapy has not been extensively characterized (Table 1).

So far, the most characterized transporters belong to ABC family and include ABCB1 (also known as MDR1 or P-glycoprotein), ABCC1 (MRP1), and ABCG2 (BCRP or MXR).

Table 1 Reports the transporters whose activity has been associated with drug resistance

Transporters	Chemotherapeutic drugs effluxed
P-gp/MDR1	Anthracyclines, taxanes, vinca alkaloids, imatinib, etoposide
MRP1	Anthracyclines, methotrexate, camptothecins, etoposide, glutathione conjugates
MRP2	Doxorubicin, methotrexate, cisplatin, vincristine
MRP3	Methotrexate, etoposide
MRP4	Methotrexate, camptothecins, thiopurines
MRP5	Methotrexate, thiopurines
MRP6	Etoposide
MRP8	5-Fluorouracil
BCRP/MXR	Doxorubicin, mitoxantrone, topotecan, flavopiridol, SN-38
LRP	Doxorubicin, cisplatin, vincristine, etoposide, paclitaxel, melphalan
Human nucleoside transporters (hENT, hCNT)	Cytarabine, gemcitabine, fludarabine, clofarabine
Copper transporters (Ctr1, Ctr2, ATP7A, ATP7B)	Cisplatin
Multidrug extrusion transporter1 (MATE1)	Cisplatin
Organic cation transporter (OCT1, -2, -3)	Platinum drugs, SN-38, mitoxantrone, melphalan
Organic anion transporter (OAT1, -2, -3)	Methotrexate, 5-fluorouracil, paclitaxel

The ATP-binding cassette (ABC) transporters superfamily is a large group of membrane proteins that are responsible for the translocation of various cytotoxic molecules out of the cell reducing their intracellular concentration and thus their cytotoxicity. ABC transporters are codified by 48 genes (divided into 7 subfamilies) and characterized by a conserved quaternary structure containing 2 transmembrane domains (TMDs) and 2 nucleotide (ATP)-binding domains (NBDs). Their ATP-dependent activity is involved in the movement of a wide variety of xenobiotics – including drugs – lipids, peptides, and metabolic products across the plasma and intracellular membranes. The ABC transporters tissue distribution reflects the complex physiological network of these proteins and reveals their important role in absorption, excretion, and distribution of drugs.

P-glycoprotein (P-gp) is a 170 kDa glycoprotein coded by ABCB1 gene mapped to chromosome 7q21.1. Its presence in normal tissue (as liver, kidney, brain, intestine, etc.) reveals the ability of modulating bioavailability and transport of wide range of substrates, especially organic molecules containing aromatic groups, and suggests its role in determining multidrug resistance (MDR) phenotype. P-gp is overexpressed in many tumors (thus causing intrinsic drug resistance) and the expression of P-gp can also be induced by chemotherapy (thus resulting in the acquired development of MDR) (Thomas and Coley 2003). Expression of P-gp fluctuates with elevated expression level in untreated cancer into higher level upon relapse after chemotherapy and undetectable or low level in the expression in drug sensitive tumors. P-gp expression has been reported in 40% of breast cancer (Kao et al. 2001), 20% of ovarian cancer (Baekelandt et al. 2000), and in more than 50% of patients with acute myelogeneous leukemia experiencing relapse (Leith et al. 1999) and is now associated with treatment failure in kidney, liver, and colon cancer (Ambudkar et al. 2003). It confers the strongest resistance to the widest variety of compounds including vinca alkaloids, anthracyclines, and taxanes contributing to the failure of chemotherapy (Szakács et al. 2006). Recent reports have suggested that molecularly targeted therapies, such as some kinases inhibitors, are also substrates for drug efflux proteins (Shervington and Lu 2008).

MRP1 is a transporter involved in glutathione-linked organic compound transport and is normally expressed in the basolateral membrane of cells from testis, kidney, oropharyngeal mucosa, etc. Recently the overexpression of MRP1 has been correlated with chemoresistance in prostate, lung, and breast cancer (Holohan et al. 2013) and confers resistance to several hydrophobic compounds that are also P-gp substrates.

Screens carried out with the NCI60 cell panel indicate that there is a strong correlation between the expression of several transporter (including ABC superfamily) and decrease in chemosensitivity.

The goal of reversing MDR in the clinic through the pharmacological inhibition of ABC transporter has been pursued for years and different generation of inhibitors have been developed. Only P-gp inhibitors have been evaluated in clinical studies. However, the results of clinical trials using modulators of P-gp have been disappointing mainly because of the required marked reduction of the anticancer drug doses. In addition it is now very clear that resistance is not only due to the

overexpression of P-gp, but also to many other mechanisms, thus making unrealistic to reverse tumor resistance by pump inhibition.

The resistance of tumors originating from tissues expressing high levels of P-gp (such as colon, kidney, or the adrenocortex) often extends to drugs that are not subject to P-gp-mediated transport, suggesting that "intrinsically resistant" cancer is also protected by non-Pgp-mediated mechanisms (Szakács et al. 2006).

Evidence linking P-gp expression with poor clinical outcome is more conclusive for breast cancer, sarcoma, and certain types of leukemia. P-gp expression in patients with AML has consistently been associated with reduced chemotherapy response rates and poor survival (Pallis and Russell 2004), in contrast with MRP1 whose expression is not a significant factor in drug resistance in AML (Leith et al. 1999).

The expression of these transporters as a mechanism of acquired resistance after a specific treatment is also responsible for the phenomenon of cross-resistance that must be taken into account especially for a clinical perspective.

2.3 DNA Damage Repair

The cellular response to DNA damage involves several mechanisms aimed at repairing the damage, thus restoring DNA integrity or alternatively activating cell death pathways.

Many chemotherapeutic drugs induce DNA damage either directly (for example, platinum-based drugs) or indirectly (for example, topoisomerase inhibitors). Without repair, this damage can result in genetic instability, acquisition of further mutations, and cytotoxic effects (Holohan et al. 2013). The different sensitivity to DNA damaging agents can be due to a different ability to repair drug-induced damage. For example, cancer cells deficient in Homologous Recombination (HR), e.g., because of mutations of BRCA1 or BRCA2 genes, are very sensitive to DNA damage caused by platinum drugs. HR deficient cancers are very sensitive to PARP inhibitors that cause an accumulation of DNA single strand breaks. In HR proficient cells, DNA single strand breaks generate DNA double strand breaks during DNA replication that are repaired before mitosis. Instead in HR deficient cells the inhibition of base excision repair by PARP inhibitors leads to unrepairable DNA double strand breaks with consequent cell death. This is the concept of synthetic lethality whose application has shown promising results in patients with breast and ovarian tumors (Farmer et al. 2005). Even in this case resistance can occur because of the acquisition of secondary mutations restoring HR normal function (Ashworth 2008).

Other pathways involved in DNA repair that have been reported to be relevant for drug resistance are nucleotide-excision repair (NER) and mismatch repair (MMR). The first one is required for the repair of DNA damage caused by many DNA-damaging drugs, such as platinum-based drugs. High expression of a key component, excision repair cross-complementing 1 (ERCC1), has been linked with poor responses to chemotherapy in non-small-cell lung carcinoma (NSCLC),

gastric, and ovarian cancer (Kirschner and Melton 2010). Notably, testicular cancers, which are very sensitive to cisplatin treatment, have very low levels of ERCC1 (Usanova et al. 2010).

The mismatch repair (MMR) system, whose key players are MLH1 and MSH2 genes, is crucial for maintaining genomic integrity, and its deficiency has been linked to high mutation rate of a large number of genes including those involved in immunogenicity and drug sensitivity. This explains why hypermethylation of MLH1 causes resistance to cisplatin and carboplatin (Fink et al. 1998). More recently, a synthetic lethal interaction between MSH2-targeted short interfering RNA (siRNA) and methotrexate was identified in MMR-deficient cancer cells; methotrexate caused the accumulation of oxidative lesions such as 8-oxoguanine (8-oxoG) in MSH2-deficient cells, which resulted in a loss of viability through apoptosis. This has led to an on-going Phase II clinical trial with methotrexate in patients with MSH2-deficient metastatic colorectal cancer using measurement of 8-oxoG lesions as a biomarker (Holohan et al. 2013).

Another DNA protein involved in resistance is *O*-6-methylguanine-DNA-methyl transferase (MGMT), whose overexpression is responsible for the resistance to methylating agents such as temozolomide. The hypomethylation of the promoter of the MGMT gene has been associated with resistance of human glioblastoma to temozolomide (Thomas et al. 2017).

2.4 Deregulation of Apoptosis

The resistance to anticancer drugs can be due to the fact that in cancer cells, apoptotic pathways are frequently dysfunctional. Numerous intrinsic adaptive responses can be triggered and promote survival of cancer cells exposed to DNA-damaging agents. Anti-apoptotic BCL-2 family members, inhibitor of apoptosis proteins (IAPs), and the caspases are key proteins of this mechanism. Mutations, amplifications, chromosomal translocations, and overexpression of the genes encoding for these proteins have been associated with various malignancies and linked to resistance to chemotherapy and targeted therapies.

BCL-2 family proteins have a pivotal role in dictating cell fate following chemotherapy treatment. The balance between the anti-apoptotic BCL-2 family members (such as BCL-XL and MCL1) and pro-apoptotic family members (such as BAX, BAD, and BAK, as well as various BH3-only proteins) is critical in determining the activation or not of a common pathway generally stimulated from all cytotoxic drugs that causes the mitochondrial outer membrane permeabilization (MOMP) and finally cell death. Overexpression of one or more anti-apoptotic proteins or underexpression of one or more pro-apoptotic proteins or a combination of both dysregulate apoptosis. For example, the overexpression of Bcl-2 protected prostate cancer cells from apoptosis while led to inhibition of TRAIL-induced apoptosis in neuroblastoma, glioblastoma, and breast carcinoma cells. In colorectal cancers bax(G)8 frameshift mutations could contribute to resistance of cells to anticancer treatments. In the case of chronic lymphocytic leukemia (CLL), the

malignant cells have an anti-apoptotic phenotype with high levels of anti-apoptotic Bcl-2 and low levels of pro-apoptotic proteins such as Bax (Wong 2011). The IAPs are a group of structurally and functionally similar proteins that regulate apoptosis by inhibiting caspase activity and promoting degradation of active caspases. Dysregulated IAP expression has been reported in many cancers. For example, drug resistance correlated with the expression of cIAP-2 in pancreatic cells while Apollon was found to be upregulated in gliomas and was responsible for cisplatin and camptothecin resistance (Chen et al. 1999).

The caspases are a large group of proteins that play a central role in apoptosis. As they work at two levels, *initiator caspases* (e.g., caspase-2, -8, -9, and -10) responsible for the initiation of the apoptotic pathway and *effector caspases* (caspase-3, -6, and -7) responsible in the actual cleavage of cellular components during apoptosis. Therefore low levels of these proteins or impairment function can decrease apoptosis thus contributing to resistance.

The p53 protein, also called tumor protein 53 (or TP 53), is one of the best known tumor suppressor proteins whose mutation acquired oncogenic property. Defects in the p53 tumor suppressor gene have been linked to more than 50% of human cancers (Bai and Zhu 2006) because it is not only involved in the induction of apoptosis but it is also a key player in cell cycle regulation, development, differentiation, gene amplification, DNA recombination, chromosomal segregation, and cellular senescence. A recent study reported that some target genes of p53 involved in apoptosis and cell cycle regulation are aberrantly expressed in melanoma cells and possibly related to resistance (Avery-Kiejda et al. 2011).

Several abnormalities in the death signaling pathways that can lead to evasion of the extrinsic pathway of apoptosis have been identified. Death receptors such as Fas, DR3, Trail-1, Trail-2, and ligands of the death receptors are key players in the regulation of extrinsic pathway of apoptosis. Downregulation or impairment of receptor function, abnormal expression of decoy receptors, as well as a reduced level in the death signals can contribute to impaired signaling and hence a reduction of apoptosis and acquisition of drug resistance.

2.5 Alteration of Drug Target

One of the most common drug resistance mechanisms involves genetic alterations of drug target such as mutations or changes in expression levels. These modifications could exist at low levels before drug treatment and undergo positive selection during exposure to chemotherapy. In oncology, genomic characterization of cancer has highlighted the importance of driver somatic mutations that give rise to an unusual reliance of cancer cells on a particular molecular pathways and their specific oncogenic kinases. These proteins are targets for many drugs and can be altered by different mechanisms.

Main categories of this type of resistance could be:

- Downregulation of gene expression (e.g., effect of doxorubicin on topoisomerase IIα) (Di Nicolantonio et al. 2005)
- Gene amplification (e.g., BCR-ABL amplification detected both in vitro and in imatinib-resistant CML specimens) (Gorre et al. 2001)
- Mutations in *gatekeeper residue* (e.g., EGFR-T790M in NSCLC after treatment with tyrosine kinase inhibitor (TKI) gefitinib and erlotinib (Bell et al. 2005); BCR-ABL T315 in CML patient treated with imatinib) (Gorre et al. 2001)
- Mutations that alter the conformation (e.g., mutations in ALK – F1174L, C1156Y, and L1152R – confer an increase in ATP affinity and clinical resistance to the ALK inhibitor crizotinib) (Choi et al. 2010)
- Alternative spliced form (e.g., $p61BRAF^{V600E}$ detected in both vemurafenib in vitro-resistant cells and from resistant patient tumor biopsies produces enhanced dimerization with other RAF family members and resistance to vemurafenib but not MEK inhibitors) (Poulikakos et al. 2011).

A possible consequence of the inhibition of specific target is also the activation of a so-called bypass signaling mechanism. This results in the activation of a critical downstream signaling effector – normally activated by the kinase and extinguished by a kinase inhibitor – through a parallel mechanism that is indifferent to the kinase-directed therapy. An illustrative example of bypass-mediated resistance has been described in EGFR-mutant NSCLC. The bypass resistance mechanisms may also involve the modulation of positive or negative feedback loops and an example could be the augmentation of AKT signaling by MEK inhibitors (Garraway and Jänne 2012).

3 Conclusion

This review article shortly summarizes the main mechanisms of resistance to anticancer drugs. In order to define possible strategies to counteract resistance mechanisms, it can be useful to divide them in *pre-target*, *target related*, and *post-target* mechanisms.

As far as *pre-target* mechanisms – that can also be classified as pharmacokinetics mechanisms – we envisage the possibility to circumvent them by increasing drug doses and improving drug delivery testing combinations with modulators of drug transport and of tumor microenvironment.

On the other side, a deeper and deeper knowledge of *target related* mechanisms, e.g., target mutations, downregulation, amplification, is instrumental to identify alternative drugs, equally effective against cancer cells, to circumvent these types of resistance.

Finally as regards *post-target* mechanisms, the modulation of apoptosis appears to be a potentially feasible approach as recently demonstrated by Croce and Reed (2016).

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Major Physiological Signaling Pathways in the Regulation of Cell Proliferation and Survival

Huifang Tang and Gongda Xue

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Abstract

Multiple signaling pathways regulate cell proliferation and survival and are therefore important for maintaining homeostasis of development. The balance between cell growth and death is achieved through orchestrated signal transduction pathways mediated by complex functional interactions between signaling axes, among which, PI3K/Akt and Ras/MAPK as well as JAK/STAT play a dominant role in promoting cell proliferation, differentiation, and survival. In clinical cancer therapies, drug resistance is the major challenge that occurs in almost all targeted therapeutic strategies. Recent advances in research have suggested that the intrinsic

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pro-survival signaling crosstalk is the driving force in acquired resistance to a targeted therapy, which may be abolished by interfering with the cross-reacting network.

Keywords
Apoptosis • Cell cycle • Drug resistance • JAK/STAT • Lung cancer • Melanoma • mTOR/PI3K/Akt • Proliferation • Ras/MAPK • Signaling crosstalk

1 Introduction

The development of a multicellular eukaryote starts from the division of a zygote that eventually produces many types of functionally specialized cells. Proper tissue formation and growth requires a homeostatic regulation of cell cycle events that control cell proliferation and the cellular metabolism that often associates with apoptosis, a process of programmed cell death that surveils a balanced cell proliferation rate and cell number. Such regulation is usually governed by defined activation and inactivation of individual genes at different developmental stages. The selective pattern of gene expression functionally directs cell proliferation, cell differentiation, cell migration, and cell–cell communication to maintain a developmental integrity of the complex formation of tissues and organs. This phenotypic complexity is precisely mirrored by the genetic control system, an orchestrated signaling network coordinated among a few key evolutionarily conserved pathways. In this chapter, we intend to discuss recent advances of two important pathways, mTOR/PI3K/Akt and mitogen-activated protein kinase (MAPK), whose activation and interaction are critical for development and are the key regulatory elements in pathological circumstances such as malignant transformation of neoplasia and drug resistance in clinical cancer therapy.

2 Signaling Pathways Involved in the Regulation of Cell Proliferation

Cell proliferation is integrated with the overall needs of the organismal development in coordination with cell growth. It is principally mediated by cell cycle, a fundamental process that occurs when a parental cell produces two daughter cells via a series of genetic events (Hartwell and Kastan 1994; Nakayama and Nakayama 2006). Each of the daughter cells theoretically obtains the same copy of the genetic material inherited from the parental cell and are therefore genetically identical and morphologically undistinguishable. Similar to their parent, the mature daughter cells may initiate and complete the cell cycle when triggered by distinct signals in favor of the needs for growth and metabolism. Under physiological conditions, the proliferative cells are generally restricted to cells that supplement the tissue. Most tissues contain a group of multipotent cells, usually called stem cells, which can not only renew themselves but also divide asymmetrically to generate a new stem cell and a progenitor cell. In a context

dependent manner, progenitor cells may undergo further divisions, or enter terminal differentiation with endued specialized functions. Thus, in order to maintain proper tissue development, cell proliferation is programmed to be tightly controlled and its deregulation is a hallmark of malignant transformation found in the clinic (Hanahan and Weinberg 2000, 2011).

Maintenance of an appropriate cell number is also contributed by programmed cell death, apoptosis, which actively eliminates unwanted cells including aged and damaged cells (Elmore 2007; Taylor et al. 2008). Importantly, these two cellular functions are under a variety of stringent controls to ensure tissue homeostasis. Aberrant alterations of each functional node, either hyperactivation of proliferation or deactivation of apoptosis, will ultimately result in uncontrolled cell proliferation and growth that leads to tumor formation.

Mechanistic studies have revealed a number of biological signaling axes important for controlling cell proliferation and apoptosis. These signaling pathways commonly form an integral signaling network through diverse functional interactions, which are mediated by specific feedback signaling loops. Recent advances from clinical studies have shown that therapeutic disruption of the indispensable signaling loops during tumorigenic development is one of the leading causes of drug resistance and is attributed to over-activation of parallel proliferative signaling pathways (Holohan et al. 2013; Wicki et al. 2016).

2.1 Biological Regulation of Proliferation by mTOR/PI3K/Akt Signaling

In response to the stimulation of a variety of extracellular signaling cues, a number of membrane receptors with kinase activities can be activated via oligomerization. Activated membranous kinases subsequently create binding motifs in their intracellular segments that recruit and further activate the essential signal-mediator kinases. One of these intracellular messengers is lipid kinase phosphoinositide 3 kinase (PI3K), an evolutionarily conserved intracellular kinase that can convert Phosphatidylinositol (4,5)-bisphosphate (PIP2) into the secondary messenger molecule Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) on the plasmic membrane (Engelman et al. 2006). This process can be reversed by the phosphatase and tensin homolog (PTEN), which pairs with PI3K to constitute a functional switch of PIP3-dependent signaling transduction. Membrane-targeted PIP3 recruits certain proto-kinases to the inner surface of cytoplasmic membrane through binding to the pleckstrin homology (PH) domain with elevated affinity. Several important kinases are dependent on PIP3-mediated membrane targeting for activation. Akt and phosphoinositide-dependent kinase 1 (PDK1), two PH-domain-containing kinases, recognize and bind to newly generated and enriched PIP3, resulting in transient membrane anchoring and consequent activation.

Akt, also called protein kinase B (PKB), is a serine/threonine kinase that belongs to the AGC protein kinase family (Pearce et al. 2010). It comprises three isoforms in mammals with a high degree of sequence identity. Although the tissue distribution pattern is slightly different, Akt isoforms are virtually expressed in all cell types and

act as one of the most important signaling nodes to direct proper responses to exogenous or endogenous stimuli (Fayard et al. 2010).

Activation of Akt is a multistep process with characteristic phosphorylation of two key amino acids, threonine 308 (T308) in the "activation loop" and serine 473 (S473) in the "carboxy-terminal hydrophobic motif," both of which are conserved in all three isoforms. T308 phosphorylation is contributed by PDK1 when both proteins are recruited to the plasma membrane by PIP3. Although not exclusively required, it is generally accepted that the full kinase activity of Akt is stimulated upon phosphorylation of S473 by the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) (Cybulski and Hall 2009). Recent studies have also uncovered another kinase, DNA-dependent protein kinase (DNA-PK), that is capable of phosphorylating Akt on the same amino acid in response to DNA double-strand break (Bozulic et al. 2008; Surucu et al. 2008). Therefore, under physiological or pathological conditions, Akt acts as a direct functional target downstream of PI3K under the stringent control of PDK1 and mTORC2 or DNA-PK, which consolidates the theoretical basis for understanding the current model (Fig. 1). Genetic knockout of each Akt isoform or pharmacological inhibition of its activity results in a universal defect in growth in mouse models, indicating a central role of the Akt family in growth control (Fayard et al. 2010). In consistence with its roles in development, Akt is widely observed to be hyperactivated in a broad panel of cancers (Fruman and Rommel 2014; Vivanco and Sawyers 2002). In this regard, the signaling axis mTOR/PI3K/Akt has become one of the most attractive targeting pathways to restrain cancer cell proliferation. In spite of its three major upstream regulators, other kinases such as serine–threonine kinases IKK (Xie

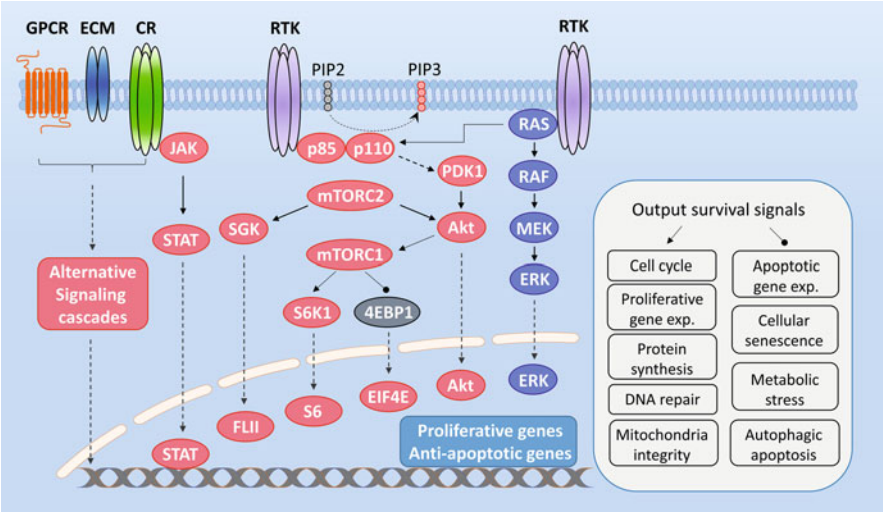

Fig. 1 Introduction to major proliferative signaling pathways. Three major signaling axes, mTOR/PI3K/Akt, Ras/MAPK, and JAK/STAT, are dissected to show their downstream targets that promote cell cycle progression. *RTK* receptor tyrosine kinase, *CR* chemokine receptor, *ECM* extracellular matrix, *GPCR* G-protein-coupled receptor

et al. 2011) and TBK1 (Ou et al. 2011; Xie et al. 2011), together with three tyrosine kinases Src (Chen et al. 2001; Haynes et al. 2003), Ack1/TNK2 (Mahajan et al. 2010; Mahajan and Mahajan 2015), and PTK6 (Zheng et al. 2010), are also reported to modulate Akt activity with distinct regulatory mechanisms. Therefore, Akt appears to be a universal intracellular mediator converged from a number of membrane signaling cascades. Indeed, not only phosphorylation but also many other types of post-translational modification of Akt have been discovered to collaboratively control Akt to maintain cellular homeostasis (Risso et al. 2015).

Akt activation triggers a number of functional events including cell proliferation, growth, survival, protein synthesis, and metabolism. These cellular functions are differentially promoted by multiple downstream targets of Akt, which can be grouped into five categories with functional specificities in regulations of cell cycle: gene synthesis and protein translation, cell survival and anti-apoptosis, developmental progress/stress and metabolism, and crosstalk with other signaling pathways. Akt-regulated cellular functions are preferentially controlled by its specific substrates with distinct roles (Manning and Cantley 2007) (Fig. 2a) as well as a few physical interacting partners that can also regulate its activity.

2.2 Ras/MAPK Signaling-Regulated Cell Proliferation, Differentiation, and Survival

In parallel to the PI3K/Akt axis, another intracellular signaling pathway can be found downstream of membrane-coupled signalosomes; this pathway is the small GTPase-mediated MAPK cascade. The MAPK family is highly conserved throughout mammalian evolution to regulate diverse developmental processes such as cell cycle regulation, differentiation, cell motility, physiological metabolism, stress response, and pro- or anti-apoptosis (Widmann et al. 1999). The mammalian MAPK family contains more than 20 protein kinases characteristically organized into three-tiered kinase cascades (Chang and Karin 2001). Activation of the last tier messenger MAPK proteins is mediated by directed phosphorylation on threonine (Thr) and tyrosine (Tyr) residues within a conserved Thr-X-Tyr motif (X represents glycine, glutamine, or proline) that is located in the activation loop of the kinase domain. Based on the extracellular stimuli and their distinct membrane-associated signaling receptors, the MAPK signaling pathway can be categorized into four major branches characterized by distinct MAPK family members including extracellular signal-regulated kinases (ERK), c-Jun NH_2-terminal kinases (JNK), p38, and ERK5 (Pearson et al. 2001; Qi and Elion 2005). These MAPK messenger kinases have been shown to implement different cellular functionalities (Fig. 2b).

In response to stimulation of growth factors, conversion of Ras^{GDP} to Ras^{GTP} activates Raf kinase, an MAPK kinase kinase (MAPKKK) that can phospho-activate its downstream target MEK, an MAPK kinase (MAPKK). MEK rapidly phosphorylates ERK1/2 and subsequently stimulates cell proliferation through activating a broad range of substrates of ERK. These substrates include intracellular kinases p90 ribosomal S6 kinases (RSK) (Carriere et al. 2008b), mitogen- and stress-activated kinases

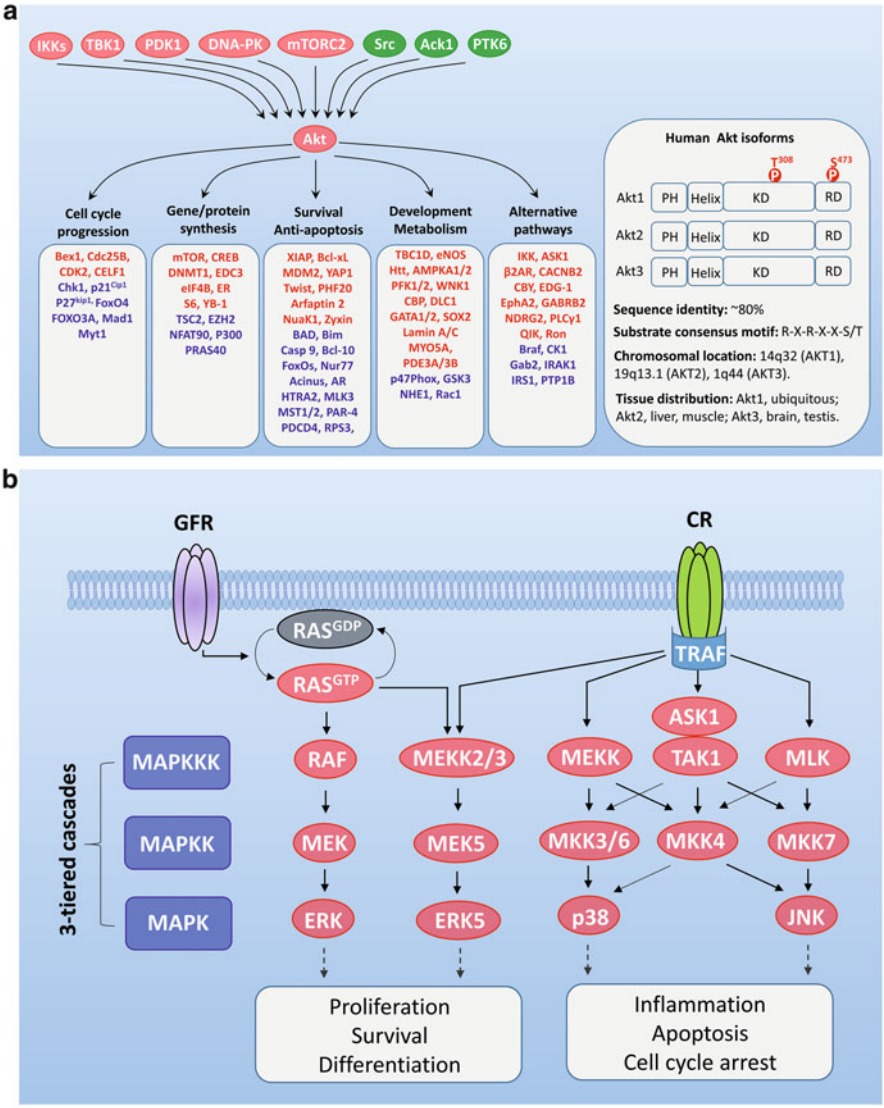

Fig. 2 Mechanistic regulation of cell proliferation by PI3K/Akt and MAPK pathways. (**a**) Up- and downstream targets of Akt that regulate differential gene expression in promoting cell proliferation and survival. Upstream kinases highlighted in *red* represent serine–threonine kinases, while kinases highlighted in *green* are phosphorylating tyrosine residues on Akt. Downstream of Akt are its substrates important in cell growth: the substrates highlighted in *red* are phospho-activated by Akt and subsequently promote cell proliferation and survival, while those highlighted in *blue* are pro-apoptotic and phospho-inactivated by Akt. (**b**) Schematic dissection of MAPK family members and their signaling interaction in regulating and determining cell fate. *GFR* growth factor receptor

(MSK) (Arthur 2008), MAPK-interacting kinases (MNKs) (Waskiewicz et al. 1997), and death-associated protein kinase (DAPK) (Chen et al. 2005). A number of transcriptional factors including NF-AT, STAT3, MEF2, c-Myc, ELK-1, and c-Fos (Cargnello and Roux 2011; Gaestel 2015) are also involved. Basal activation of the ERK1/2 cascade is important for efficient G1-S transition during cell cycle. Therefore, ERK1/2 conventionally acts as a positive regulator of cell cycle progression contributing to cell proliferation under physiological conditions. In fact, this proliferative role of activated ERK is directly reflected in many types of clinical disorders, such as cancer, in which ERK activation is dramatically deregulated to favor an uncontrolled proliferative phenotype of cancer cells.

In contrast to ERK activation in response mainly to growth factors (Ramos 2008), other classes of stimuli like cytokines potently induce JNK and p38 activation through three well-defined signaling routes: MEKKs-MKK3/6-p38, ASK1/TAK1-MKK4-JNK/p38, and MLK-MKK7-JNK. Similar to ERK, JNK and p38 also amplify the transduced signals in response to stimulation of pro-inflammatory factors and environmental stress through their specific substrates including c-Jun, p53, Bax, Tau, ATF-2, HuR, EST1, HSF-1, JunB, cPLA2, GADD153, and MK2/3 in spite of some shared substrates with ERK kinases such as ELK-1, NF-AT, STAT3, c-Myc MNK, MEF2, and MSK. Unsurprisingly, both proliferative and proapoptotic phenotypes can be triggered due to the complexity of these substrates that may induce both apoptosis and survival (Wada and Penninger 2004). Therefore, the cell fate regulated by MAPK signaling pathways is possibly determined by the equilibrium of the input signals of proliferation and apoptosis, where overexpression of either interferes with cellular homeostasis (Royuela et al. 2002). Another branch in the canonical MAPK signaling is mediated by ERK5, previously called BMK1 (big MAPK kinase 1). Knockout of ERK5 in mice induces early embryonic lethality due to impaired angiogenesis and cardiac development. Tissue-specific depletion of ERK5 leads to impaired endothelial cell development and vascular integrity (Hayashi and Lee 2004). Further studies dissect its role in the regulation of cell survival in response to the stimulation of both growth factor and pro-inflammatory pathways (Drew et al. 2012).

Although the canonical MAPK pathway is ubiquitously present, biochemical studies have revealed a set of unique kinases remotely linked to the conventional branches. These kinases fall into three subgroups based on individual messenger MAPKs. It is not clear whether these kinases can be interpreted by the characteristic three-tiered cascades as many of the components have not been identified (or do not exist at all). However, it is demonstrated that the unique messenger MAPKs, such as ERK3/4, ERK7/8, and NLK, do not harbor the same consensus phosphorylation motif Thr-X-Tyr (Coulombe and Meloche 2007). Because of many less-understood observations, it is still too early to review the functional relevance and importance of these atypical MAPKs in physiology, although recent studies have shed some light in this field (Aberg et al. 2009; Al-Mahdi et al. 2015; De la Mota-Peynado et al. 2011; Long et al. 2012; Sirois et al. 2015). Taken together, the complex signaling networks established by MAPKs are capable of generating highly specific signals to induce remarkable diversities of cellular responses to regulate the machinery of cell fate control.

2.3 JAK/STAT Signaling in the Regulation of Cell Proliferation and Survival

The third appreciated intracellular signaling axis is Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Notably, recent studies from clinical disorders reveal that JAK/STAT is one of the most deregulated signaling routes in secondary hyperactivation of parallel survival signaling in acquired therapeutic resistance to cancer therapies (Britschgi et al. 2012; Sun and Bernards 2014). Despite that the deregulated activation by RTKs frequently occurred during oncogenic progression (Wicki et al. 2016), JAK kinases are classical non-receptor tyrosine kinases that transduce cytokine-mediated signals. Compared to the MAPK signaling network, the JAK/STAT module seems to be simple; upon binding of individual cytokines to their corresponding receptors, four JAK family members (JAK1–3 and TYK2) can be differentially recruited to cytokine receptors and activated by trans- and/or autophosphorylation. Activated JAK kinases subsequently induce specific tyrosine phosphorylation on the intracellular tail of cytokine receptors that serve as a docking site for inactive cytosolic STAT proteins. To date, seven STAT family members have been identified in mammals. Direct phospho-activation by JAKs leads to oligomerization of STAT proteins, either homo- or heterodimerization, and rapidly translocates into the nucleus to activate distinct transcriptional programs (Levy and Darnell 2002). Several well-studied genes are directly regulated by STAT transcription factors. For example, STAT3 can upregulate MMP2 and MMP9 that increase cell motility, and VEGF, bFGF, and HIF-1α that stimulate angiogenesis. Importantly, activated STATs can directly upregulate anti-apoptotic genes such as Bcl-X_L and cyclin D1, providing an enhanced anti-apoptotic potential to cells under stress conditions, implying a pro-survival role of JAK/STAT-mediated cytokine signaling (Rawlings et al. 2004).

3 Signaling Interaction Network Between PI3K/Akt, Ras/MAPK, and JAK/STAT Axes

Homeostatic development requires a balanced activity of each signaling cascade. Multiple physiological mechanisms have been shown to regulate cell growth and aging. In principle, these mechanisms can be characterized into two categories: autonomous signaling feedback loop and dependent signaling crosstalk loop, both of which can be integrated at transcriptional and translational levels. When this physiological functionality is taken by the cancer cells in clinical treatment, the pharmacologically stressed cancer cells may evolve to neglect the single-pathway-suppression-induced cytotoxicity. If such aptitude is (epi-)/genetically established, the acquired resistance of cancer cells to pathway inhibitors will inevitably be developed. Notwithstanding the well-understood negative self-feedback signaling mediated by the docking proteins insulin receptor substrate (IRS), GRB2-associated binder (GAB), and FoxO along the mTOR/PI3K/Akt axis (Chandarlapaty et al. 2011; Hsu et al. 2011; Laplante and Sabatini 2012; Yu et al. 2011), clinical observations evidently confirm that the interaction network

between PI3K/Akt, Ras/MAPK, and JAK/STAT has been implicated to play a vital survival role in promotion of cancer cells defending apoptotic induction, particularly in the context of therapeutic treatments (Fig. 3).

Signaling crosstalk can be either cross-activation or cross-inhibition. Activation of Akt may directly phospho-inhibit Raf (Moelling et al. 2002; Reusch et al. 2001) to maintain adequate MAPK activity, while preventing from stress-induced apoptosis by phosphorylating ASK1 (Kim et al. 2001; Zhang et al. 2005) and MLK (Figueroa et al. 2003). Meanwhile, Akt can promote its activation by transcriptional reprogramming through its downstream substrates, such as Twist (Cheng et al. 2007; Xue et al. 2012; Yang et al. 2015), that complies a feedforward signaling loop; in contrast, to avoid unwanted hyperactivation, it can suppress mTORC1-directed IRS1/GAB-mediated upstream RTK signalosomes. Moreover, mitogen-activated ERK was also reported to enhance mTORC1 activation through direct phosphorylation of Raptor, a key regulatory subunit of mTORC1 (Carriere et al. 2011), and indirectly through its downstream kinase RSK that may phosphorylate Raptor (Anjum and Blenis 2008; Carriere et al. 2008a). Such cross-activation-resulted enhancement of the mTOR pathway is also reflected by ERK-dependent inactivation of the intrinsic inhibitory Tuberous Sclerosis Complex 2 (TSC2) that behaves similar to the mode of Akt and suppresses mTOR activity (Huang and Manning 2009; Inoki et al. 2002; Ma et al. 2005). Reciprocal elevation of ERK activity can also be triggered by inactivation of GSK3 in an Akt-dependent manner (Ding et al. 2005). Interestingly, in vitro kinase assay revealed that PDK1 is able to promote MAPK activation by phosphorylating MEK1 and MEK2 (Lee et al. 2012).

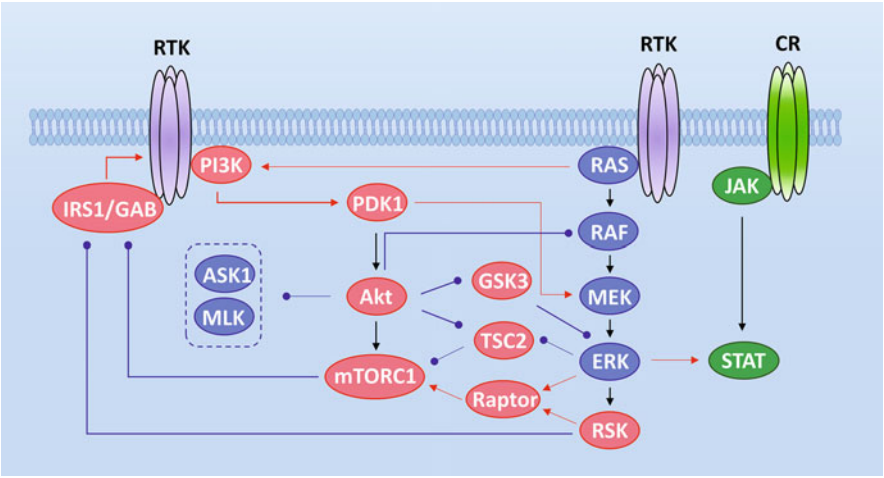

Fig. 3 Molecular interactions between three pathways of PI3K/Akt, Ras/MAPK, and JAK/STAT. The molecules highlighted in *red* are key components in the PI3K/Akt pathway, while in *blue* and in *green* belong to the MAPK and JAK/STAT pathways, respectively. The *arrows* in *red* represent "phospho-activating" and in *blue* represent "phospho-inactivating"

4 Output Signals of Integrated Signaling Pathways

These experimental studies demonstrate that activation of both mTOR/PI3K/Akt and Ras/MAPK reciprocally regulates each other in favor of a physiological response to extracellular cues. Clearly such interplay is strictly maintained in a context dependent manner. This is also supported by molecular modeling that signaling interaction is triggered by the special-temporal expression level of the signaling components such as stimuli, receptors, and effectors (Borisov et al. 2009).

4.1 Redundant Targeting of Shared Substrates in Cell Growth and Metabolism

Interaction of multiple pathways is to enable the cells optimally adapting the developmental conditions to promote their proliferation and/or survival. Signals can vary dramatically: higher level of growth factor stimulation may trigger negative feedback signaling, whereas decrease of one axis activity may stimulate other parallel signaling axes to maintain a balanced signaling altitude to timely determine the cell fate. This is best revealed in cell line-based studies in vitro when one axis is chemically blocked as well as in animal models upon treatment with pharmacological inhibitors. Therefore, crosstalk network rewires the signal transduction path through inducing amplification or attenuation of the activities of effector molecules. Interestingly, the endpoint effector kinases, Akt, S6K and RSK, all are AGC kinase family members (Manning et al. 2002). Of note, the consensus sequences of their substrates are almost identical with a core phosphorylating motif "RXRXXS/T" (Pearce et al. 2010). Unsurprisingly, they are capable of phosphorylating the same substrate characteristically depending on the nature of stimulation and consequence. In fact, the outcome from signaling crosstalk is mirrored by cooperated regulation of a large substrate network. One of the best studied proteins involved in protein synthesis is ribosomal protein S6. S6 participates in activation of a translational initiation complex that promotes cell proliferation (Jastrzebski et al. 2007; Magnuson et al. 2012). Activation of S6 is shown to be regulated by RSK- and S6K-mediated phosphorylation on serine 235/236 and serine 240/244 residues that promote its binding to cap complex (Roux et al. 2007). Another key regulator involved in protein synthesis is the eukaryotic initiation factor 4B (eIF4B), which is required to modulate assembly of the translational initiation complex. Phosphorylation on serine 422 mediated by Akt (van Gorp et al. 2009), RSK (Shahbazian et al. 2006), and S6K (Holz et al. 2005) promotes eIF4B-dependent maturation of pre-initiation complex and subsequently leads to efficient translation. A set of transcription factors directly activating a number of proliferative genes are also co-regulated by Akt, RSK, and S6K kinases. Estrogen receptor (ER) is crucial to drive expression of Myc and Cyclin D1. Similar to S6, ERα activity is also co-regulated by direct phosphorylation on serine 167 by the above three kinases to enhance the transcription of its downstream target genes (Sun et al. 2001; Yamnik and Holz 2010). Similar actions also occur on other transcription factors such as Y-box binding protein 1 (YB1) and Mad1. In contrast, to establish the positive activating loops, Akt, RSK,

and S6K can also phospho-inactivate intracellular proliferation suppressors such as Glycogen synthase kinase 3 (GSK3) which is important in the regulation of cell metabolism and proliferation. In response to different stimuli, serine 9/21 may be phosphorylated by Akt, RSK, and S6K (Cohen and Frame 2001; Cross et al. 1995) which coordinately suppress its function, for example, by activation of its downstream antiproliferative complex TSC2 (Inoki et al. 2006).

4.2 Differential Phospho-Regulation of Shared Substrates in Cell Proliferation and Survival

In spite of phosphorylation on the same amino acids, Akt, RSK, ERK, and S6K can also target different sites on the same substrate to meticulously define its biological output. For example, the transcription factor forkhead box O (FoxO) family actively inhibits cell cycle progression by transcriptionally regulating a number of proapoptotic proteins, such as p21, p27, and p130, that induce cell cycle arrest at G1 phase, and GADD45 and Cyclin G2 at G2 phase, despite that FoxO family members can promote BIM-1, bNIP3, Bcl-6, FasL, and Trail, all of which are directly involved in cell death induction and enhancement (Greer and Brunet 2005). Akt targets FoxO3A on threonine 32 serines 253/315, whereas ERK can phosphorylate FoxO3A on serines 294/344/425, all of which trigger FoxO3A degradation (Tang et al. 1999; Yang et al. 2008). Similar to FoxO proteins, both RSK-mediated phosphorylation of BAD on serine 112 (Shimamura et al. 2000) and S6K-targeted serine 136 (Harada et al. 2001) cooperatively sequester BAD in the cytoplasm in a 14-3-3-dependent manner, which stimulates its apoptotic signaling from mitochondria thus promoting cell survival.

5 Impact of Signaling Interaction Network in Clinical Cancer Therapies

Akt is widely known for its pro-growth/survival roles via a large number of its substrates involved in regulation of many cellular events. mTOR/PI3K/Akt is one of the most deregulated signaling pathways in human malignancies and is often linked to aberrant cellular metabolism (Garcia-Echeverria and Sellers 2008; Hennessy et al. 2005). Under physiological conditions, depletion of each Akt isoform remarkably impairs normal development of mammals, phenotypically exhibiting retarded growth and smaller body size. Akt, being a cross-road signaling node, responds to growth signals and transduces through mTORC1 to direct gene transcription and translation. To avoid out-of-control cell proliferation and growth, physiological activation of protein phosphatases and attenuation of PI3K by lipid phosphatase PTEN are essential to repress excess Akt activity. In many types of cancer cells, PTEN is either genetically absent or inhibited, which results in a constitutive hyperactivation of PI3K/Akt that drives uncontrolled cancer cell proliferation. In addition, gain-of-function (GOF) mutations on PI3K and Akt, or their upstream kinase such as EGFR, are always dominant and contribute to oncogenesis and cancer progression. In non-small cell lung

cancer (NSCLC), in addition to highly deregulated EGFR activation, PTEN protein expression is inhibited in almost 70% of tumors (Marsit et al. 2005) together with oncogenic mutation on PI3K (~10%) (Spoerke et al. 2012) and Akt (~2%) (Scrima et al. 2012). Frequent gene amplification of PI3KCA (30%~50%) (Ji et al. 2011) and overexpression of Akt protein (~35%) (Scrima et al. 2012) are also associated with advanced disease progression. Therefore, inhibitors specifically targeting these components along the PI3K pathway are suggested to provide therapeutic effects. However, due to its extensive interacting network with other pathways, monotherapeutic inhibition of PI3K/Akt activity inevitably induces compensatory activation of crosstalked circuits to favor cancer cell survival. Buparlisib is a pan-PI3K inhibitor that is under investigation in clinic. Although its efficacy was demonstrated in preclinical models, a recent phase II study failed in confirming its benefit in NSCLC patients due to acquired resistance (Vansteenkiste et al. 2015). Even when both PI3K and mTOR are simultaneously inhibited, for example, by BEZ235, which is tested in patients with advanced solid tumors (Mayer and Arteaga 2016), JAK/STAT pathway is activated in parallel to promote cancer cell survival (Britschgi et al. 2012; Jin et al. 2014).

On the other hand, inhibition of oncogenic Ras-driven MAPK activation also frequently elevates PI3K/Akt activity. A current model in clinical first-line therapy is the melanoma harboring an oncogenic BRAF mutation on valine 600. Although therapeutic targeting of mutated BRAF with either dabrafenib or vemurafenib improved clinical benefit compared with conventional pan-DNA-damaging reagent, almost all the melanoma patients developed resistance within 10 months. Many mechanisms have been revealed and widely reported. Of note, all these reactivating mechanisms including Ras mutation and RTK activation converge on hyperactivation of the central node PI3K/Akt, which fuels cancer cells to resist to apoptosis.

6 Final Remarks

These clinical observations indicate an important role of signaling compensation by functional interaction between pathways, especially under stressed conditions. In fact, this is the origin where acquired resistance starts. Subsequently it is hypothesized that simultaneous targeting of two or three oncogenic drivers or key pathways upregulated in resistant tumors theoretically could synergize to kill cancer cells. Based on current understandings of drug resistance, a great number of clinical trials are underway to mechanistically combine different inhibitors in order to overcome tumor relapse. Although more data is needed, a few of the combinatory strategies have proven beneficial. In particular, combined inhibition of PI3K/Akt and Ras/MAPK pathways is shown to induce significant cytotoxicity compared with targeting a single component (Posch et al. 2013; Shi et al. 2011; Will et al. 2014). Therefore, orchestrated signaling crosstalk between PI3K/Akt, Ras/MAPK, and JAK/STAT (Chung et al. 1997) ultimately determines the cell fate with a fine tuned balance between proliferation and apoptosis.

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Immune-Mediated and Hypoxia-Regulated Programs: Accomplices in Resistance to Anti-angiogenic Therapies

Diego O. Croci, Santiago P. Mendez-Huergo, Juan P. Cerliani, and Gabriel A. Rabinovich

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Abstract

In contrast to mechanisms taking place during resistance to chemotherapies or other targeted therapies, compensatory adaptation to angiogenesis blockade does not imply a mutational alteration of genes encoding drug targets or multidrug resistance mechanisms but instead involves intrinsic or acquired activation of compensatory angiogenic pathways. In this article we highlight hypoxia-regulated and immune-mediated mechanisms that converge in endothelial cell programs and preserve angiogenesis in settings of vascular endothelial growth factor (VEGF) blockade. These mechanisms involve mobilization of myeloid cell populations and activation of cytokine- and chemokine-driven circuits operating during intrinsic and acquired resistance to anti-angiogenic therapies. Particularly, we focus on findings underscoring a role for galectins and glycosylated ligands in promoting resistance to anti-VEGF therapies and discuss possible strategies to overcome or attenuate this compensatory pathway. Finally, we highlight emerging evidence demonstrating the interplay between immunosuppressive and pro-angiogenic programs in the tumor microenvironment (TME) and discuss emerging combinatorial anticancer strategies aimed at simultaneously potentiating antitumor immune responses and counteracting aberrant angiogenesis.

Keywords

Angiogenesis • Anti-angiogenic therapy • Galectins • Hypoxia • Immunotherapy • Resistance

1 Introduction

Vascular programs leading to the development of blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis) entail the synchronized action of different cells and the convergence of a complex network of stimulatory and inhibitory factors. Angiogenesis, defined as the formation of new blood vessels from preexisting ones, is a critical process for the establishment of solid tumors. Blood vessels deliver oxygen and nutrients, remove waste, and represent the central traffic route for immune cells (Potente et al. 2011). Early mechanisms of angiogenesis in tumors or in normal tissue (e.g., during wound healing) are similar, involving vessel destabilization, guided endothelial cell (EC) migration, proliferation, and sprouting (Carmeliet and Jain 2000). However, in normal angiogenesis, these events are followed by a stabilization phase of the newly formed vessels in which ECs change their metabolism due to the dominance of negative factors and become quiescent (Stockmann et al. 2014). In contrast, pathological angiogenesis lacks this inhibitory phase, resulting in the generation of a highly dense, aberrant vascular network that generates a hostile microenvironment characterized by low pH, hypoxia, and interstitial leakage. This particular setting, in turn, creates a barrier to immune surveillance, fueling disease progression, metastasis, and resistance to treatments

(Motz and Coukos 2011). Thus, abnormal angiogenesis represents an important target for the development of novel anticancer therapies (Carmeliet and Jain 2011; Croci et al. 2014b). The concept of "anti-angiogenesis" as the elimination of blood vessel formation in tumors was first proposed by Judah Folkman in 1971. Folkman and colleagues put forward the hypothesis that tumor growth relies on the formation of new blood vessels to obtain oxygen and nutrients and for elimination of metabolic waste, while providing gateways for metastasis (Folkman 1971; Ferrara and Adamis 2016). These early observations set the bases for inhibition of angiogenesis as a mean to halt tumor growth and prevent metastatic dissemination.

Although more than 40 molecules have been identified to play key roles in the angiogenesis cascade, most studies have focused on vascular endothelial growth factor (VEGF) originally discovered by Ferrara's group in 1989 and its receptor signaling pathways (Leung et al. 1989; Jain 2014). In 2004, the US Food and Drug Administration (FDA) approved the first recombinant humanized anti-VEGF-A monoclonal antibody – bevacizumab – for first-line treatment of metastatic colorectal cancer (Ferrara et al. 2005). Thus far, several therapeutic strategies have been implemented including antibodies that target VEGFR2 (ramucirumab) (Krupitskaya and Wakelee 2009) and receptor tyrosine kinase (RTK) inhibitors (sunitinib, sorafenib, pazopanib, vandetanib, cabozantinib, tivozanib, linifanib, and axitinib) that inhibit VEGFR signaling through direct competition with adenosine triphosphate (ATP) for the intracellular tyrosine kinase-binding domain (Loges et al. 2009; Jeong et al. 2013). In addition, a soluble VEGF receptor 1 (VEGFR1)–VEGFR2 chimeric protein that antagonizes VEGF-A (aflibercept) has been designed (Holash et al. 2002). The aforementioned targeted therapies have improved the clinical outcome of several types of tumors including metastatic colorectal cancer, non-small cell lung carcinoma, renal cell carcinoma, and hepatocarcinoma (Ferrara and Adamis 2016). However, whereas preclinical studies targeting pro-angiogenic factors have shown considerable effects on tumor growth, the clinical outcome of most anti-angiogenic modalities is variable, and patients respond with different degrees of sensitivity, with many of them developing progressive resistance (Ellis and Hicklin 2008). These clinical observations suggest that compensatory pathways may contribute to trigger VEGF-independent pro-angiogenic programs during tumor growth (Bergers and Hanahan 2008; Carmeliet and Jain 2011).

2 Mechanisms of Resistance to Anti-angiogenic Therapies

Tumors and their adjoining microenvironment display a variety of mechanisms that thwart anti-angiogenic treatments. Whereas some tumors are inherently refractory to anti-angiogenic therapies (*intrinsic resistance*), most of them transiently respond to angiogenic blockade and further develop compensatory pathways (*evasive resistance*) (Bergers and Hanahan 2008; Sharma et al. 2017). Pathways of evasive resistance could be grouped into three types: (a) expression of alternative angiogenic mediators, (b) mechanisms involving adaptive responses to hypoxia, and

(c) mobilization of angio-competent myeloid cells that preserve vascularization programs. Immune-mediated resistance to anti-angiogenesis therapies will be discussed in detail in Sect. 3.

2.1 Expression of Alternative Pro-angiogenic Mediators

In contrast to mechanisms taking place during resistance to chemotherapies or other targeted therapies (Dobbelstein and Moll 2014), compensatory adaptation to angiogenesis blockade does not imply mutational alterations of genes encoding the drug target or multidrug resistance (MDR) mechanisms but instead involves activation of alternative pro-angiogenic pathways (Bergers and Hanahan 2008). Tumors can evade VEGF blockade by inducing the synthesis of alternative pro-angiogenic factors (Casanovas et al. 2005; Shojaei et al. 2007b; Potente et al. 2011; Croci et al. 2014a; Carbone et al. 2016) that compensate for the absence of VEGF signaling, including fibroblast growth factor-2 (FGF2/bFGF), stromal cell-derived factor-1α (SDF-1α also known as CXCL12) (Batchelor et al. 2007), placental growth factor (PIGF) (Fischer et al. 2007), platelet-derived growth factor (PDGF) (Song et al. 2009), interleukin (IL)-8 (Mizukami et al. 2005), IL-17 (Chung et al. 2013), Bv8 (Shojaei et al. 2007b), galectin-1 (Gal1) (Croci et al. 2014a), angiopoietins, and hepatocyte growth factor (HGF)/c-Met (Shojaei et al. 2010), which fuel revascularization programs and limit the efficacy of anti-VEGF treatment. In this regard, pericytes, which are perivascular cells that wrap around blood capillaries, can activate compensatory PDGFR-mediated pro-angiogenic signaling in the absence of VEGF (Song et al. 2009). However, current PDGFR-β inhibitors are neither specific nor sufficient to induce a complete regression of tumor vasculature (Arrondeau et al. 2015). Recently Carbone and colleagues identified interleukin-1 (IL-1), CXC receptor (CXCR)1/2 ligands, and transforming growth factor-β (TGF-β) as soluble factors that are overexpressed in anti-VEGF refractory tumors (Carbone et al. 2016).

2.2 Adaptation to Hypoxia

Vessel pruning, a typical hallmark of anti-angiogenic therapies, induces severe hypoxia that in turn favors revascularization programs and promotes tumor growth and metastasis (Paez-Ribes et al. 2009; Ebos et al. 2009). Certainly, in Darwinian terms, hypoxia acts as a selection-pressure mechanism that selects tumor cell variants with increased aggressiveness and lower sensitivity to anti-angiogenic therapy. Hypoxia is the most important stimulus when it comes to inducing secretion of pro-angiogenic factors in the TME but also contributes to tumor escape by favoring growth advantage of cancer stem cells (Semenza 2017). Interestingly, most hypoxic tumors are also refractory to current chemotherapy and radiotherapy approaches as only tumor cell populations surviving in poorly oxygenated niches, such us pro-angiogenic cancer stem cells, are selected under these adverse

conditions (Heddleston et al. 2010; Myszczyszyn et al. 2015). Furthermore, VEGF blockade aggravates hypoxia, which upregulates the production of other pro-angiogenic factors and increases tumor cell invasiveness and metastasis (Paez-Ribes et al. 2009). Tumor cells respond to hypoxia by becoming tolerant and modifying their metabolism to overcome low oxygen levels (Heddleston et al. 2010). Increased tumor hypoxia induces the selection of highly invasive metastatic clones arising from cancer cells that are resistant to anti-angiogenic agents, through the synthesis of pro-migratory proteins, such as SDF1-α, hepatocyte growth factor-scatter factor (HGF-SF), and pro-invasive extracellular matrix proteins (Tan et al. 2004; Ju et al. 2017). Thus, hypoxia generated by angiogenesis inhibitors triggers pathways that make tumors more aggressive and less sensitive to anti-angiogenic treatment. In this regard, Paez-Ribes and colleagues demonstrated enhanced invasiveness and metastasis of tumors following interruption of VEGF signaling in models of pancreatic neuroendocrine carcinoma and glioblastoma (Paez-Ribes et al. 2009). Interestingly, hypoxic colon cancer cells lacking HIF-1α preserved angiogenesis by inducing the synthesis of IL-8, a chemokine implicated in innate immune responses, whose expression is regulated by prolyl hydroxylase 2 (PHD2) in an NF-κB-dependent manner (Mizukami et al. 2005; Chan et al. 2009). In this sense, hypoxia also induced expression of Gal1, an endogenous immunomodulatory lectin, through both HIF-dependent or HIF-1-independent mechanisms involving reactive oxygen species (ROS) and NF-κB signaling pathways (Zhao et al. 2010; Croci et al. 2012). Interestingly, Gal1 interaction with specific *N*-glycans on the surface of endothelial cells has demonstrated to play a key role in linking tumor hypoxia to VEGF-independent angiogenesis in several cancer types (Croci et al. 2014a). These results highlight the central role of tumor hypoxia in modulating resistance to anti-angiogenic therapies through multiple mechanisms.

3 Immune-Mediated Mechanisms of Resistance to Anti-angiogenesis

Experimental evidence over the past decade has demonstrated a key role of immune-mediated circuits, including inflammatory cells, cytokines, and growth factors, in promoting angiogenesis and sustaining tumorigenesis and metastasis (Hanahan and Coussens 2012) (Fig. 1). In response to hypoxic conditions and tumor-derived soluble factors, immune regulatory cells are recruited to the tumor microenvironment (TME) and secrete large amounts of pro-angiogenic factors including pro-inflammatory cytokines (IL-1β, IL-6, TNF), chemokines (IL-8, SDF-1α/CXCL12), growth factors (VEGF, bFGF), and metalloproteases (MMP-9), among others (Grunewald et al. 2006; Gabrilovich et al. 2012; Croci et al. 2014b). These cells include $Tie2^{+}$ monocytes (De Palma et al. 2005), Bv8-expressing $CD11b^{+}Gr1^{+}$ myeloid-derived suppressor cells (MDSCs) (Shojaei et al. 2007a), M2-type tumor-associated macrophages, regulatory T cells (Tregs), and NK cells expressing pro-angiogenic mediators such as VEGF, FGF2, TGF-β, and PDGF-α (Murdoch et al. 2008; Facciabene et al. 2011; Bruno et al. 2013).

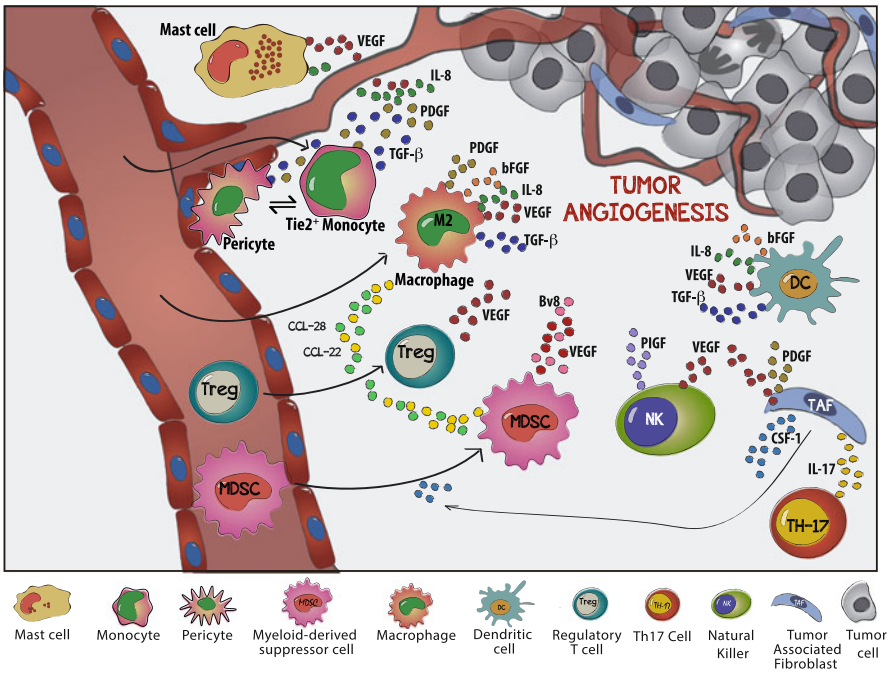

Fig. 1 Immune-mediated mechanisms of tumor angiogenesis. Tumor recruits a variety of regulatory cells to the tumor microenvironment (TME) which not only contribute to generate an immunosuppressive milieu but also promote angiogenesis. Hypoxia favors recruitment of immune cells and release of pro-angiogenic and immunosuppressive cytokines. $Tie2^+$ monocytes, M2-type macrophages, dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs) secrete several pro-angiogenic factors that influence EC signaling. Indeed, these myeloid cells may also interact with lymphoid cells including regulatory T cells (Tregs), regulatory B cells (Bregs), Th17 cells, and natural killer (NK) cells which directly or indirectly contribute to sustain angiogenesis and immunosuppression

3.1 Myeloid Cell-Dependent Resistance to Anti-angiogenic Therapies

Bone marrow-derived myeloid cells, such as immature monocytes and macrophages, dendritic cells (DCs), mast cells (MCs), and neutrophils, have a prominent role in orchestrating and/or resolving innate and adaptive immunity. Solid tumors recruit distinct populations and subsets of myeloid cells which support tumor progression by promoting angiogenesis and suppressing antitumor immune responses (Hanahan and Coussens 2012; Gabrilovich et al. 2012; Stockmann et al. 2014). These immune cells exhibit a remarkable plasticity with selective pro-angiogenic potential displayed at specific differentiation or polarization stages. For example, a population of pro-angiogenic Tie2-expressing monocytes ($Tie2^+$ $CD11b^+$) that are specifically recruited to the TME is also capable of differentiating into mesenchymal progenitors, which serve as a source of pericytes that control

aberrant angiogenesis (De Palma et al. 2005). Moreover, other studies suggest that MDSCs in the TME can differentiate into M2-type macrophages (Gabrilovich et al. 2012). As myeloid cell subpopulations can interconvert into each other quite easily, deeper functional analysis is needed to better discriminate cells that belong to functionally different subsets from those that functionally overlap but express different surface patterns. Here, we will focus on different myeloid subsets that are directly or indirectly involved in tumor angiogenesis.

3.1.1 Pericytes

Pericytes are involved in vascular stability through the release of factors that maintain ECs quiescence such as angiopoietin-1 (Ang-1), a ligand for the Tie2 receptor, and expression of receptors for transforming growth factor-β (TGF-β) and platelet-derived growth factor-β (PDGF-β) (von Tell et al. 2006). Several genes encoding immunosuppressive factors have been reported to be upregulated in pericytes by the PDGF-β signaling pathway in the absence of VEGF signaling (Kale et al. 2005), suggesting critical roles for these cells in modulation of both immune and vascular programs. In fact, pericytes have been considered an important immunological component of the mammalian central nervous system (CNS) (Winkler et al. 2011; Barbi et al. 2013). Paradoxically, pericyte depletion in tumor-bearing mice suppressed growth of primary tumors but enhanced metastasis (Cooke et al. 2012), highlighting the complexity of these cells. In this sense, targeted deletion of regulator of G-protein signaling-5 (Rgs5), a master regulator of pericyte function, induced pericyte maturation and vascular normalization, enhancing infiltration of $CD8^+$ T cells into tumor parenchyma and promoting tumor regression (Hamzah et al. 2008). Moreover, tumor-derived pericytes have been shown to negatively control $CD4^+$ T cell activation and proliferation, promoting anergy in an Rgs5- and IL-6-dependent manner (Bose et al. 2013). Interestingly, fully differentiated pericytes maintain their phenotypic plasticity, enabling differentiation into various cell types, including smooth muscle cells, neural cells, neutrophils, adipocytes, fibroblasts, and other mesenchymal stem cells (Birbrair et al. 2013), suggesting multiple physiologic roles for these cells. However, at present, the pathophysiological relevance of this functional plasticity has not been examined in the context of resistance to anti-angiogenic therapies.

3.1.2 $CD11b^+Gr1^+$ MDSCs

Myeloid-derived suppressor cells represent a heterogeneous population of myeloid progenitor cells that are reminiscent of immature neutrophils, DCs, and macrophages. These cells exhibit strong immunosuppressive functions, as they can inhibit T-cell and NK-cell activation and proliferation as well as DC maturation (Gabrilovich et al. 2012). Besides their well-known immunosuppressive potential, several studies have demonstrated that $CD11b^+Gr1^+$ MDSCs can also promote tumor growth and angiogenesis (Yang et al. 2004; De Palma et al. 2005) through mechanisms involving the signal transducer and activator of transcription 3 (STAT3) transcription factor (Kujawski et al. 2008). Ferrara and collaborators identified a major role for MDSCs in mediating tumor resistance to VEGF-targeted

therapies (Shojaei et al. 2007a). The authors identified an immunological circuit through which tumor-infiltrating IL-17-producing (Th17) cells promoted recruitment of pro-angiogenic $CD11b^{+}Gr1^{+}$ myeloid cells into the TME. Specifically, IL-17 induced expression of granulocyte colony-stimulating factor (GCSF) on tumor-associated fibroblast (TAFs) and upregulated secretion of IL-6 and SDF-1α by both TAFs and tumor cells. This cytokine network stimulated the mobilization of vasculogenic $CD11b^{+}Gr1^{+}$ myeloid cells from the bone marrow, conferring resistance to VEGF-targeted therapies (Shojaei et al. 2008, 2009; Chung et al. 2013). Interestingly, some tumors intrinsically recruited $CD11b^{+}Gr1^{+}$ cells into the TME, while others activated this mechanism only in response to VEGF blocking antibodies (Shojaei et al. 2008). The mechanism underlying the pro-angiogenic activity of $CD11b^{+}Gr1^{+}$ MDSCs involves expression of the pro-angiogenic factor Bv8, a protein related to endocrine gland-derived VEGF that facilitates the mobilization of these cells and stimulation of VEGF-independent tumor angiogenesis (Shojaei et al. 2007a). Treatment with anti-Bv8-neutralizing antibodies in preclinical models reduced GCSF-mediated $CD11b^{+}Gr1^{+}$ cell mobilization, inhibiting tumor growth and suppressing the vasculogenic phenotype (Shojaei et al. 2007b). Moreover, hypoxia-induced recruitment of myeloid cells promoted resistance to sunitinib treatment in glioblastoma (Piao et al. 2012), metastatic renal cell carcinoma (mRCC), and breast adenocarcinoma (Finke et al. 2011) via suppression of Th1 responses and production of pro-angiogenic factors in the TME. In accordance, depletion of $CD11b^{+}Gr1^{+}$ myeloid cells in preclinical models using a colony-stimulating factor (CSF)-1 signaling inhibitor sensitized tumors to anti-VEGFR2 therapy (Priceman et al. 2010), highlighting the central role of MDSCs in mechanisms of resistance to anti-angiogenic therapy.

3.1.3 Macrophages

Macrophages are specialized phagocytic cells that display remarkable plasticity, capable of shaping their phenotype and function in response to environmental variations. These changes can give rise to different macrophage subpopulations with distinct functions (Mosser and Edwards 2008). Depending on their activation status, tissue localization, and polarization profiles, macrophages can display pro-tumoral or antitumor activities. In response to Th1-type cytokines, macrophages are polarized toward an M1 phenotype releasing high amounts of IL-12 and displaying antitumor responses, whereas a Th2 cytokine secretion partner (IL-4, IL-13) endows macrophages with an M2 immunosuppressive potential, leading to enhanced tumor cell invasion, angiogenesis, and metastasis (Siveen and Kuttan 2009; Mantovani et al. 2017).

The selective angio-modulatory role of macrophages under hypoxic conditions has been originally proposed in 1983 by Knighton et al. (1983). Afterward, several studies demonstrated that tumor-associated macrophages (TAMs) promote angiogenesis of solid tumors mostly via VEGF secretion (Noy and Pollard 2014). Moreover, studies in human tumors demonstrated a positive correlation between blood vessel density and the number of M2-type, but not M1-type, TAMs in vascularized tumor areas (Jetten et al. 2014). Whereas depletion of TAMs led to

decreased tumor angiogenesis, restoration of macrophage infiltration rescued vessel sprouting (Lin et al. 2006). Recent evidence indicated that this effect was not exclusively mediated by VEGF, as TAMs release a variety of pro-angiogenic factors including PlGF, bFGF, M-CSF, PDGF, TGF-β, and several cytokines including IL-1, IL-8, TNF, and MCP-1 (Rolny et al. 2011; Stockmann et al. 2014).

In spite of the well-established role of macrophages in promoting tumor angiogenesis (Mantovani et al. 2017), there are few direct evidences showing a role for these cells in resistance to anti-angiogenic therapies. Indirect evidence shows that PlGF mediates resistance to anti-VEGF(R) inhibitors by promoting angiogenesis and inducing recruitment of TAMs (Fischer et al. 2007). Moreover, human monocytes cultured with macrophage colony-stimulating factor (M-CSF) increased Tie2 expression and became more pro-angiogenic. In vivo, tumor-bearing mice treated with M-CSF showed expansion of $Tie2^{+}$ macrophages, leading to enhanced tumor angiogenesis (Forget et al. 2014). In the presence of hypoxic microenvironments, TAMs develop metabolic adaptation through upregulation of HIF-1α, an effect that ultimately led to VEGF-dependent angiogenesis and tumor progression (Burke et al. 2002; Lewis and Pollard 2006). Moreover, hypoxia-induced tumor cell apoptosis engages TAMs in an angiogenesis program through a prostaglandin E2 (PGE2)-dependent mechanism (Brecht et al. 2011).

3.1.4 Dendritic Cells

Dendritic cells (DCs) are terminally differentiated myeloid cells specialized in antigen processing and presentation. In spite of their well-established roles in orchestrating adaptive immunity, DCs can also trigger inhibitory circuits that ensure immunological tolerance and promote angiogenesis (Curiel et al. 2004; Murdoch et al. 2008; Ilarregui et al. 2009; Rabinovich and Conejo-Garcia 2016). Due to their remarkable plasticity, these cells respond to a plethora of environmental inputs that signal the occurrence of pathogens, tumors, or tissue inflammation by migrating from peripheral tissues into secondary lymphoid organs to empower T cells with stimulatory or regulatory potential (Cerliani et al. 2016). In addition, DCs have an extraordinary capacity to produce a wide array of pro-angiogenic mediators, including VEGF, bFGF, IL-8, TGF-β, TNF, GM-CSF, CXCL1, CXCL8, and CCL2, among others (Stockmann et al. 2014). This wide spectrum of pro-angiogenic mediators controls vascularization through indirect mechanisms involving hierarchical upregulation of pro-angiogenic growth factors by other cell types (Sozzani et al. 2007). As an example, TNF production by activated DCs induces the synthesis of VEGF in different cell types (including ECs), which influences angiogenesis. In fact, DCs themselves express both VEGFR1 and VEGFR2 (Mimura et al. 2007) and respond to PlGF and VEGF signaling by inhibiting their differentiation into functional DCs (Dikov et al. 2005; Mimura et al. 2007). Tumor-derived VEGF also induces a significant decrease in the number and functionality of spleen and lymph node DCs (Gabrilovich et al. 1996, 1998). These data suggest that DCs may rely on VEGF to promote angiogenesis, suggesting an indirect modulatory role of these cells in controlling anti-VEGF resistance. Supporting this assumption, anti-VEGF therapy enhanced the antitumor

activity of DCs and increased their infiltration in TME (Gabrilovich et al. 1999; Osada et al. 2008).

3.1.5 Mast Cells

In addition to their well-established roles in regulating innate and adaptive immunity, mast cells (MCs) have been recognized as potent inducers of tumor angiogenesis (Hanahan and Coussens 2012). Mobilization of these cells to virally induced squamous cell carcinomas and pancreatic tumors accentuated tumor growth (Coussens et al. 1999; Soucek et al. 2007), whereas impaired MC function inhibited tumor angiogenesis. Mast cells have been shown to act as reservoirs of pro-angiogenic mediators including VEGF, angiopoietin-1 (Ang-1), and IL-8 (Coussens et al. 1999; Marone et al. 2016) which are released to the TME under hypoxic conditions. Thus, MCs play key roles during tumorigenesis and metastasis by modulating tumor-promoting inflammation and vascularization, although evidence is still lacking on the role of these cells in mechanisms of resistance to anti-angiogenic therapies.

3.2 Lymphoid Cell-Dependent Resistance to Anti-angiogenic Therapies

Given the crucial role of regulatory lymphoid populations, including regulatory T cells (Tregs), regulatory B cells (Bregs), and different subsets of natural killer (NK) or natural killer T (NKT) cells in tumor-induced immunosuppression, it is likely that these cells may also contribute to tumor progression through nonimmune mechanisms. However, at present, there is no direct evidence implicating tumor-infiltrating lymphocytes (TILs) in resistance to anti-angiogenic therapies. However, early evidence showed that T cells contributed to sustain tumor angiogenesis by expressing VEGF (Freeman et al. 1995).

Although accumulation of Tregs responsible of suppressing activation of effector T cells has been correlated with the number of blood vessels in cancer (Gupta et al. 2007; Giatromanolaki et al. 2008; Gasparri et al. 2013) and wound healing (D'Alessio et al. 2015), a direct role for these cells in promoting tumor angiogenesis and conferring resistance to anti-angiogenic therapies has not been demonstrated. Nevertheless, it has been shown that human and mouse $CD4^+CD25^+$ Tregs secrete high amounts of VEGF-A in response to hypoxia (Curiel et al. 2004; Facciabene et al. 2011; Gasparri et al. 2013). In turn, depletion of Tregs suppressed VEGF-A expression in the TME and decreased tumor angiogenesis (Facciabene et al. 2011). Interestingly, activated $CD4^+$ T cells may capture neuropilin-1 (NRP1), a VEGF co-receptor from DCs (Bourbie-Vaudaine et al. 2006), thus allowing interactions with VEGF following recruitment by CCL22 and CCL28 (Curiel et al. 2004; Facciabene et al. 2011).

As mentioned above, Ferrara and colleagues identified and dissected an immune-vascular circuitry which controls compensatory angiogenesis. In the TME, secretion of IL-17 led to GCSF production by TAFs and subsequent

recruitment of MDCSs which in turn promoted compensatory angiogenesis and conferred resistance to anti-VEGF therapy (Shojaei et al. 2007a; Chung et al. 2013). Additionally, IL-17 accentuated the pro-angiogenic function of MDSCs through expression of VEGF and Bv8 (Chung et al. 2013), indicating an unexpected role of tumor-infiltrating Th17 cells in facilitating VEGF-independent vascularization.

In contrast to myeloid cells and T cells, limited information is available regarding the angiomodulatory roles of B cells. B cell-derived VEGF has been associated with both lymphangiogenesis and angiogenesis in lymph nodes from healthy mice (Shrestha et al. 2010). Moreover, tumor-associated B cells have been shown to promote angiogenesis via a STAT3-dependent mechanism in melanoma and Lewis lung carcinoma models (Yang et al. 2013). Interestingly, a subpopulation of immature B cells has recently been associated with response to anti-angiogenic treatment (Fagiani et al. 2015). Interfering with VEGF or PDGF signaling regulated the frequency of immature B cells in tumor bearing but not in healthy mice, indicating that a $CD45^{dim}VEGFR1^{-}CD31^{low}$ B-cell subpopulation may serve as a predictor of responses to anti-angiogenic therapies (Fagiani et al. 2015).

Natural killer (NK) cells are prototypical innate lymphoid cells (ILCs) with crucial roles in cancer immunosurveillance. These cells can terminally differentiate in peripheral tissues depending on the presence of stimulatory or inhibitory signals that shape their functionality (Mamessier et al. 2011; Platonova et al. 2011). Accordingly, tumors may release a variety of soluble factors that could influence the ability of NK cells to promote tumor elimination or escape. Tolerogenic NK cells, originally described as decidual NK cells (dNKs) at the fetomaternal interface, play a key role in generating tolerogenic microenvironments and inducing placental angiogenesis by releasing VEGF and IL-8 (Hanna et al. 2006). In this regard, Bruno and colleagues found a tumor-specific subset of $CD56^{+}CD16^{-}$ NK cells that produce functional VEGF, PIGF, and IL-8 (Bruno et al. 2013). The mechanism underlying this pro-angiogenic phenotype resembled that exhibited by decidual NK cells. Supporting this notion, a STAT5-dependent mechanism of VEGF regulation in tumor-associated NK cells has been shown to control tumor angiogenesis (Gotthardt et al. 2016). Yet, there is still no evidence of the role of NK cells in resistance to anti-angiogenic therapies and the cross talk of these cells with other components of the TME. Further studies are needed to further understand the role of innate and adaptive lymphoid populations in regulating EC biology and resistance to anti-angiogenic therapies.

4 Hypoxia Bridges Immune Tolerance and Angiogenesis in TME

In the past few years, most studies have focused on the TME (Hanahan and Coussens 2012), mainly because (1) stromal and inflammatory cells are key players in tumor progression (Quail and Joyce 2013; Coussens et al. 2013); (2) therapies targeting tumor cells have shown limited clinical efficacy (Jain 2014); (3) tumor stroma plays a central role in acquired resistance to targeted therapies (Junttila and

de Sauvage 2013); and (4) new therapies targeting immune, endothelial, or other stromal components of the TME (such as checkpoint blockers, anti-angiogenic agents, or targeted compounds) have demonstrated clinical success in a variety of tumors (Pardoll 2012; Junttila and de Sauvage 2013). In fact, the TME continually changes over the course of cancer progression, and non-transformed cells from the TME coevolve with tumor cells.

Hypoxia emerges as a major driving force that influences both cancer cells and cells of the TME to promote tumor progression and metastasis. Given its central role in angiogenesis, immunosuppression, and resistance to therapies, tumor hypoxia represents one of the most important targets to be exploited for the development of new cancer therapies (Keith et al. 2011; Pan et al. 2012). In spite of significant progress in understanding the molecular components of hypoxia-regulated programs (LaGory and Giaccia 2016), the high plasticity of these responses and the presence of tumor divergence in oxygen-sensing mechanisms make hypoxia-related targets difficult to be identified. Adding complexity to this scenario, the molecular mechanisms coupling tumor hypoxia and compensatory angiogenesis remain poorly understood.

Targeting the vascular compartment of the TME induces an imbalance of pro- and anti-angiogenic factors that impairs tumor blood perfusion. Despite reducing blood supply which generates an apparently unfavorable setting for tumor growth, it may also enhance metastatic potential and generate resistance to radiotherapy, chemotherapy, and targeted therapies, including anti-angiogenic therapies (Jain 2005; Bergers and Hanahan 2008). An explanation of this phenomenon is that reducing oxygen supply generates an abnormal microenvironment characterized by hypoxia and acidosis, a setup that affects several pathways of tumor progression (Jain 2014). In this sense, tumor hypoxia not only promotes reexpression of soluble factors that promote compensatory angiogenesis (Chouaib et al. 2012; Jain 2014) but also downmodulates immune responses contributing to tumor-driven immunosuppression (Hanahan and Coussens 2012; Motz and Coukos 2013). Recently, Shehade and colleagues demonstrated that hypoxia affects T-cell activity by blunting the capacity of Th1 cells to produce IFN-γ through STAT3-dependent regulation of IL-10 expression (Shehade et al. 2015). Moreover, after T-cell receptor (TCR) engagement, HIF-1α deficiency shifts T-cell differentiation toward a pro-inflammatory cytokine secretion profile (Lukashev et al. 2006), suggesting that HIF-1α may serve as a negative regulator of T-cell differentiation. Hypoxia promotes inhibition of antitumor immune responses via induction of $FOXP3^+$ Tregs in gastric cancer (Deng et al. 2013) and glioblastoma (Wei et al. 2011) through common mechanisms involving a TGF-β1/STAT3-regulated pathway. Indeed, supernatants derived from gastric cancer cells cultured under hypoxic conditions induced expression of the FOXP3 transcription factor on T cells through modulation of TGF-β_1 signaling (Deng et al. 2013). In addition, hypoxia has been associated with mobilization of tolerogenic myeloid cells toward the tumor parenchyma in several tumor models. In gastric cancer, tumor hypoxia directly correlated with Treg cell infiltration (Deng et al. 2013). In ovarian tumors, hypoxia recruited Tregs to the TME through induction of the C-C motif chemokine ligand

28 (CCL28) by tumor cells (Facciabene et al. 2011) and CCL22 by TAMs (Curiel et al. 2004). Moreover, tumor cell expression of VEGF contributed to recruit Tregs to the TME in melanoma models (Hansen et al. 2012). Hypoxia and acidosis also endowed tumor-associated myeloid cells with pro-angiogenic and immunosuppressive potential (Murdoch et al. 2008). MDSCs are rapidly differentiated in a hypoxic TME into TAMs or immature DCs through mechanisms mediated by HIF-1α activation (Corzo et al. 2010). Upon differentiation, these cells acquired pro-angiogenic properties while maintaining their immunosuppressive phenotype (Motz and Coukos 2011). Hypoxia further aggravated immunosuppression by attracting macrophages into hypoxic areas via HIF-1α-mediated expression of VEGF, endothelin-1, and CCL2 chemoattractant (Murdoch et al. 2004; Gabrilovich et al. 2012; Kitamura et al. 2015). These TAMs produced several anti-inflammatory mediators including IL-10, TGF-β_1, VEGF, and PGE_2 (Triner and Shah 2016). Interestingly, Colegio and colleagues demonstrated a cross talk between tumor cells and macrophages in which lactic acid produced by tumor cells under anaerobic conditions promoted angiogenesis by inducing HIF-1α-dependent VEGF expression and M2 polarization of TAMs (Colegio et al. 2014). Increased levels of lactate induced adenosine accumulation and lowered pH in the TME, thereby impairing DC migration and function. DCs differentiated in the presence of adenosine and hypoxia had impaired allostimulatory activity and expressed higher levels of immunosuppressive/pro-angiogenic molecules including VEGF, IL-6, IL-8, IL-10, cyclooxygenase 2 (COX2), TGF-β_1, and indoleamine 2,3-dioxygenase (IDO) (Yang et al. 2010; Gabrilovich et al. 2012). In addition, HIF-1α but not HIF2-α activation under hypoxic conditions upregulated PD-L1 expression in MDSCs, TAMs, and DCs, endowing these cells with tolerogenic activity (Noman et al. 2014). These results suggest that simultaneous blockade of inhibitory checkpoints such us PD-L1, CTLA-4, or BTLA-4 along with inhibition of HIF-1α may represent a novel approach for combinatorial cancer immunotherapy.

However, in spite of the well-known pro-angiogenic and anti-inflammatory roles of HIF-1α, other studies revealed that under specific circumstances, this transcription factor may also display pro-inflammatory activity by promoting Th17 cell differentiation through mechanisms involving reprogramming of glycolytic metabolism and mTOR/RORγt activation (Shi et al. 2011; Dang et al. 2011). These apparently controversial data highlight the versatility and the context-dependent roles of hypoxia in inflammation and cancer. Although hypoxia promotes tolerance and angiogenesis by modulating the immune component of the TME, there is still no direct information about the role of hypoxia in generating resistance to anti-angiogenic therapies through immune-mediated mechanisms. Further studies are still needed to better understand the mechanisms by which local immune tolerance and angiogenesis are interconnected and cooperate to sustain tumor resistance to anti-angiogenic therapies.

5 The Galectin-Glycan Axis: Linking Immunosuppression, Hypoxia, and Resistance to Anti-angiogenic Treatment

Galectins (Gals) are evolutionarily conserved proteins that function in the extracellular milieu by interacting with a myriad of glycosylated receptors, or intracellularly by controlling signaling pathways through protein-glycan or protein-protein interactions (Cerliani et al. 2016; Mendez-Huergo et al. 2017). Galectins are defined by a conserved carbohydrate recognition domain (CRD) that recognizes glycans containing the disaccharide *N*-acetyllactosamine [Galβ1-4GlcNAc or LacNAc] (Liu and Rabinovich 2005; Thiemann and Baum 2016). Some galectins (i.e., Gal1 and Gal3) are expressed in a wide range of immune cell types with high expression in macrophages (Rabinovich et al. 1996; Sato and Nieminen 2004), DCs (Ilarregui et al. 2009; Tesone et al. 2016), eosinophils (Ge et al. 2016), and Tregs (Garin et al. 2007), as well as in ECs (Thijssen et al. 2006), whereas others (including Gal7 and Gal12) show more restricted tissue localization (Cerliani et al. 2016). Although binding of galectins to glycans involves low-affinity interactions, multivalency results in high-avidity binding, promoting cross-linking and clustering of surface glycoreceptors, regulating their endocytosis and signaling (Nabi et al. 2015). Particularly within immune and vascular compartments, galectin-glycan complexes control signaling thresholds of relevant receptors including the T-cell receptor (TCR), pre-B-cell receptor (BCR), cytokine receptors like TGF-βR (Rabinovich and Croci 2012), immune checkpoint molecules including lymphocyte-activation gene-3 (LAG-3), cytotoxic T lymphocyte antigen-4 (CTLA-4) and T-cell immunoglobulin domain and mucin domain-3 (TIM-3) (Zhu et al. 2005; Lau et al. 2007; Rangachari et al. 2012; Kouo et al. 2015), tyrosine kinase receptors such as VEGFR2 (Croci et al. 2014a; Markowska et al. 2011), and integrins including $\alpha_1\beta_1$ and $\alpha_5\beta_1$ (Chen et al. 2016). Given their capacity to regulate signaling programs by modulating canonical pathways that govern angiogenic processes (Croci et al. 2014a), together with their roles in regulating immune responses (Cerliani et al. 2016) and their marked expression in the TME (Rabinovich and Conejo-Garcia 2016), galectins have emerged as alternative players bridging tumor vascularization and immunosuppression (Croci et al. 2014b). The critical role of galectins in regulating immune response has been recently revised (Thiemann and Baum 2016; Mendez-Huergo et al. 2017). Here we will focus on the role of galectins in tumor neovascularization and resistance to anti-angiogenic therapies.

5.1 Galectins as Extracellular Mediators of Angiogenesis

An increasing number of studies support the central role of galectins in the control of vascular programs (Thijssen et al. 2013) and tumor immunity (Rabinovich and Croci 2012). Galectins (Gal1, Gal3, Gal8, and Gal9) can influence angiogenesis by cross-linking EC surface glycoproteins and activating distinct signaling pathways (Croci et al. 2014b). In this regard, Gal1 interacts with neuropilin-1 (NRP1) and/or

VEGFR2, where it modulates receptor segregation, internalization, and trafficking through glycan-dependent mechanisms, leading to VEGFR2 phosphorylation and signaling via the Raf/extracellular signal-regulated kinase (ERK) and Akt (Hsieh et al. 2008; Thijssen et al. 2010; Mathieu et al. 2012; Croci et al. 2012, 2014a; D'Haene et al. 2013). On the other hand, Gal3 acts through binding to *N*-glycans on $\alpha_v\beta_3$ integrin and modulating cell surface retention of VEGFR2 (Nangia-Makker et al. 2000; Markowska et al. 2010; Markowska et al. 2011), whereas Gal8 triggers angiogenesis through binding to the activated leukocyte-cell adhesion molecule (ALCAM, CD166) (Delgado et al. 2011). Interestingly, Gal8 also contributes to pathological lymphangiogenesis through binding to VEGF-C, podoplanin, and integrins $\alpha_1\beta_1$ and $\alpha_5\beta_1$ (Chen et al. 2016). Moreover, Gal9Δ5, a splice variant isoform of Gal9, induces a dose- and context-dependent effect on EC morphogenesis (Heusschen et al. 2014).

Tumor hypoxia, induced in response to anti-angiogenic therapies, upregulates Gal1 expression in different tumor types through HIF-1-dependent or ROS/NF-κB-dependent (Le et al. 2005; Zhao et al. 2010; Croci et al. 2012) mechanisms. Targeting Gal1 expression attenuated vascularization and suppressed growth of several tumors including melanoma (Thijssen et al. 2006; Mathieu et al. 2012; Croci et al. 2014a), Kaposi's sarcoma (Croci et al. 2012), prostate carcinoma (Laderach et al. 2013), lung adenocarcinoma and T-cell lymphoma (Croci et al. 2014a), hepatocarcinoma (Manzi et al. 2016), pancreatic adenocarcinoma (Martinez-Bosch et al. 2014), glioblastoma (Verschuere et al. 2014), and gastric cancer (Tang et al. 2016). Moreover, Gal1 expression correlated with blood vessel density in human prostate adenocarcinoma (Laderach et al. 2013), non-small cell lung adenocarcinoma (NSCLC) (Carlini et al. 2014), and Kaposi's sarcoma (Croci et al. 2012). Interestingly, Gal1-induced angiogenesis appeared to be independent of canonical pro-angiogenic factors including VEGF, FGF2, oncostatin M, angiopoietin-like 4 (ANGPTL4), and platelet-derived growth factor (PDGF)-α (Croci et al. 2012; Laderach et al. 2013) emphasizing the role of this lectin as a main player in VEGF-independent angiogenesis. In contrast, Gal3 acts as a pro-angiogenic factor through mechanisms involving VEGF and TGF-β signaling (Machado et al. 2014). These findings are consistent with the ability of Gal3 to potentiate VEGFR and FGFR-mediated angiogenesis through *N*-glycan-dependent mechanisms (Markowska et al. 2010). Furthermore, LGALS3BP, a protein known to specifically bind Gal3, functions as a pro-angiogenic factor through a dual mechanism involving induction of tumor VEGF and direct stimulation of EC morphogenesis (Piccolo et al. 2013). Moreover, other studies have shown an indirect role of Gal2, Gal4, and Gal8 in modulating angiogenesis programs by inducing the secretion of EC-derived cytokines and chemokines (GCSF, IL-6, MCP-1, and GROα) which in turn stimulate EC signaling (Chen et al. 2014).

5.2 Galectins as Mechanisms of Resistance to VEGF Blockade

Signaling events triggered by Gal1 recapitulated those activated by the canonical cytokine VEGF including VEGFR2, ERK1/2, and Akt phosphorylation (Croci et al. 2014a). At the molecular level, Gal1 preferentially bounds to branched *N*-glycans present in immunoglobulin domains-3, 4, and 7 of VEGFR2, leading to glycosylation-dependent clustering of this receptor on the surface of ECs (Croci et al. 2014a). Interestingly, exposure of ECs to immunosuppressive or hypoxic conditions led to significant changes in the EC glycome, which facilitated Gal1 binding and triggered angiogenesis, suggesting a dynamic interplay between immunosuppression and hypoxia in the regulation of EC signaling. Interestingly, VEGFR2 glycosylation pattern was recently confirmed by structural MS/MS analysis (Chandler et al. 2017), confirming the relevant role of glycosylation in lectin-receptor signaling and angiogenesis.

Given the striking similarities of VEGF and Gal1 signaling, we proposed that glycosylation-dependent binding of Gal1 to VEGFR2 might preserve angiogenesis in settings of VEGF blockade, especially in tumors that are resistant to anti-VEGF treatment. Supporting our in vitro findings, tumors produced high amounts of Gal1 in response to anti-VEGF treatment, while their associated vasculature changed its glycosylation profile facilitating Gal1-VEGFR2 interactions (Croci et al. 2014a). In contrast, vessels associated to anti-VEGF-sensitive tumors displayed glycosylation patterns that prevented Gal1 binding and angiogenesis even in the presence of VEGF blockade. Accordingly, genetic modification of EC glycosylation (lack of β1,6-GlcNAc-branched *N*-glycans) or silencing of tumor-derived Gal1 converted refractory into anti-VEGF-sensitive tumors (Croci et al. 2014a). These findings highlight the critical importance of tumor-derived Gal1 as a potential therapeutic target to overcome anti-VEGF compensatory programs.

5.3 Targeting Gal1-*N*-Glycan Interactions Limits the Efficacy of Anti-VEGF Treatment

Based on the aforementioned evidence, we proposed that selective Gal1-VEGFR2 interactions may serve as an alternative compensatory mechanism that preserves angiogenesis in settings of VEGF sequestration (Croci et al. 2014a) (Fig. 2). This evidence together with the role of Gal1 as an immune modulator (Rubinstein et al. 2004; Juszczynski et al. 2007; Rutkowski et al. 2015; Tesone et al. 2016) highlights the role of Gal1 as an attractive target to attenuate aberrant angiogenesis and circumvent tumor-induced immunosuppression. Blockade of Gal1-*N*-glycan interactions using an anti-Gal1 monoclonal antibody eliminated resistance to anti-VEGF treatment, suppressed the formation of aberrant vascular networks, and enhanced antitumor immunity in several tumor models including melanoma, lung cancer, and T-cell lymphoma (Croci et al. 2014b). Noteworthy, Gal1 blockade induced normalization of blood vessels early after treatment, as shown by reduced vessel diameter, increased pericyte coverage and maturation, and alleviation of

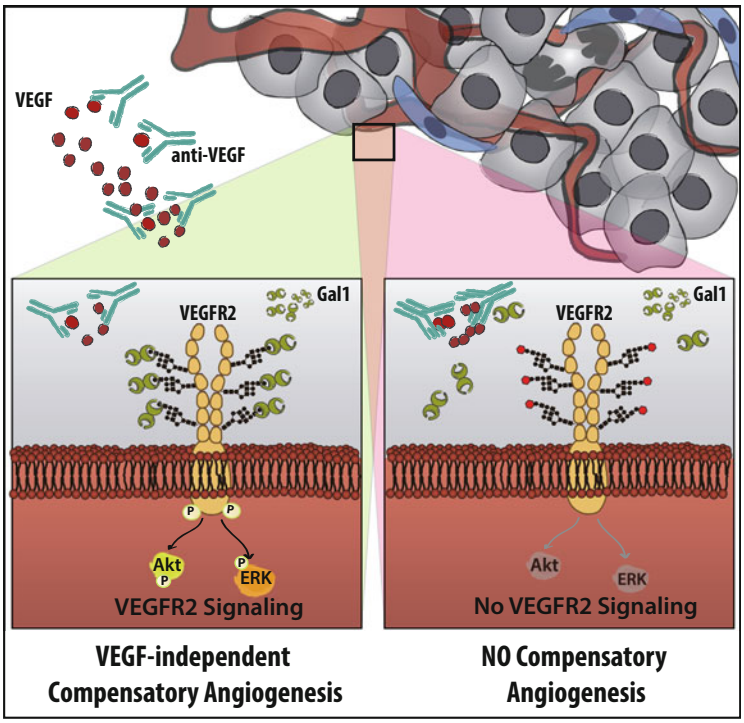

Fig. 2 Resistance to anti-VEGF treatment mediated by Gal1-*N*-glycan interactions. In anti-VEGF resistant tumors, hypoxia generated by VEGF blockade induces Gal1 expression in tumor cells and dynamic remodeling of the repertoire of glycans decorating VEGFR2 in tumor-associated ECs. Interaction between Gal1 and complex branched *N*-glycans lacking α2,6-linked sialic acid promotes VEGFR2 signaling and preserved tumor angiogenesis (*left panel*). In contrast, vessels associated to anti-VEGF-sensitive tumors (*right panel*) exhibit high amounts of α2,6-linked sialic acid, which prevent Gal1 binding and compensatory angiogenesis even in the absence of VEGF signaling

tumor hypoxia; this effect favored mobilization of immune cells and potentiated the antitumor response (Croci et al. 2014a). Supporting these findings, treatment of tumors with both bevacizumab and anginex, an anti-angiogenic peptide that binds to Gal1, normalized tumor vessels, increased oxygenation, and improved responses to radiation therapy (Dings et al. 2007). Moreover, administration of OTX008, an anginex-derived synthetic compound, potentiated the activity of the TKI sunitinib in nude mice inoculated with tumor xenografts (Zucchetti et al. 2013). These results support the use of combination therapies containing Gal1-blocking agents to maximize the efficacy of anticancer treatments.

6 Combinatorial Therapies: Anti-angiogenesis as a Partner of Immunotherapy

Combining anti-angiogenic therapy with immunotherapies is not a new idea, although it recently gained particular attention as clinical evidence revealed a reciprocal paradox: immunotherapy can also lead to anti-angiogenic effects, and anti-angiogenesis can stimulate immune responses (Garber 2014). Over the last 20 years, considerable data has accumulated showing that in addition to its pro-angiogenic role, VEGF also suppresses adaptive immunity (Ferrara and Adamis 2016). In several preclinical models, tumor-derived VEGF was associated with decreased immune cell activity by preventing DC maturation and promoting expression of inhibitory checkpoints (Voron et al. 2015), decreasing recruitment of lymphocytes, and increasing the Treg/CD8 ratio in the TME, thus hampering presentation of tumor antigens (Rabinovich et al. 2007; Melero et al. 2015). On the other hand, low-dose anti-angiogenic therapies have increased immune cell infiltration in TME (Huang et al. 2013). Therefore, normalizing tumor vasculature with low-dose anti-angiogenic therapies (Jain 2014) emerges as a new therapeutic option to improve the efficiency of immunotherapy by promoting infiltration of specific T cells in adoptive T-cell therapies, tumor vaccination approaches, and checkpoint blockade therapies. In this regard, targeting tumor vasculature with low vascular-normalizing doses of DC101 (an anti-VEGFR2 antibody) results in a more homogeneous distribution of functional tumor vessels, polarization of TAMs toward an immune stimulatory M1 phenotype, and higher influx of $CD4^+$ and $CD8^+$ T cells into the tumor parenchyma, events that overall improved cancer vaccination (Huang et al. 2012). Furthermore, targeting RTK signaling with sunitinib not only blocked proliferative signaling in ECs but also inhibited proliferation in tumor cells and reduced accumulation of MDSCs (Huang et al. 2013). Although anti-angiogenic therapy of kidney cancer increased infiltration of $CD4^+$ and $CD8^+$ T lymphocytes, it also augmented infiltration of Tregs and enhanced expression of programmed death-ligand 1 (PD-L1) (Liu et al. 2015), suggesting that anti-angiogenic therapy may positively or negatively regulate immune responses.

Detection and destruction of malignant cells by cytolytic T lymphocytes (CTLs) are a hallmark of cancer immunotherapy. Hence, targeting pathways that not only increase T-cell activation but also enhance intratumoral trafficking and persistence of tumor-specific T cells has the potential to become a highly effective antitumor strategy. In a murine cancer model of colon adenocarcinoma, anti-PD-1 and anti-VEGFR2 (DC101) antibodies acted synergistically to inhibit tumor growth (Yasuda et al. 2013). Moreover, in a recent study, Wallin and collaborators explored the mechanisms underlying the therapeutic activity of anti-PD-L1 (atezolizumab) in combination with bevacizumab in patients with metastatic renal cell carcinoma (mRCC). Despite the small cohort of patients, the authors found an increase of genes and markers associated with Th1-driven chemokines and $CD8^+$ T-cell effectors, as well as natural killer (NK) cell functions following simultaneous administration of both therapeutic antibodies. Additionally, the authors found that anti-VEGF and anti-PD-L1 combination improved antigen-specific T-cell

migration via a CX3CL1-mediated mechanism (Wallin et al. 2016). Increased intratumoral homing of lymphocytes following combination therapy was also observed in other types of tumors (Hughes et al. 2016). The combination of ipilimumab (anti-CTLA-4 monoclonal antibody) and bevacizumab in metastatic melanoma showed an increase of activated vessels accompanied by an extensive $CD8^+$ T-cell and $CD163^+$ macrophage infiltration (Hodi et al. 2014). Furthermore, the authors showed an increase of anti-Gal1, Gal3, and Gal9 antibodies in the peripheral blood from patients treated with bevacizumab or bevacizumab plus ipilimumab (Hodi et al. 2014). Moreover, Hodi's group demonstrated that combination of bevacizumab and ipilimumab leads to increased vascular expression of cell adhesion molecules including intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) and promoted lymphocyte recognition and tumor infiltration via upregulation of circulating CXCL10, IL-1α, TNFα, CXCL1, IFN-α, and IL-8 and downregulation of VEGF (Wu et al. 2016). These studies provided the basis for further investigation of the dual roles of angiogenic factors and immune regulators, suggesting the design of novel combinatorial modalities that strike both the immune and vascular compartments (Manegold et al. 2017). Since most combinatorial strategies are based on modulation of the VEGF-VEGFR axis, further studies should be aimed at exploring other agents capable of generating vascular normalization and facilitating recruitment of immune cells to the tumor parenchyma. In this sense, Gal1-blocking antibodies appear as attractive agents to simultaneously target vascular normalization and reinforce antitumor immune responses, particularly those mediated by Th1, Th17, and $CD8^+$ CTLs (Croci et al. 2014a).

7 Conclusions and Future Perspectives

In this article, we highlight the relevance of hypoxia-regulated programs and immune-mediated circuits that converge in EC signaling to control vascularization and resistance to anti-angiogenic therapies. We underscore the relevance of myeloid and lymphoid subsets as well as cytokine and chemokine networks as potential compensatory angiogenic pathways and discuss the role of galectin-glycan lattices in sustaining angiogenesis in settings of VEGF blockade. Interestingly, galectins not only mediate resistance to anti-angiogenic therapies, but they can also regulate sensitivity to other anticancer modalities including immunotherapy by rituximab (Lykken et al. 2016), targeted therapy with imatinib (Glivec) (Luo et al. 2016), and chemotherapy with paclitaxel and adriamycin (Wang et al. 2017). Given the importance of unleashing tumor immunity to eradicate tumor cells, we also highlighted the bi-directional and reciprocal cross talk between immunosuppression and angiogenesis in the TME. Future studies should be aimed at dissecting the molecular basis involved in this intriguing interplay and to further explore combinatorial strategies aimed at attenuating aberrant angiogenesis, normalizing tumor vasculature, and potentiating antitumor immune responses. Furthermore, innovative structure-function studies are required to further dissect the mechanisms

underlying the therapeutic action of novel and currently available anticancer drugs, to further validate new targets and combinatorial modalities. Finally, identification of biomarkers that could predict responsiveness to different anti-angiogenic treatments and/or acquisition of resistance mechanisms is of critical importance to increase the number of patients who will benefit from vessel-targeting therapies.

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Mechanisms of Resistance to Target Therapies in Non-small Cell Lung Cancer

Francesco Facchinetti, Claudia Proto, Roberta Minari, Marina Garassino, and Marcello Tiseo

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Abstract

Targeted therapies are revolutionizing the treatment of advanced non-small cell lung cancer (NSCLC). The discovery of key oncogenic events mainly in lung adenocarcinoma, like *EGFR* mutations or *ALK* rearrangements, has changed the treatment landscape while improving the prognosis of lung cancer patients. Inevitably, virtually all patients initially treated with targeted therapies develop resistance because of the emergence of an insensitive cellular population, selected by pharmacologic pressure. Diverse mechanisms of resistance, in

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particular to EGFR, ALK and ROS1 tyrosine-kinase inhibitors (TKIs), have now been discovered and may be classified in three different groups: (1) alterations in the target (such as *EGFR* T790M and *ALK* or *ROS1* mutations); (2) activation of alternative pathways (i.e. *MET* amplification, *KRAS* mutations); (3) phenotype transformation (to small cell lung cancer, epithelial–mesenchymal transition). These basic mechanisms are informing the development of novel therapeutic strategies to overcome resistance in the clinic. Novel-generation molecules include osimertinib, for EGFR-T790M-positive patients, and new ALK-TKIs. Nevertheless, the possible concomitant presence of multiple resistance mechanisms, as well as their heterogeneity among cells and disease localizations, makes research in this field particularly arduous. In this chapter, available evidence and perspectives concerning precise mechanisms of escape to pharmacological inhibition in *oncogene-addicted* NSCLC are reported for single targets, including but not limited to EGFR and ALK.

Keywords
ALK • EGFR • NSCLC • Resistance mechanisms • ROS-1 • T790M

1 Introduction

Lung cancers currently figure among the most frequent tumor diagnoses and are the most relevant in terms of mortality worldwide (Siegel et al. 2016).

Before the year 2000 the dichotomy between small cell and non-small cell lung cancer (SCLC and NSCLC, respectively) was sufficient to address treatment strategies. Further histologic definition within NSCLC (squamous cell carcinoma and adenocarcinoma) was therefore recognized as clinically meaningful (Scagliotti et al. 2008). Since the last decades, molecular sub-typing of NSCLC (with an almost exclusive regard to adenocarcinoma) is providing a drastic refinement in the detection of alterations suitable of specific inhibition, generating a dramatic evolution in patients' management. Such aberrations (whose incidence in western population is showed in Fig. 1), in general mutually exclusive, normally represent the very funding oncogenic event (Gainor et al. 2013). The targeting of such altered tyrosine-kinase (TK) receptors by means of specific inhibitors (TKIs, actively competing against ATP-binding) usually generates extremely rapid and profound tumor responses, defining thus far the scenario of *oncogene addiction* (Lynch et al. 2004; Paez et al. 2004; Kwak et al. 2010). In this field, the superiority of targeted agents over standard chemotherapy, in the advanced setting, is at this point evident (Mok et al. 2009; Solomon et al. 2014).

Albeit targeted therapies are revolutionizing the treatment of advanced NSCLC, sooner or later resistance appears in virtually every patient. Molecular treatment exhaustion denotes the emergence of a cellular population insensitive and selected by the pharmacologic pressure. In parallel to the crucial recognition of specific mechanisms on the diagnostic samples, the detection of molecular reasons explaining treatment resistance at the moment of disease progression, obtaining

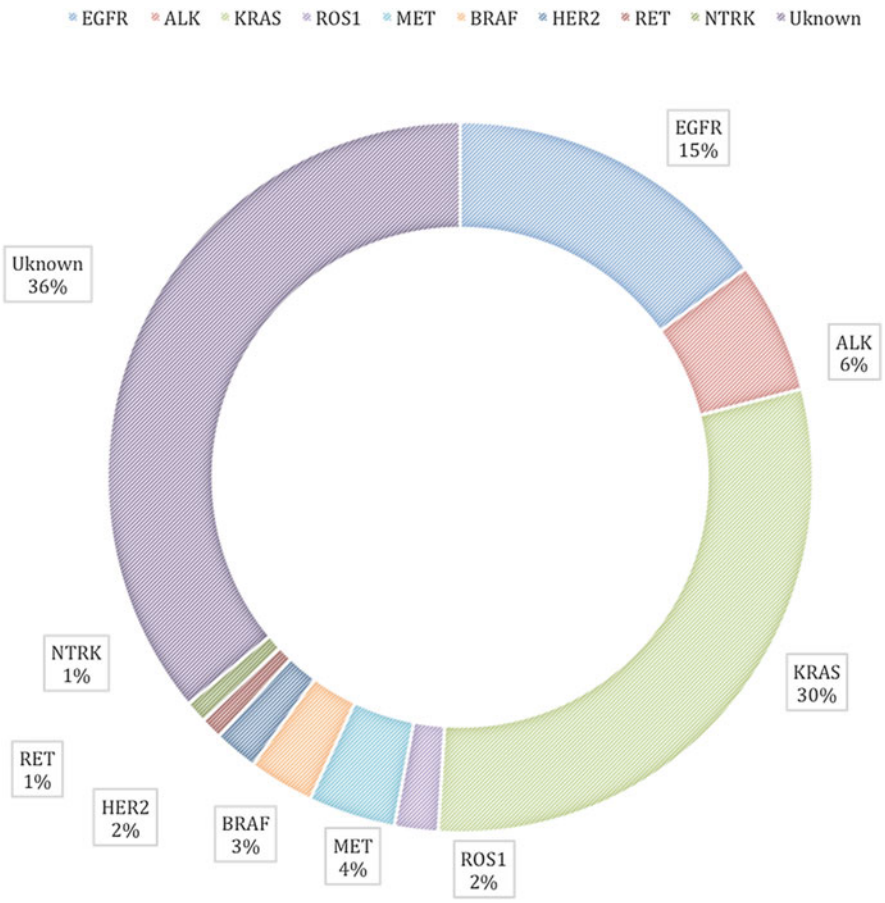

Fig. 1 Distribution of molecular aberrations responsible for *oncogene addiction* in lung adenocarcinoma affecting Western populations

of novel tumor material (*re-biopsy*) harbours a similar pivotal importance. The role of re-biopsies in the clinical setting is currently gaining more and more relevance due to the development of novel-generations Epidermal Growth Factor Receptor (EGFR)-TKIs, like osimertinib (AZD9291), active against T790M *EGFR* mutation, whose emergence is the most common mechanism of resistance to first-and second-generation anti-EGFR compounds (Kobayashi et al. 2005; Cross et al. 2014; Mok et al. 2016) (see next paragraphs).

Patterns of resistance to tailored therapies are shared among different activating aberration, and lessons regarding targets rare in lung cancers can be driven from other tumors. The general molecular ways lung cancer cells find to escape directed targeting are resumed in Table 1. The possible concomitant presence of multiple mechanisms, as well as their heterogeneity among cells and disease localizations (Suda et al. 2016; Hata et al. 2015), makes research in this field particularly

Table 1 Schematic description of the main resistance mechanisms to targeted treatments in non-small cell lung cancer

Mechanisms	Examples	Oncogene
Alterations in the target	*EGFR* T790M mutations	*EGFR*
	ALK TKD mutations – *ALK* amplification	*ALK*
Activation of bypass pathways	*MET* or *HER2* amplification	*EGFR*
	EGFR hyperactivation	*ALK*
Morpho-phenotypic evolutions	EMT and transformation from ADC to SCLC	Both

TKD tyrosine kinase domain, *EMT* epithelial–mesenchymal transition, *ADC* adenocarcinoma, *SCLC* small cell lung cancer

arduous. Available evidence and perspectives concerning precise mechanisms of escape to pharmacological inhibition in *oncogene-addicted* NSCLC are reported for single targets in the next paragraphs.

2 Resistance Mechanisms to EGFR-Driven NSCLC

Mutations in the *EGFR* gene are the most frequent oncogenic drivers in NSCLC, reported in approximately 10–15% of Caucasian NSCLC patients (Rosell et al. 2009) and 30–50% of Asians ones (Mok et al. 2009). The development of EGFR-TKIs, such as erlotinib, gefitinib (belonging to the first generation) and afatinib (second generation), shaped a great shift in the therapeutic management of *EGFR*-mutated NSCLC patients resulting in improved response rate (RR), progression free survival (PFS) and quality of life compared to first-line platinum-based chemotherapy (Mok et al. 2009; Rosell et al. 2012; Yang et al. 2015).

Unfortunately, prognosis remains unfavourable because of the occurrence of treatment resistance.

However, the identification of some mechanisms of resistance improved the therapeutic chances of these patients. In particular, the point mutation p.Thr790Met (T790M) occurring in *EGFR* exon 20 is responsible of resistance in about 50–60% of the patients when progression occurs (Sequist et al. 2011). Recently, the third-generation TKI osimertinib improved outcomes in patients harbouring this new mutation (Mok et al. 2016). Some other molecular resistance mechanisms have already been identified, but other information are needed to better understand and effectively overcome resistance to EGFR-TKIs in the remaining 40–50% lacking T790M mutation. Although exciting survival data and response rates have been registered in patients treated with osimertinib, acquired resistance unfortunately still occurs also during this therapy (Minari et al. 2016). Here, we will review principle mechanisms of resistance described during NSCLC treatment with both first-/second- and third-generation EGFR-TKIs.

2.1 Resistance to First- and Second-Generation EGFR-TKIs

Today erlotinib and gefitinib, together with the second-generation afatinib, are recognized as the standard first-line therapy in NSCLC patients with activating *EGFR* mutations (Mok et al. 2009; Rosell et al. 2012; Yang et al. 2015). Despite these important results, some patients with confirmed mutations in the EGFR-TK domain do not respond to EGFR-TKIs at all (de novo/*intrinsic resistance*). The remaining *EGFR*-mutated patients, after favourable and prolonged responses, inevitably exhibit disease progression (*acquired resistance*), usually after 10–14 months of treatment. Although the large majority of evidence concerns tumor evasion of targeted treatments represented by erlotinib and gefitinib, afatinib exhaustion seems to share the same molecular mechanisms (Campo et al. 2016). Several mechanisms of resistance have been identified and they may be classified in three different groups, as indicated in the introduction: (1) *EGFR* mutations; (2) activation of alternative pathways; (3) phenotype transformation (Table 1 and Fig. 2).

2.1.1 Preclinical Evidence and Clinical Relevance of Resistance Mechanisms

Mechanisms of primary resistance are still not fully understood, but several cases of de novo inefficacy of EGFR-TKIs are the consequence of the presence of

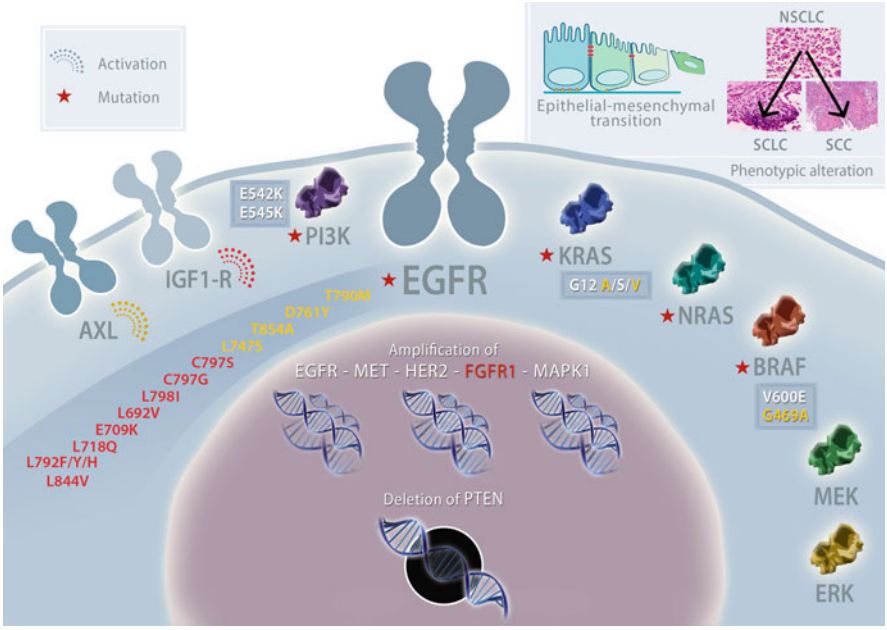

Fig. 2 Mechanisms of resistance to first/second (*yellow*) and third (*red*)-generation EGFR-TKIs; shared mechanisms among the three TKI generations are depicted in *white*. *NSCLC* non-small cell lung cancer, *SCLC* small cell lung cancer, *SCC* squamous cell carcinoma

non-sensitive *EGFR* mutations. Exon 20 insertions, which represent the 1–10% of the total number of *EGFR* mutations, adding residues at the N-lobe of EGFR (M766 to C775) in particular in the C-helix (A767 to C775), frequently reduce affinity for EGFR-TKIs (Yasuda et al. 2013). New sequencing technologies are able to detect cases of concomitant (double or multiple) *EGFR* mutations. Patients with a combination of typical and atypical mutations reported less favourable outcomes compared to patients with a single typical mutation (Wu et al. 2011). Also the coexistence of different driver alterations in other genes, such as *ALK* rearrangements and *KRAS* mutations, resulted associated with worse prognosis after EGFR-TKI treatment in *EGFR*-mutated NSCLC (Ulivi et al. 2016).

The most common mechanism of resistance is the development of acquired T790M *EGFR* gene mutation (Sequist et al. 2011), a secondary point mutation in exon 20, engendering the substitution of methionine (T) for threonine (M) at codon position 790, that sterically prevents the EGFR-TKI binding in the TK domain (TKD), allowing the ATP-mediated activation of the receptor (Kobayashi et al. 2005). Nevertheless, T790M mutation has been also identified as a de novo mutation (Inukai et al. 2006). In this case of primary resistance, it is predictive for poor survival outcomes under EGFR-TKI treatment (Su et al. 2012). Moreover, the T790M impact on responsiveness to EGFR-TKI therapy may depend on the proportion of pre-treatment EGFR T790M-positive clones (Hata et al. 2016).

Third-generation EGFR-mutant selective inhibitors (such as osimertinib and rociletinib) have been developed for patients whose cancers acquire the T790M mutation. These third-generation EGFR-TKIs realize selective inhibition of activating as well as T790M alterations, by means of an irreversible covalent binding to the target while sparing wild-type EGFR (Cross et al. 2014), with important efficacy results and reduced toxic effects (Mok et al. 2016; Jänne et al. 2015; Sequist et al. 2015).

Other rare resistance *EGFR* point mutations including D761Y, T854A and L747S have been reported in less than 10% of mutated NSCLC patients. The mechanism underlying resistance conferred by these mutations is still unclear (Nguyen et al. 2009).

The activation of alternative pathways is now recognized as a different mechanism of resistance (Niederst and Engelman, 2013; Yu et al. 2013a).

The *MET* gene amplification is the second most common mechanisms of acquired resistance, affecting about 5–20% of NSCLC patients during EGFR-TKI treatment, irrespective of the T790M mutation status (Sequist et al. 2011; Engelman et al. 2007). *MET* amplification, accompanied by HGF (MET ligand) autocrine signalling, drives resistance to EGFR-TKIs acting upon molecular elements regulating critical intracellular pathways (Engelman et al. 2007; Turke et al. 2010). MET inhibition has proven to be effective in cell lines with *MET* gene amplification and many preclinical and clinical data demonstrate that contemporary inhibition of MET and EGFR may be a strategy to overcoming resistance (Engelman et al. 2007; Bahcall et al. 2016; Gainor et al. 2016a).

HER2 amplification is a rare event in lung adenocarcinoma at diagnosis, accounting for about 1–2% of cases, but it has been reported in up to 13% of

NSCLC with acquired resistance to EGFR-TKIs (Yu et al. 2013a; Takezawa et al. 2012), whereas resulting absent in other series (Sequist et al. 2011). Mutated EGFR has the tendency to heterodimerize with HER2, the resulting heterodimers being resistant to degradation (Takezawa et al. 2012). Therefore, HER2 heterodimerization could support EGFR-TKIs resistance in presence of both T790M mutation and *HER2* amplification itself as acquired mechanisms of drug exhaustion.

Boosting of cell signalling pathways due to activation of BRAF, PIK3CA and AXL has been proposed as mechanism of drug resistance in cancer cells and in *EGFR*-mutated NSCLC patients, in which their emergence can be overall detected in up to 20–25% of cases (Sequist et al. 2011; Ohashi et al. 2012; Wang et al. 2014; Zhang et al. 2012).

A third resistance mechanism is the phenotypic transformation of lung cancer cells. Histological transformation in SCLC has been observed after the development of acquired resistance to EGFR-TKI in about 3–14% of patients (Sequist et al. 2011; Yu et al. 2013a). The mechanism underlying this histological modification is still not completely known: minor pre-existent cells under the selection pressure of EGFR-TKIs could originate SCLC cells or adenocarcinoma cells could transdifferentiate in SCLC cells (Oser et al. 2015); alternatively, SCLC cells could develop from multi-potent pre-existing stem cells (Oser et al. 2015). Whatever the funding cellular evolution, the loss of Rb protein seems a common and necessary event for this kind of transformation (Niederst et al. 2015a).

Loss of E-cadherin expression and upregulation of mesenchymal proteins such as vimentin, fibronectin and N-cadherin are the main features of epithelial–mesenchymal transition (EMT). In the EMT setting, AXL upregulation and alterations in the Hedgehog pathway have been recently recognized as mechanisms of resistance to targeted agents in EGFR-mutated NSCLC (Zhang et al. 2012; Thomson et al. 2005).

Moreover, transformation from adenocarcinomas to squamous cell carcinomas during the administration of anti-EGFR molecules has been reported as a mechanism of acquired drug resistance (Haratani et al. 2016).

Anyway, the cause of resistance remains still unknown in 18–30% of NSCLC patients resistant to anti-EGFR targeted therapy (Sequist et al. 2011; Yu et al. 2013a).

2.1.2 Detection of T790M Mutation

According to current guidelines, after progression to first-line EGFR-TKI treatment, carrying out a new biopsy to identify the molecular mechanism of acquired resistance and to select patients for targeted therapies is a reasonable procedure (Novello et al. 2016). The feasibility and utility of re-biopsies have been evaluated in several clinical experiences (Mok et al. 2016; Campo et al. 2016; Arcila et al. 2011).

However, serial tumor sampling to monitor cancer evolution is not always feasible in clinical practice. An alternative approach in NSCLC patients may be indeed the use of the so-called *liquid biopsy*, whereby circulating cell-free tumor DNA (ctDNA), DNA fragments passively released into the blood by primary cancer cells, or circulating tumor cells (CTCs), viable or apoptotic cells released from the

primary tumor, can be analysed in the peripheral blood to detect *EGFR* mutations (Crowley et al. 2013; Douillard et al. 2014). Dynamic changes of *EGFR* mutational status in ctDNA seem to predict the clinical outcome to EGFR-TKI treatment (Tseng et al. 2015). A meta-analysis showed a sensitivity of 61% and a specificity of 90% for blood (plasma and serum) analysis compared to tissue evaluation in identifying *EGFR* mutations with a concordance rate of 79% (Mao et al. 2015).

Many studies confirmed the utility and validity of plasma DNA in detection T790M mutation in patients with NSCLC who progressed under EGFR-TKI therapy (Mok et al. 2016; Sundaresan et al. 2016; Remon et al. 2017). According to results of many studies, this method it is today approved by the FDA (http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/DeviceApprovalsandClearances/Recently-ApprovedDevices/ucm519922). Considering the level of sensitivity, when a liquid biopsy is negative for the detection of *EGFR* T790M, this result should be confirmed on tissue biopsy specimen (Oxnard et al. 2016). In addition, a study recently demonstrated satisfying agreements in T790M status definition between urine, plasma and tissue (Reckamp et al. 2016); further urine-based tests are indeed under study.

As mentioned before, T790M mutations account for up to 60% of resistant cases to first- and second-generation EGFR-TKIs (Sequist et al. 2011; Yu et al. 2013a). The remaining T90M-negative cases, for which, in addition, molecular treatment strategies are less developed, can be less characterized by liquid biopsy, with special regard to morpho-phenotypic changes. Recently nevertheless, a high quote of *KRAS* activating mutations has been uncovered in ctDNA in EGFR-mutant NSCLC patients progressing to first or second-generation TKIs (Del Re et al. 2016).

2.1.3 Potential Strategies to Overcome Resistance

Some clinical strategies have indeed been developed in order to *deal with* or overcome resistance to first- and second-EGFR-TKIs.

Because of cancer heterogeneity, once the onset of resistance is manifest, some clones may continue to remain sensitive to EGFR-TKIs, whose continuation can slow down disease progression. For these reasons, in selected patients with slow-growing and low-volume disease, progression in non-critical or asymptomatic sites, no clinical deterioration or intolerable toxicity, first-line EGFR-TKI treatment can be continued beyond progression, as several retrospective studies and some prospective experience showed (Park et al. 2016; Yap et al. 2017).

In the case of clinical progression in circumscribed localization, disease behaviour reflects the spatial heterogeneity of resistance. Retention of the targeted treatment with the addition of local approaches (including surgical resection or radiotherapy) to the dimensionally increasing lesions results a suitable option in order to achieve long-term disease control, acting directly against the resistant counterparts while maintaining active EGFR suppression (Weickhardt et al. 2012; Yu et al. 2013b).

Brain metastases interest around 20% of *EGFR*-mutated NSCLC patients at diagnosis, while 30–60% of them, during an effective EGFR-TKI administration,

develop central nervous system lesions, often representing the isolated site of disease recurrence (Heon et al. 2010; Khalifa et al. 2016). If the progression after first-line EGFR-TKI therapy is characterized by the development of isolated brain metastases, stereotactic radiotherapy or surgery, when possible, is recommended, while multiple lesions require whole-brain radiotherapy. In order to keep extracerebral disease control, EGFR-TKI treatment should be continued (Khalifa et al. 2016).

These reported are effective clinical ways to delay the requirement of novel (cytotoxic or targeted) treatment. In virtually every patient indeed, disease progression not allowing such approaches sooner or later occurs. The research of T790M mutation on tumor specimens or ctDNA is crucial, both for the quote of patients harbouring it and for the actual possibility to overcome it with the novel molecules of third generation, such as osimertinib, rociletinib, HM61713 (olmutinib), ASP8273, EGF816 and PF-06747775. The clinical development of rociletinib and olmutinib has been recently interrupted and ASP8273, EGF816 and PF-06747775 are under early investigation.

Osimertinib is an oral, irreversible EGFR-TKI that is selective for both activating and T790M-resistance mutations (Cross et al. 2014), with significant activity against central nervous system metastases too (Mok et al. 2016; Ballard et al. 2016). In the phase 1 trial (AURA) the RR for osimertinib in patients with T790M-positive tumors was 61%, with a median PFS of 9.6 months (Jänne et al. 2015). These findings were confirmed in a pooled analysis of two subsequent phase 2 studies (Yang et al. 2016), one of which recently published (AURA2) (Goss et al. 2016). On the basis of these results, FDA approved osimertinib in T790M-positive NSCLC patients. A confirmatory, randomized, open-label, international phase 3 trial (AURA3) was conducted and osimertinib showed significantly greater efficacy than platinum plus pemetrexed chemotherapy in patients with T790M-positive cancers after progression under first- or second-generation EGFR-TKIs (Mok et al. 2016). Median PFS for osimertinib was 10.1 months, compared to 4.4 months for chemotherapy (HR: 0.30; 95% CI 0.23–0.41; $p < 0.001$) (Mok et al. 2016). Under the AURA development, osimertinib became the standard of care in second-line treatment for *EGFR*-mutated patients harbouring the T790M mutation.

At the time of progression for patients who are not candidate to osimertinib due to the absence of *EGFR* T790M resistance mutation, different therapeutic options have been or are under investigation. The randomized phase III IMPRESS trial compared gefitinib with versus chemotherapy alone in 265 EGFR-mutated NSCLC resistant to first-line gefitinib (Soria et al. 2015). The underlying objective was to sound out the potential contribution of maintaining inhibition of the driver molecule in addition to standard cytotoxic treatment. No benefit in survival was observed when gefitinib was associated with chemotherapy, suggesting that the EGFR-TKI should be discontinued in resistant patients when the switch to chemotherapy is required (Soria et al. 2015, 2016).

In order to overcome the resistance mediated by specific bypass mechanisms, targeting the detected drivers of resistance itself in combination with EGFR-TKIs may be a sound therapeutic possibility. In particular, the use of MET inhibitors in

combination with EGFR-TKI recently revealed as a promising strategy for *EGFR*-mutated and *MET* amplified NSCLC. Cabozantinib, capmatinib and tepotinib reported significant results in terms of disease response if associated with anti-EGFR agents in this subgroup of NSCLC (Bahcall et al. 2016; Wu et al. 2016; Soo et al. 2015).

In cases of rapid systemic progression, performing a new biopsy is recommended because in presence of a phenotypic transformation to SCLC, to squamous cell carcinoma or when EMT is evident, the use of the chemotherapy could be more beneficial than the use of target therapies.

2.2 Resistance to Third-Generation EGFR-TKIs

The introduction of third-generation EGFR-TKIs resulted in a further outcome improvement for a selected group of NSCLC patients. Nevertheless, despite the high RR and the significant prolongation of survival, after 9–13 months, unfortunately, acquired resistance occurs again (Mok et al. 2016; Jänne et al. 2015; Goss et al. 2016). Several (and not fully recognized) underlying molecular mechanisms have been described (Minari et al. 2016).

2.2.1 Preclinical Evidence and Clinical Relevance of Resistance Mechanisms

In the case of third-generation EGFR-TKIs too, we can classify the mechanisms of resistance in three different categories: (1) EGFR-dependent mechanisms; (2) activation of alternative pathways; (3) phenotypic transformation (Table 1 and Fig. 2).

The emergence of tertiary *EGFR* mutations has been repeatedly reported in the presence of acquired resistance to third-generation TKIs and it has been well characterized in cell lines models (Minari et al. 2016). The *EGFR* p.Cys797Ser (C797S) mutation in the exon 20 is the most common mutation responsible for resistance to osimertinib. Firstly, C797S was identified in ctDNA of 6 out of 15 (40%) patients progressing to osimertinib in the AURA phase I/II study (Thress et al. 2015). It seems also responsible of acquired resistance to other third-generation EGFR-TKIs such as HM61713 and WZ4002, but it is rare after rociletinib (Niederst et al. 2015b; Ercan et al. 2015; Chabon et al. 2016). The substitution of a cysteine with a serine in the position 797 of the tyrosine kinase domain reduces the inhibitory effect of third-generation TKIs by interfering with their covalent binding to EGFR (Thress et al. 2015). Interestingly, according to preclinical evidence, the location of C797S mutation among other *EGFR* alleles (in *cis* vs. in *trans*) may affect the efficacy of subsequent treatments (Niederst et al. 2015b).

After the report of this first mutation responsible of resistance third-generation EGFR-TKIs, several other single-site alterations, such as L718Q and L844V, have been reported in patients and in cellular models treated with osimertinib or other third-generation TKIs (Minari et al. 2016). Importantly, liquid biopsy confirmed its value in detecting such mutations in ctDNA, reinforcing its importance as a clinical

tool (Thress et al. 2015; Ercan et al. 2015; Chabon et al. 2016; Piotrowska et al. 2015).

Again, EGFR-independent mechanisms of resistance during third-generation TKI treatment can emerge. *HER2* amplification was discovered in a NSCLC patient with disease progression after 12 months of osimertinib in the AURA trial (Planchard et al. 2015). It appeared to be mutually exclusive with *EGFR* T790M mutation, as described for first-generation TKIs (Takezawa et al. 2012), and not associated with C797S. Similar findings were reported in other patients treated with osimertinib (Minari et al. 2016), while in the case of resistance to rociletinib, *HER2* amplification was associated with T790M persistence (Chabon et al. 2016). *MET* amplification was first reported in a single case of NSCLC after 10 months of osimertinib treatment, in the absence of T790M or C797S mutations (Planchard et al. 2015), and it was documented too as a mechanism of acquired resistance, both in preclinical in vitro models and clinical cases (Ortiz-Cuaran et al. 2016; Ou et al. 2016a).

In preclinical studies of acquired resistance to osimertinib, an increased dependence on RAS signalling was reported. *NRAS* mutations, including a novel E63K mutation, and *NRAS* or *KRAS* amplification have been described as mechanisms of acquired resistance to osimertinib (Eberlein et al. 2015). The emergence of three *KRAS* activating mutations (p.G12A, p.Q61H and p.A146T), alone or in combination with other resistance mechanisms, has been reported after rociletinib (Chabon et al. 2016). Moreover, at the time of progression, p.E542K and p.E545K mutations in *PIK3CA* gene have been described in five patients treated with rociletinib (Chabon et al. 2016). Other reported resistance mechanisms include *BRAF* p.V600E mutation (Oxnard et al. 2015) and *EGFR* amplification (Chabon et al. 2016; Piotrowska et al. 2015).

Finally, after third-generation TKIs too, in some cases resistant tumors showed phenotypic changes, such as SCLC transformation or EMT (Piotrowska et al. 2015; Kim et al. 2015; Ham et al. 2016).

2.2.2 Potential Strategies to Overcome Resistance

Currently, different therapeutic strategies to overcome the above-described heterogeneous resistance mechanisms to third-generation TKIs are under development.

A new era of fourth-generation TKIs is coming (Minari et al. 2016). EAI045 is the first of a new class of inhibitors, able to overcome T790M and C797S mutations, being selective against mutant-EGFR while sparing the wild-type forms. The combination of EAI045 and cetuximab showed efficacy in mouse models of lung cancer carrying EGFR L858R/T790M/C797S mutations (Jia et al. 2016).

Combinations with third-generation TKIs are being investigated in several studies to avoid the occurrence or overcome resistance (Minari et al. 2016). The association of the MEK inhibitor trametinib with the third-generation EGFR-TKI WZ4002 was able to prevent the emergence of resistance in *EGFR*-mutant lung cancer models (Tricker et al. 2015). The association of osimertinib and another MEK inhibitor, selumetinib, prevented the onset of resistance in cellular lines and

reported in vivo cancer regression in an *EGFR*-mutated, T790M-positive, osimertinib-resistant transgenic model (Eberlein et al. 2015).

Some evidences suggest that patients with C797S and T790M mutations *in trans* could be sensitive again to the association of first/second-generation TKIs with third-generation ones, while the *in cis* disposition results in resistance to all molecules, both alone and in combination (Niederst et al. 2015b). Moreover, the occurrence of C797S in T790M wild-type cells is responsible of resistance to third-generation TKIs, despite the sensitivity to first-generation TKIs (Niederst et al. 2015b). Patients progressing on rociletinib achieved response with osimertinib (Sequist et al. 2016), suggesting a slight different activity of the molecules developed against the T790M mutation.

For patients whose tumors undergo SCLC transformation or EMT, switching platinum-based chemotherapy could be recommended.

Surely, other escape mechanisms are likely to emerge, highlighting the importance of molecular characterization at the time of progression, aiming at the definition of the most correct therapeutic strategy.

3 Resistance Mechanisms to ALK- and ROS1-Driven NSCLC

ALK and *ROS1* rearrangements are present in approximately 4–7% and 1–2% of NSCLC, respectively (Barlesi et al. 2016; Bergethon et al. 2012). These two oncogenes share profound similarities in phylogenesis, biology, genomic sequences, profiles of pharmacological inhibition and tumor clinical features (Ou et al. 2012). Importantly, tumors driven by either ALK or ROS1 manifest similar mechanisms of resistance to targeted agents, which will be approached in parallel. Several mechanisms of drug escape have been identified and they may be classified, similarly to EGFR-TKIs, in three different groups, as indicated in the introduction: (1) involving the target (ALK or ROS1); (2) activation of alternative pathways; (3) phenotype transformation (Table 1 and Fig. 3).

3.1 Mechanisms of Crizotinib Resistance

3.1.1 Mechanisms of Crizotinib Resistance Involving ALK and ROS1

Crizotinib, firstly developed as a MET inhibitor (Kwak et al. 2010), is currently registered by FDA and EMA for patients suffering from *ALK*- and *ROS1*-rearranged NSCLC. Similarly to EGFR-driven tumors, mutations in the target have been reported as the first mechanism of resistance to crizotinib for both oncogenes (Choi et al. 2010; Awad et al. 2013). The number of reported *ALK* mutations responsible of acquired resistance is high, whereas altogether their detection is present in around 30% of clinical samples (Gainor et al. 2016b).

Concomitantly with the first report of crizotinib clinical activity (Kwak et al. 2010), Choi and colleagues described *ALK* C1156Y and L1196M mutations as responsible of acquired resistance to the drug (Choi et al. 2010). L1196 corresponds

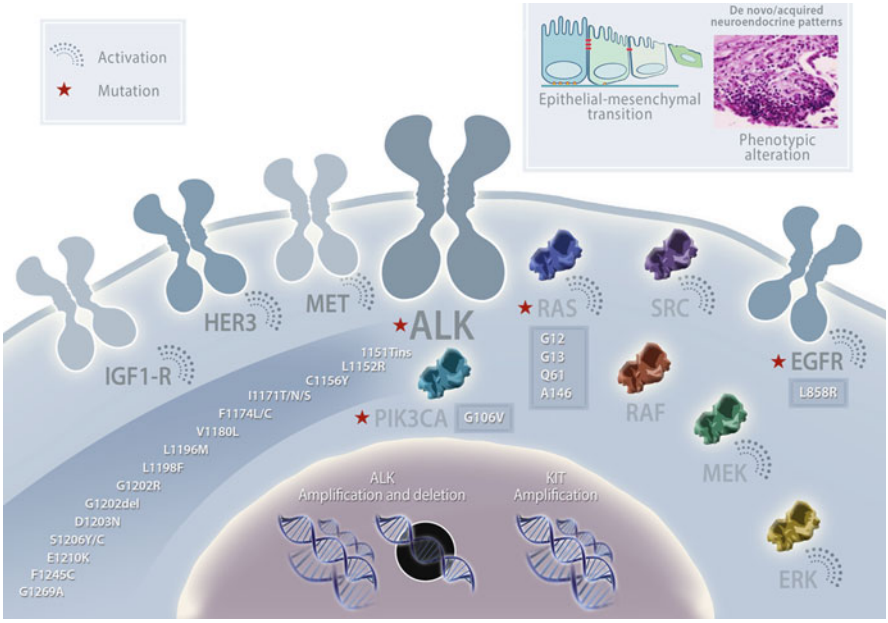

Fig. 3 Thorough representation of mechanisms of resistance to crizotinib and novel-generation inhibitors in ALK-rearranged non-small cell lung cancer. See Gainor et al. Cancer Discovery 2016 (Gainor et al. 2016b) to distinguish the differential mechanisms for single molecules

to the gatekeeper residue in ALK tyrosine kinase domain and this substitution is among the most frequently reported single-nucleotide alteration responsible of crizotinib resistance in NSCLC, together with G1269A (Gainor et al. 2016b). Biopsies obtained in *ALK-positive* NSCLC patients at the moment of disease progression to crizotinib (Katayama et al. 2012; Doebele et al. 2012) allowed the detection a conspicuous number of mutations in ALK TKD. These latter engender crizotinib resistance by means of two main mechanisms: by increasing enzymatic activity at a level not suitable of crizotinib inhibition or interfering with its binding (Gainor et al. 2016b; Friboulet et al. 2014). Considering also isolated case reports, single-nucleotide substitutions reported thus far include 1151Tins, L1152R, C1156Y, I1171N/S/T, F1174C/V, L1196M, G1202R, D1203N, S1206Y, F1245C and G1269A (Gainor et al. 2016b; Facchinetti et al. 2016a) (Fig. 3).

Due to the more limited rate of patients harbouring the oncogenic aberration and to the particularly recent introduction of crizotinib for ROS1-positive advanced NSCLC, information concerning resistance emerging in this setting is barely less abundant. Given the homology between ALK and ROS1 TKD (which share >80% sequence identity within their ATP-binding sites), every mutation reported so far to negatively affect crizotinib activity in ROS1-positive patients find its corresponding with comparison to *ALK* (Facchinetti et al. 2016b). G2032R (Awad et al. 2013), D2033N (Drilon et al. 2016a) and S1986Y/F (Facchinetti et al. 2016b), here reported in order of discovery, can be indeed aligned with the

corresponding *ALK* G1202R, D1203N and C1156Y, respectively. Moreover, the gatekeeper L2026M substitution, together with L1951R, has been recently reported in a patient biopsy after crizotinib resistance (McCoach et al. 2016). The clinical relevance of other mutations (L2155S, K2003I, L1951R and M2128V) reported in vitro only has yet to be established (Katayama et al. 2015; Song et al. 2015).

ALK activation in NSCLC is a consequence of gene fusions and the most frequently partner gene is represented by *EML4*. Several fusion variants are possible, showing slight differential sensitivity to crizotinib in cellular models (Heuckmann et al. 2012) and potentially accounting to the variable durations of disease control in the clinics (Yoshida et al. 2016; Woo et al. 2016).

Crizotinib exhaustion can depend from the amplification/copy number gain of the *ALK* gene itself (Doebele et al. 2012; Shaw et al. 2014) and the possibility of loss of the driver alteration under selective pressure has been proposed in the clinics (Doebele et al. 2012).

These mechanisms have not yet been reported in ROS1-postive NSCLC, neither in preclinical studies nor in the clinics.

3.1.2 Activation of Bypass Pathways Explaining Crizotinib Resistance

Occurrence of either *ALK* or *ROS1* rearrangements together with *KRAS* mutations in NSCLC can explain primary (Mengoli et al. 2016; Schmid et al. 2016) or acquired (Doebele et al. 2012) resistance to crizotinib. Moreover, KRAS and NRAS activation through mutations or amplifications leads to the exhaustion of first-generation inhibitors activity in ROS1-positive cellular models (Cargnelutti et al. 2015).

EGFR signalling has been reported as the responsible of crizotinib resistance in a non-negligible rate of patients-derived biopsies and in cellular models (Katayama et al. 2012; Doebele et al. 2012). Similarly to what observed for RAS alterations, EGFR signalling could augment, with or without the evidence of classical activating mutations (Katayama et al. 2012; Doebele et al. 2012; Kim et al. 2013). Several experiences indeed shown the amplification of *EGFR* gene and implementation of EGFR phosphorylation have been functionally related to acquired resistance to crizotinib, in both ALK- and ROS1-dependent NSCLC (Katayama et al. 2012; Song et al. 2015; Kim et al. 2013; Davies et al. 2013).

KIT gene amplification or activating mutations in *ALK* and *ROS1*-rearranged NSCLC patients can, respectively, explain the acquisition of crizotinib resistance (Katayama et al. 2012; Dziadziuszko et al. 2016).

Moreover, IGF1R, SRC and MEK/ERK activation can mediate resistance to specific inhibitors in ALK-dependent cell models, suggesting thus far the potential of combinatorial strategies in the clinics (Lovly et al. 2014; Crystal et al. 2014; Hrustanovic et al. 2015).

3.1.3 Further Mechanisms of Crizotinib Resistance

The largest part of ALK-positive NSCLC patients exposed to crizotinib experiences intracranial disease progression in spite of extra-cerebral disease control. Pharmacokinetics issues concerning the low blood–brain barrier penetration of the

compound account for its reduced, albeit present, activity in CNS lesions (Costa et al. 2011). Data concerning ROS1-driven disease are still too limited to infer such similar conclusions.

Morpho-phenotypic tumor changes in ALK-positive disease are more limited than *EGFR* mutated, while in *ROS1*-rearranged cells they are limited to preclinical reports (Song et al. 2015). The neuroendocrine phenotypes of NSCLC can be responsible of either de novo crizotinib resistance (Omachi et al. 2014) or for the exhaustion of drug efficacy after initial responses, as transformation from adenocarcinoma to SCLC (Caumont et al. 2016).

In vitro data suggests EMT contribution to the establishing of resistance to ALK inhibitors (Kogita et al. 2014). Experimental data are sustained by clinical reports (Kobayashi et al. 2013).

3.1.4 Overcoming Crizotinib Resistance

In the very recent years, several other molecules have been developed aiming to maintain specific inhibition of ALK and ROS1 when crizotinib runs out of activity. Second-generation compounds include ceritinib, alectinib and brigatinib, while lorlatinib belongs to the third-generation of drugs. If ceritinib, brigatinib and lorlatinib are characterized by half maximal inhibitory concentration (IC50) values suitable of clinical application in both ALK- and ROS1-driven diseases, according to cellular assays alectinib exclusively hits ALK (Gainor et al. 2016b; Facchinetti et al. 2016b; Davare et al. 2015).

The mentioned inhibitors shown crucial properties, such as the ability to inhibit the target more potently than crizotinib, the activity against multiple mutant forms of ALK and ROS1 and the good brain penetration, confirming their relevant activity in the setting of crizotinib resistance (Facchinetti et al. 2016a, 2017). Data from clinical trials and patients' cohorts suggests the clear advantage of administering new-generation compounds after crizotinib (Facchinetti et al. 2016a, 2017). There is moreover an efficacy gradient from the less recent to the newest inhibitors, as the latest are more potent and active against a larger number of mutations in the targets conferring resistance, as long as the brain penetration and the selectivity increase (Gainor et al. 2016b; Zou et al. 2015).

Among the cited novel inhibitors, only ceritinib and lorlatinib ostensibly harbour a significant role as ROS1 TKIs, considering the reduced potency of brigatinib against the wild-type and mutated forms of the enzyme (Chong et al. 2017), together with the inefficacity of alectinib.

Other wide-spectrum tyrosine-kinase inhibitors (e.g. cabozantinib, foretinib, entrectinib) shown activity against ROS1 and/or ALK (Facchinetti et al. 2017). The current availability of the mentioned specific inhibitors with important anti-ALK activity makes questionable the utilization of the latter ones in ALK-driven diseases. Among these less-specific molecules, cabozantinib only could have a role, as the sole drug potentially active against the G2032R *ROS1* mutation, the most frequent mechanism of crizotinib resistance reported so far, albeit in very small series (Katayama et al. 2015; Gainor et al. 2016c). Nevertheless, the toxicity spectrum of cabozantinib at the systemic concentrations required to inhibit mutant

ROS1 forms, still leave doubts about its potential clinical proposition; beside, all other crizotinib-resistance mutations seem to be overcome by the more manageable drug lorlatinib (Facchinetti et al. 2017).

3.2 Resistance to Next-Generation ALK and ROS1 Inhibitors

Recently, Gainor and colleagues reported the results from a wide series of ALK-positive tumor biopsies obtained at progression to crizotinib or second-generation inhibitors (Gainor et al. 2016b). Some of the codons involved in TKD mutations were previously unreported in the clinics, as E1210K, conferring crizotinib resistance, and V1180L, occurring after alectinib administration and already approached in in vitro studies (Gainor et al. 2016b; Katayama et al. 2014).

After ceritinib and alectinib, amino-acidic substitutions mediating resistance were observed in more than 50% of the samples, compared with the 20–30% target alterations responsible of crizotinib exhaustion (Gainor et al. 2016b). Although post-brigatinib biopsies were limited in the series, an enrichment in *ALK* G1202R mutation was reported after the onset of resistance for all the three new inhibitors compared to post-crizotinib samples. Other *ALK* mutations responsible of new-generation inhibitors resistance, unreported after crizotinib, are emerging, as reported in a patient developing ceritinib exhaustion due to the G1123S substitution (Toyokawa et al. 2015), underlying the differential selective pressure exerted by the inhibitors.

PIK3CA G106V activating mutation was detected in an alectinib-resistant specimen, thus allowing to envisaging the involvement of the AKT-mTOR pathway in resistance (Redaelli et al. 2016), as seen in *EGFR*-mutant NSCLC (Sequist et al. 2011).

Bypass signalling activation mediated by IGF1R, HER3 (with the concomitant overexpression of its ligand neuregulin-1) and MET has been proven as mechanism of resistance to alectinib in cellular models (Isozaki et al. 2016).

Morpho-phenotypic changes driving EMT were clearly depicted in one ceritinib-resistant sample and present, with different levels of intensity, in up to 42% (five out of 12) specimens, often in the presence of ALK mutations (Gainor et al. 2016b). Two cases of SCLC transformation were reported in alectinib-resistant tumors (Fujita et al. 2016; Takegawa et al. 2016).

Great interest was raised by the first and, to date, the only report of specific lorlatinib resistance in an ALK-rearranged NSCLC patient. Tumor cells, already harbouring the crizotinib and ceritinib resistant mutation C1156Y, developed the previously unknown L1198F substitution, determining in vitro resistance to all available ALK inhibitors except for crizotinib (Shaw et al. 2015). Administration of the first-generation compound led indeed to disease response. Data concerning resistance to new-generations inhibitors in ROS1-positive NSCLC models or patients are still lacking.

4 Resistance Mechanisms to Targeted Drugs in NSCLC Driven by Other Oncogenes

As several other targets and corresponding pharmacological compounds are emerging in lung cancer, mechanisms of resistance in this new field are rising and lessons can be inferred from tumor models other than NSCLC, sharing driving molecular aberrations and specific inhibition.

4.1 MET

Beside its role in mediating resistance to EGFR inhibition (Engelman et al. 2007), MET is known as a meaningful driver oncogene in NSCLC since around a decade. Nevertheless, its precise mechanisms of activation, harbouring biological and clinical relevance, have been profitably elucidated in the last 2 years (Drilon et al. 2017). *MET* gene amplification needs a precise definition for achieving a meaningful role in predicting response to specific inhibitors, the most relevant one to date again represented by crizotinib (Drilon et al. 2017). Moreover, MET can be biologically activated by a newly recognized mechanism represented by the loss of its exon 14 (*exon 14 skipping*), coding for the juxtamembrane domain, that leads to a meaningful increase in MET signalling by means of the decrease of its degradation (Awad 2016). As in this case MET TKD remains intact, crizotinib is indeed active (Drilon et al. 2017). Nevertheless, mutations occurring in *MET* TKD (D1228N, D1228V, Y1230C, the two latter founded in ctDNA too) have been recently reported as putative responsible of resistance to MET inhibitors in the clinics (Bahcall et al. 2016; Heist et al. 2016; Ou et al. 2016b). Strategies to overcome resistance to type I MET inhibitors (which preferentially bind to the active conformation of the protein, e.g. crizotinib and savolitinib) with type II compounds (which preferentially bind to the inactive molecule conformation, e.g. cabozantinib and capmatinib) yield an in vitro and clinical crucial meaning (Bahcall et al. 2016). If MET activation can explain resistance to EGFR inhibitors, as seen above, the reverse situation has been reported, with the onset of crizotinib resistance in a MET-driven NSCLC associated to the appearance to the activating *EGFR* L861A mutation (Benderra et al. 2016).

4.2 BRAF

BRAF-mutated NSCLC is similar in biology (in terms of role of oncogenic agent), clinical and therapeutic approaches to melanomas harbouring *BRAF* activating mutations (Nguyen-Ngoc et al. 2015). According to *BRAF*-mutant melanomas, the co-inhibition of BRAF and MEK in NSCLC generates better outcomes compared to BRAF blockade alone (Planchard et al. 2016a, b). With regard to *BRAF*-mutated lung adenocarcinoma, the onset of mutations in *KRAS*, *TP53* and *CDKN2A*

has been proposed as a resistance mechanism to the BRAF inhibitor dabrafenib in the clinics (Rudin et al. 2013).

Clear evidence concerning resistance to BRAF and MEK inhibitors in NSCLC is yet to be provided, but mechanisms could be the same observed in melanoma. Nevertheless, if *BRAF* mutations in melanoma occur for the largest part in codon V600 (of which V600E is the archetypal), in NSCLC the involvement of different BRAF activating sites in up to 50% of the cases (Nguyen-Ngoc et al. 2015). Non-V600 *BRAF* mutants are globally less potently inhibited by available anti-BRAF molecules (Gatalica et al. 2015; Noeparast et al. 2016), making the association with MEK inhibitors even more recommended.

4.3 RET

RET rearrangements drive oncogenesis in 1–2% of lung adenocarcinomas (Kohno et al. 2012). Responses to cabozantinib and vandetanib have currently been systematically recognized in phase II clinical trials (Drilon et al. 2016b; Yoh et al. 2017). Moreover, clinical activity of sunitinib (Wu et al. 2015) and alectinib (Lin et al. 2016) has been documented in RET-driven NSCLC. No clinical demonstration of molecular mechanisms of targeted treatment exhaustion is available thus far. An extensive preclinical study recently identified *RET* mutations conferring differential resistance to cabozantinib and vandetanib, while overcome by ponatinib, the most potent RET inhibitor (Huang et al. 2016), whose activity in patients is currently under study. Another experience revealed the hyperactivation of *SRC*, a central gene in focal adhesion, as a suitable mechanism of acquired resistance to dovitinib (Kang et al. 2015); specific inhibition of SRC with sarcatinib allowed the re-sensitization to RET inhibition, as robustly seen in *ALK*-rearranged models (Crystal et al. 2014).

5 Conclusions

The obtaining of the most prolonged disease control with targeted therapies represents nowadays the primary goal in oncogene-driven advanced NSCLC. The profound knowledge of the molecular mechanisms driving resistance to specific inhibitors is basilar in order to develop further treatment strategies. Nevertheless, in experiences when re-biopsies are performed once treatment exhaustion manifests, mechanistic reasons for this clinical behaviour remain biologically uncovered in up to 30–50% of the cases.

Adaptive strategies with novel inhibitors are showing outstanding results both after and in comparison with first-generation molecules when administered upfront, suggesting a scenario in which the most potent drugs would be given immediately. Nevertheless, combinatorial strategies aiming at bypass collapse, achievable with the fruitful blocking of both the primary molecular alteration and the alternative signalling tracks responsible of resistance, are still lacking. Given the relevant

potency of novel molecules against their respective targets, together with the emergence of novel resistance mutations thus far uncommon (*EGFR* C797S, *ALK* L1198F), activation of bypass pathways are expected to arise in a significant quote of cases and this therapeutic gap would need to be filled.

Inner tumor heterogeneity manifests in this field with the various mechanisms adopted by tumors to find escapes under specific therapeutic pressure, in different individuals as well as in the diverse lesions of the same patient. This represents one of the greatest issues to deal with, hampering the potential of pharmacologic developments. Strategies to face the limits imposed by biologic tumor variability are lately emerging (Suda et al. 2017).

The detection of mutations responsible of resistance to old and novel EGFR inhibitors in the blood represents a major improvement (hopefully applicable to other drivers) as a proof of principle and for practical reasons. Nevertheless, its application still requires additional adjustments.

Taken together, the evidence contained in this chapter depicts a scenario in continuous evolution, in which the search for the best-targeted treatment option in lung cancer strictly relies upon the digging towards the deeper and widest knowledge concerning biologic resistance. Questions to be solved are still copious and complex; nevertheless, the recent advances in both clinical and preclinical research, allowed by the impressive developments in experimental methods and techniques, generate enthusiasm and hope for the very next future.

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Mechanisms of Drug Resistance in Melanoma

Matthew Winder and Amaya Virós

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Abstract

Metastatic melanoma is associated with poor outcome and is largely refractory to the historic standard of care. In recent years, the development of targeted small-molecule inhibitors and immunotherapy has revolutionised the care and improved the overall survival of these patients. Therapies targeting BRAF and MEK to block the mitogen-activated protein kinase (MAPK) pathway were the

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first to show unprecedented clinical responses. Following these encouraging results, antibodies targeting immune checkpoint inhibition molecules cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death (PD)-1, and PD-ligand1(PD-L1) demonstrated sustained tumour regression in a significant subset of patients by enabling an anti-tumour immunologic response. Despite these landmark changes in practice, the majority of patients are either intrinsically resistant or rapidly acquire resistance to MAPK pathway inhibitors and immune checkpoint blockade treatment. The lack of response can be driven by mutations and non-mutational events in tumour cells, as well as by changes in the surrounding tumour microenvironment. Common resistance mechanisms bypass the dependence of tumour cells on initial MAPK pathway driver mutations during targeted therapy, and permit evasion of the host immune system to allow melanoma growth and survival following immunotherapy. This highlights the requirement for personalised treatment regimens that take into account patient-specific genetic and immunologic characteristics. Here we review the mechanisms by which melanomas display intrinsic resistance or acquire resistance to targeted therapy and immunotherapy.

Keywords

Acquired resistance • BRAF inhibitor • Checkpoint inhibitor • Immunotherapy • Intrinsic resistance • MAPK pathway • MEK inhibitor • Melanoma • Targeted therapy

1 Melanoma Incidence and Clinical Subtypes

Malignant melanoma accounts for >80% of skin cancer-related deaths despite representing <1% of cases (NICE 2015). The current incidence and mortality of melanoma in Europe is over 100,000 new cases and over 22,000 deaths each year (http://www.IARC.fr). In most European countries rates are doubling every decade and in many countries melanoma will soon be the fourth most common cancer (Ferlay et al. 2013). The yearly incidence in the UK is 12,000 cases, which is rising by ~2% per year, and approximately 2,000 patients die from advanced disease. Approximately one third of melanoma patients are under 50 years of age, so this cancer disproportionately affects the elderly population (NICE 2015).

Melanoma stems from the malignant transformation of melanocytes, which are neural crest-derived, and migrate during development to colonise the skin, eye, and in scarce numbers, other tissues of the body. The most common type of melanoma arises from melanocytes in the skin and predominantly affects the population of European descent (Whiteman and Green 1999). The only known environmental risk factor for cutaneous melanoma is ultraviolet radiation (UVR) and informing the population at risk remains a public health challenge (Gilchrest et al. 1999). Previous efforts to use environmental factors to classify cutaneous melanoma include proposals for a divergent pathway model (Whiteman and Green 1999), where UVR exposure pattern, host susceptibility, and the site of the primary lesion are

used as criteria to identify epidemiologically uniform subsets of patients. Indeed, cutaneous melanoma arising at sites chronically exposed to UVR (head and neck) is more likely to occur in adults with a high lifetime accumulation of UVR exposure, whereas melanomas arising at sites that are only intermittently exposed to UVR (trunk) appear in younger patients who received a lower lifetime exposure. This group of patients were more likely to report intermittent UVR exposure on the trunk through recreational activities (Whiteman et al. 2003; Curtin et al. 2005; Chang et al. 2009).

The most common founder mutation in melanomas arising in patients younger than 55 years of age is ^{V600E}BRAF (Davies et al. 2002; Cancer Genome Atlas Network 2015). In contrast, melanomas arising in the elderly are more likely to be driven by mutations in *NRAS*, *NF1* and *BRAF* mutations that do not affect the codon V600 (Cancer Genome Atlas Network 2015; Krauthammer et al. 2015; Shain and Bastian 2016). Thus, the protein kinase BRAF is mutated in almost half of all cutaneous melanomas, and *BRAF* mutations are more frequently found to drive melanomas arising over skin that has been only intermittently exposed to sun such as the trunk and upper limbs (Cancer Genome Atlas Network 2015; Shain and Bastian 2016; Shain et al. 2015).

2 Melanoma Treatment Overview

Until the last decade, the standard of care for advanced disease included surgical resection, chemotherapy with the alkylating agent dacarbazine (DTIC), and high-dose interleukin 2 (IL-2), which only led to durable responses in a few patients with metastatic disease. The only approved adjuvant treatment for patients at high risk of progression was interferon-α-2b. Despite these treatments, metastatic melanoma remained associated with extremely poor prognosis and only curable if detected at an early, pre-metastatic stage (Balch et al. 2009). More recently, insights into the molecular mechanisms driving melanomagenesis have led to the development of targeted therapies, and the advances in immune checkpoint inhibitor therapies have also contributed to a revolution in melanoma care.

3 The Rationale for Targeted Therapies in Melanoma

One of the principal signalling pathways in melanoma is the MAPK pathway, which signals via RAS (Young et al. 2009; Stephen et al. 2014) and RAF (Davies et al. 2002; Wan et al. 2004; Marais et al. 1997). The signalling cascade is triggered by stimulatory input from extracellular growth factors, cytokines and hormones that bind to the receptor tyrosine kinases (RTK) on the cell membrane (Schlessinger 2000). Once activated, the RTKs form stable dimers that undergo phosphorylation at multiple tyrosine residues that are in the cytosolic component of the protein. This chemical change drives activation of RAS proteins, and downstream proteins BRAF and CRAF, leading to activation and phosphorylation of the dual-specificity

kinases MEK1 and MEK2; which in turn phosphorylate ERK1 and ERK2 (Holderfield et al. 2014). ERK proteins then phosphorylate approximately 50 cytoplasmic and nuclear proteins to control cell proliferation, differentiation, adhesion and migration (Roberts and Der 2007). This powerful signalling pathway presents negative regulators that limit ERK effects supplying feedback mechanism systems (Lito et al. 2013). In addition to the MAPK pathway, however, extracellular stimuli may affect multiple other pathways. Of particular significance to melanoma is the PI3K/AKT/mTOR pathway, which is upregulated across many cancer types (Fruman and Rommel 2014). Critically, these signalling pathways not only drive signals in a single direction from the extracellular space to the nucleus, but also interconnect at different levels creating a complex network (Fey et al. 2012). Thus, targeted therapies aim to not only interfere with a single signalling axis but also aim to target secondary signalling streams that the tumour may use to circumvent the target of a single node within the network.

Nearly half of all cutaneous melanomas present a gain-of-function (GOF) mutation in the *BRAF* gene at the codon V600, which constitutively drives MAPK pathway activation via MEK1/2 and ERK1/2 (Davies et al. 2002; Wan et al. 2004). A single activating mutation in the V600 codon of *BRAF*, which usually leads to substitution of a valine residue to glutamic acid residue (V600E) (Dhomen et al. 2009), renders BRAF constitutively active but is not enough to drive tumour initiation (Pollock et al. 2003). Additional hits in tumour suppressors *PTEN* (Dankort et al. 2009), *TP53* (Goel et al. 2009) or *CDKN2A* (Sharpless et al. 2003) are required to progress melanomagenesis. Critically, in physiological conditions where BRAF is wild type, RAF proteins dimerise upon activation (Wan et al. 2004; Luo et al. 1996; Farrar et al. 1996). By contrast, ^{V600E}BRAF is able to dimerise with CRAF (Wan et al. 2004), a RAF family protein, and function as a monomer vulnerable to inhibition (Poulikakos et al. 2011).

The second most common mutation is a GOF at codons G12/13 (approximately 5% of melanomas) or Q61 (17% of melanomas) of NRAS (Sekiya et al. 1984; Albino et al. 1989; van't Veer et al. 1989). BRAF and NRAS mutations occur in a mutually exclusive pattern in pre-treatment samples (Cancer Genome Atlas Network 2015). NRAS presents GTPase activity and when wild type, following the phosphorylation of RTKs, is activated by a guanine nucleotide exchange factor (GEF), which permits the exchange of GDP for GTP (Young et al. 2009). When RAS is mutated, it remains in GTP-bound state (Katz and McCormick 1997). RAS signals downstream by a variety of pathways, including both the MAPK and PI3K/Akt/mTOR pathways, and in human melanoma and mouse models of NRAS melanoma, like in BRAF, there is synergy to drive tumour progression when RAS alterations are combined with additional genetic hits in *CDKN2A*, *TP53* and *PTEN* (Feng et al. 2013; Conde-Perez and Larue 2014). Critically, there are currently no successful drugs in clinic targeting oncogenic RAS.

In the tumours that are wild type for both BRAF and NRAS mutations, the more frequent identified drivers are *NF1* loss-of-function (LOF) mutations, as well as alterations in *KIT*, *TERT* and CCND1 (Cancer Genome Atlas Network 2015; Krauthammer et al. 2015; Shain et al. 2015; Hodis et al. 2012).

4 RAF Inhibition in Melanoma

The discovery of the BRAF oncogene and MAPK pathway signalling led to the development of targeted BRAF inhibition therapy (vemurafenib (Chapman et al. 2011; Sosman et al. 2012; McArthur et al. 2014) and dabrafenib (Hauschild et al. 2012)) and targeted MEK kinase inhibition (trametinib (Flaherty et al. 2012)), although MEK inhibitor trials have shown less impressive responses than BRAF inhibitors. More recently, combination therapies have improved initial monotherapy results (Long et al. 2014; Ascierto et al. 2016). These new drugs achieve improved progression-free and overall survival, and about 90% of patients show some improvement and tumour regression, with up to approximately 50% achieving partial or complete responses. Although these responses are encouraging, the response to these drugs is limited, with only a small subset of patients developing long-term, durable responses. Unfortunately, approximately 50% of patients that present an initial improvement generally relapse within 7 months of treatment (acquired resistance) (Chapman et al. 2011; Hauschild et al. 2012). Additionally, there are about 10% of patients who do not respond to the targeted inhibition at all, and these are termed intrinsic or primary resistant to targeted therapy. Importantly, BRAF inhibitors lead to characteristic side effects, including photosensitivity, which can limit treatment, and the rapid development of cutaneous squamous cell carcinoma (cuSCC) (Su et al. 2012). Keratinocytic secondary neoplasia are thought to arise due to the paradoxical activation of the MAPK pathway in keratinocytes that are wild type for BRAF but present upstream RAS activation in chronically damaged skin (Su et al. 2012). In this cellular context, the BRAF inhibitor leads to increased downstream ERK signalling in BRAF wild type keratinocytes that give rise to cuSCC (Heidorn et al. 2010). Thus, combining BRAF and ERK inhibitors was predicted to decrease this side effect, and indeed, this combination treatment not only was demonstrably linked to improved progression free and overall survival compared to BRAF inhibitor in monotherapy (Larkin et al. 2014; Robert et al. 2015) but also decreased development of cuSCC. However, as in BRAF inhibitor monotherapy, resistance develops in most melanoma patients, and great efforts have been invested to understand how and when tumours resist or stop responding.

5 Tumour Cell Intrinsic or Autonomous Resistance to RAF Inhibition in Melanoma due to Mutational or Genetic Events

Resistance to BRAF inhibitors is predominantly linked to reactivation of the MAPK pathway (Lito et al. 2013; Nazarian et al. 2010; Maertens et al. 2013; Whittaker et al. 2013). As monomeric ^{V600E}BRAF is the relevant target of RAF inhibitors, all changes that increase the proportion of RAF dimerisation likely decrease sensitivity to BRAF inhibitors (Poulikakos et al. 2011). Following the rationale that increasing RAS signalling to drive RAF heterodimer formation should decrease therapy

response, we find that upstream, acquired GOF mutations in *RAS* (Nazarian et al. 2010), LOF mutations in *NF1* (Maertens et al. 2013) and several GOF mutations in RTKs (Whittaker et al. 2013) have all shown to drive tumour resistance. Apart from these mechanisms that reactivate RAS, there are structural changes in oncogenic BRAF due to aberrant splicing that can lead to resistance (Poulikakos et al. 2011; Solit and Rosen 2011). For example, p61-^{V600E}BRAF splice variants retain an active kinase activity but are unable to bind RAS. They dimerise regardless of RAS status and drive constitutive signalling to ERK, uncoupled from upstream regulation (Poulikakos et al. 2011). More recently, a screen for resistance mechanisms using patient derived xenografts (PDX) found that duplications of the BRAF kinase domain led to resistance in approximately 10% of PDX (Kemper et al. 2016). This was found to hold true in a validation cohort of resistant patient samples. Additional drivers of resistance are the increased expression of CRAF or copy number increase of BRAF, which possibly also drive dimer formation and increase signalling throughput.

There are also mechanisms of resistance that arise downstream of the inhibition site BRAF within the MAPK cascade, that bypass the effect of the drug. Importantly, the specific mutations in *MEK1* (*MAP2K1*) coding amino acid changes P124L and Q56P are able to decrease response to BRAF inhibition (Wagle et al. 2011), but not all MEK1 somatic mutations lead to equivalent reactivation of the pathway downstream of MEK1 (Shi et al. 2012). Additionally, the increased expression of the MAPK kinase kinase COT (*MAP3K8*) is thought to lead to direct activation of MEK1, circumventing RAF inhibition (Johannessen et al. 2010). More recently, the role of ERK reactivation driving resistance has been further highlighted by massively parallel sequencing efforts revealing mutations in genes encoding for proteins in the cohesion complex that participate in the organisation of chromatids, *STAG2* and *STAG3* (Shen et al. 2016). Mutant proteins are shown to drive the reactivation of ERK signalling to drive resistance by inhibiting the expression of the phosphatase DUSP6, an inhibitor and regulator of ERK activity in melanoma. Other implicated negative regulators include the LOF of DUSP4 (Shen et al. 2016).

Further molecular alternatives driving resistance are the activation of parallel signalling, such as the activation of PI3K/AKT/mTOR pathway by deletion or inactivating mutations of the negative pathway regulator and tumour suppressor *PTEN* (Xing et al. 2012), and the inactivation of the tumour suppressor *RB1* (Xing et al. 2012) to decrease the requirement for BRAF/MEK signalling. The discovery of these contributing signalling pathways provides new rationales of second-line therapies, as demonstrated by the success of combinations of MAPK and PI3K/AKT/mTOR pathway inhibitors to overcome acquired resistance to monotherapy targeting BRAF alone (Greger et al. 2012).

To add further complexity, patients who present disease progression following initial therapeutic response to BRAF inhibition are more likely to progress presenting metastasis at previously uninvolved sites, and there is a selection for more aggressive clones (Paraiso et al. 2015). Importantly, tumour heterogeneity and heterogeneous mechanisms of resistance are present in tumours and at different

metastatic sites simultaneously (Shi et al. 2014). Recent efforts to dissect the degree of heterogeneity and its clinical implications have led to transcriptional studies targeting thousands of single tumour cells, and show that at the cellular level in all tumours, there are distinct transcriptional patterns within each tumour that display varying degrees of predicted responsiveness to BRAF inhibition (Tirosh et al. 2016). Thus, there are subclones of cells, in varying proportions within each tumour, expressing molecular programmes that make them less likely to respond to therapy and vulnerable to selection during disease progression (Tirosh et al. 2016).

6 Tumour Cell Intrinsic or Autonomous Resistance to RAF Inhibition in Melanoma due to Non-mutational Events

As described above, additional genetic damage to *PTEN* or *RB1* leads to activation of alternative signalling pathways that will lead to therapeutic failure (Xing et al. 2012). However, we already observe the activation of parallel signalling pathways, leading to progressive "dampening" (adaptation) of response to BRAF inhibitors during therapy in the absence of additional genetic hits. This occurs because there is a progressive switch of cells to rely on other signalling pathways such as PI3K/AKT as a natural consequence of the new pressures exerted by BRAF inhibition (Lito et al. 2012). This rewiring or switch happens because BRAF inhibitors will initially shut down transcription of key targets of proliferation, leading to response, via suppression of ERK function, but will also decrease the expression of negative pathway regulators downstream of ERK such as DUSP6 (negative regulator of ERK activity) and Sprouty (*SPRY* (Tsavachidou et al. 2004); inhibitor of GRB2, an adaptor protein transducing signals between RTKs and RAS) (Lito et al. 2013; Lito et al. 2012). Thus, inherent tumour cell adaptation to targeted therapy, in the absence of additional genetic events, per se provides an explanation for progressive decline in therapeutic response.

Another way tumour cells co-opt established biological cellular signalling pathways to advance tumour progression in the absence of genetic mutations is by up-regulation or activation of RTKs (Whittaker et al. 2013). Tumour cells express multiple RTKs that integrate extracellular signals to modulate intracellular events feeding multiple cascades that will affect the fundamental MAPK and PI3K/AKT pathways, and influence proliferation and survival. Therefore, up-regulation of RTKs and increases in soluble RTK ligands and growth factors are all described mechanisms to reduce innate drug sensitivity and drive acquired resistance. Critical RTKs that are implicated in reducing response to treatment due to activation or up-regulation include MET, IFG-1R (Villanueva et al. 2010), EGFR and PDGFRbeta (Wilson et al. 2012). In melanoma, the expression of the RTK ligand HGF is a well-known driver of poor response (Straussman et al. 2012).

Both the expression and loss of MITF, the critical melanoma lineage survival factor, are additional mechanisms of resistance to BRAF pathway inhibition. MITF

plays a central role in ensuring melanocyte survival, and regulates multiple critical survival and antiapoptotic genes (Levy et al. 2006).

Different MITF expression levels have been linked to distinct melanoma tumour and cell behaviour, where high levels mediate differentiation, moderate levels promote proliferation, and low/absent levels drive a more invasive phenotype (Levy et al. 2006). Therefore, the multifaceted role of MITF as a consequence of varying expression levels, with seemingly opposing effects, is further mirrored during the development of resistance. Specifically, overexpression of MITF reduces the therapeutic effect of BRAF inhibitors (Johannessen et al. 2013; Smith et al. 2016; Haq et al. 2013; Van Allen et al. 2014), MEK inhibitors (Smith et al. 2013) and combination treatments (Shi et al. 2014) via increased cAMP pathway signalling (Johannessen et al. 2013).

However, at the other end of the expression spectrum, loss of MITF is also a common occurrence in acquired resistance (Muller et al. 2014). Critically, in cell clones down-regulating MITF, there is an inversely correlated up-regulation of the RTK AXL that enhances tumour resistance. The expression programme in MITF low/AXL high tumours is implicated in driving the resistant phenotype and has now been found to exist in a continuum in subsets of tumour cells that may otherwise express a predominating "AXL low" expression programme that would predict response. A recent study has found the expression programme observed in "MITF low/AXL high" resistant samples can already be detected at the single cell level in treatment-naïve samples, and these transcriptomic features are subsequently increased in resistant, progressive disease (Muller et al. 2014; Konieczkowski et al. 2014). Additionally, the neighbouring stromal cells also influence the expression programme at the single cell level (Tirosh et al. 2016).

A landmark 2015 study performed transcriptome and methylome analysis of matched melanoma samples taken before treatment and during disease progression. One of the critical findings in this paper is that resistant samples displayed a more homogeneous pattern of expression that was in contrast to heterogeneity shown at the DNA level. The acquired resistance transcriptome correlated with YAP1 enrichment, MET high and LEF1 low expression levels. Moreover, MAPK pathway inhibition with targeted therapies led to direct methylation changes that were therapy time-dependent, affecting key regulatory genes and the transcriptome across the resistant samples. One of the other striking findings in the paper is that in resistant samples, there is a predominance of NF-Kβ inflammation that correlates with monocyte expression and M2 macrophages (tumour associated macrophages), suggesting MAPK pathway inhibition affects the inflammatory context of the tumour. Indeed, as a consequence of the M2 switch in macrophage phenotype, the researchers observed that as MAPK pathway inhibitor resistance arose, there was a parallel increase in $CD8^+$ T cell deficiency, exhaustion of the cell population and down-regulation of the antigen presentation molecules in about half the resistant samples (Hugo et al. 2015). Thus, this shows that the study of resistance must extend beyond DNA analysis to incorporate non-genomic approaches with a focus on the consequences on the tumour as well as on the immune tumour ecosystem. Unravelling these changes impacts the therapeutic options of patients, as this work

shows that when patients progress on targeted therapies they likely respond less to immunotherapy. Providing further evidence of how a deep understanding of the immune context underpins therapeutic choice, there are data to support the up-regulation of PD-L1 and the tumour-infiltrating immune populations in metastatic melanoma before MAPK inhibition are associated with progression and survival (Hugo et al. 2017; Massi et al. 2015).

7 Tumour Cell Extrinsic or Microenvironmental Resistance to RAF Inhibition in Melanoma

Research focus into the mechanisms of drug resistance in melanoma has predominantly centred on properties intrinsic to tumour cells, but disease progression and resistance to targeted therapies is no longer considered an exclusive function of genomic and non-genomic modifications of tumour cells. The importance of the tumour microenvironment in supporting resistance to MAPK inhibition is slowly unravelling, and a more complex, comprehensive interpretation must incorporate knowledge of cross talk between discrete cellular compartments including the tumour and supporting stroma (Tape et al. 2016). As we have already alluded to, recent studies delineate a clear contribution of macrophages and fibroblast-derived factors that are well known to confer resistance to MAPK pathway inhibitors.

Fibroblasts are well-known facilitators of melanoma progression, and there is a bidirectional communication through direct cell-to-cell contact and via the secretion of soluble factors to promote melanoma invasion, survival and growth (Li et al. 2003). In melanoma, an extrinsic, non-cell autonomous signal leading to HGF secretion by the stromal cells may lead to resistance (Straussman et al. 2012). HGF can bind to RTKs that will increase intracellular signalling to drive up-regulation of RAS, and ultimately, reactivation of MAPK pathway (Straussman et al. 2012). Furthermore, HGF is also known to contribute to resistance in human cell lines treated with a BRAF inhibitor by down-regulating the pro-apoptotic response. A more recent paper investigated how genetically unharmed stroma evolves under treatment with targeted therapy. Importantly, they demonstrated that in areas of high stromal density, fibroblasts present a hyperactivation of MAPK pathway that elicits a qualitative change in the tumour matrix via integrin β1 and FAK to induce ERK, providing an early subset of melanoma cells with the capacity to rapidly tolerate treatment (Hirata et al. 2015). Moreover, the effect of fibroblasts may vary in patients who are elderly, as melanoma cells in contact with aged fibroblasts are more invasive (Kaur et al. 2016). A recent study has shown that aged fibroblasts increase the secretion of sFRP2, a β-catenin inhibitor that decreases MITF expression, leading to downregulation of the redox regulator APE1, thus rendering melanoma cells more sensitive to oxidative stress and secondarily driving resistance to BRAF inhibition (Kaur et al. 2016). Together, all these studies demonstrate that melanoma cells and fibroblasts are both under pressure to adapt to targeted inhibitors. Thus, fibroblasts change their matrix providing melanoma

cells with a new microenvironment that will enable adjacent tumour cells to evade therapy at a very early stage.

Additional stromal cells that play a demonstrated role in the development of resistance are macrophages (Ruffell and Coussens 2015). Tumours from patients treated with MAPK pathway inhibitors present an increased density of tumour-associated macrophages. These tumour macrophages secrete the melanoma growth factor TNFα that drives, in an NF-kβ-dependent manner, the expression of MITF, leading to resistance (Smith et al. 2014). Moreover, studies show that combination of MAPK and NF-kβ inhibitors delay the appearance of resistance (Smith et al. 2014). TNFα is known to block apoptosis in cells where BRAF is inhibited, and additionally contributes to melanoma invasion and vascularisation of tumours (Gray-Schopfer et al. 2007). As TNFα and NF-kβ play context-specific roles within cancer progression, it will be critical to further investigate whether the signalling pathways described to promote therapy failure secondary to macrophage infiltration hold true across different cancer types and other targeted therapies. These studies, together with the recent discovery that MAPK pathway inhibition leads to $CD8^+$ T cell depletion, highlight how the pressure exerted by novel MAPK inhibitor therapies not only impact tumour evolution by genomic and non-genomic events, but also shape the behaviour of the supporting connective tissue and immune cell populations and function.

8 Immunotherapies in Melanoma

Another landmark change in melanoma therapy has been the development of antibodies to elicit an antitumor immunologic response (Brahmer et al. 2012; Hodi et al. 2010; Topalian et al. 2012; Kaufman et al. 2013; Mellman et al. 2011; Topalian et al. 2014; Wolchok et al. 2013). These novel therapies target the immune checkpoints that exist in physiological conditions to counterbalance immune activation. In cancer, these critical signalling barriers inhibit the activation of antitumoural immune responses. The inhibition of these checkpoints allows the immune system to target cancer cells and leads to long-term disease control. In melanoma, there is improved survival by targeting the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) checkpoint molecule, the inhibitory T-cell receptor programmed death 1 (PD-1) receptor and the PD-1 ligand PD-L1. The mechanisms of action have been extensively reviewed. Other novel immunotherapies include adoptive T cell therapy, which is based on the isolation and ex vivo expansion of T cells that are tumour-specific (Restifo et al. 2012). This therapy allows the vast expansion of T cells, which are then infused into patients to target tumour cells. However, there is great variability of response to immunotherapies across different cancer types, and even within cancer types where immunotherapy is successful, such as melanoma, lung cancer and renal cell cancer, there is a proportion of patients who never respond. Similarly to targeted therapy patients, these are termed intrinsically resistant patients. There is also a subset of patients who initially respond but later progress, indicating the development of acquired resistance to

immunotherapy. Thus, there are tumours that cannot be detected by the immune system (intrinsically resistant), and tumours where the cells adapt to immunotherapy and progressively outgrow the inhibition (adaptive immune resistance).

9 Intrinsic and Adaptive Resistance to Immunotherapies in Melanoma

Intrinsic resistance arises in cells that present genetic or non-genetic changes that afford the tumour natural resistance, as seen, for example, in tumours that express few molecular changes that are recognised as foreign by the immune system (Snyder et al. 2014; McGranahan et al. 2016). This affords one explanation as to why tumours with fewer mutation loads are less likely to respond to immunotherapy. During tumour evolution, there is a tendency for tumours to lose a proportion of their non-silent mutations, which can potentially lead to a lower ratio of antigenic epitopes, leading to immunoadaptation of tumours (Rooney et al. 2015). One of the necessary elements linked to response is the presence of pre-existing T cells within the tumour. The absence of effector T cells within a melanoma has been linked in humans and mice to melanoma-cell-intrinsic oncogenic activation of the WNT/β-catenin pathway (Spranger et al. 2015; Spranger and Gajewski 2016). Importantly, analysis of pre-anti-PD-1 treatment and post-treatment samples has shown a greater proportion of mutations in the DNA repair gene *BRCA2* in responding patients (Hugo et al. 2017). By contrast, the samples that are intrinsically resistant to anti-PD-1 therapy have upregulation of genes involved in a non-melanocytic, more mesenchymal behaviour (*AXL*, *ROR2*, *WNT5A*, *LOXL2*, *TWIST2*, *TAGLN*, *FAP*), as well as genes involved in angiogenesis and wound healing (Hugo et al. 2017). Curiously, this expression profile significantly overlaps with the expression profile observed in samples that have developed resistance to MAPK pathway inhibition (Hugo et al. 2017), implying convergence of resistance-expression programmes.

Cooperating with the loss of antigenicity and the intrinsic characteristics in the tumour that render them more or less vulnerable to immunotherapy are additional mechanisms arising during immune response that directly inhibit tumour-targeting T cells. For example, resistances can be acquired by new resistance-driving mutations in genes involved in interferon-receptor signalling and in antigen presentation (Zaretsky et al. 2016). Additionally, during the initial phase of the interaction between a tumour cell and an antigen-specific T cell, the T cell produces interferon-γ (Ribas 2015; Peng et al. 2012). Interferon-γ signalling has a dual anti-tumour and immune inhibitor role in this interaction (Shankaran et al. 2001). It first serves as a mediator and amplifier of the immune effect, exerting a chemoattraction role to recruit further leukocytes, macrophages and natural killer T cells, but also has a second effect dampening the immune response to cancer cells by expressing factors aimed to reduce the immune anti-tumour effect (Rooney et al. 2015; Bald et al. 2014), such as indolamin 2.2 dioxygenase (IDO) (Peng et al. 2016). IDO is a critical enzyme that when expressed, interferes with

appropriate T cell function. Most critically, following an interferon-γ stimulus, T cells express the ligand PD-L1 that binds to the PD1 receptor leading to inactivation of T cells (Spranger et al. 2013; Tumeh et al. 2014; Pardoll 2012). Other interferon-γ-dependent checkpoints that have also been described to mediate inhibitory immune loops are most prominently the carcinoembryonic antigen cell adhesion molecule-1 (CEACAM1) that forms heterodimers with TIM-3, an activation-induced inhibition molecule involved in immune tolerance and T-cell exhaustion, to inhibit T cell function (Huang et al. 2015). This interaction is an attractive target for additional immune checkpoint blockade in cancers expressing CEACAM1 and TIM-3 (Huang et al. 2015).

There are additional interferon-independent mechanisms of adaptation to immunotherapy. For example, in some tumours, mutations in cancer cells affecting major signalling pathways per se can also lead to expression of PD-L1 in tumours (Parsa et al. 2007; Mittendorf et al. 2014; Marzec et al. 2008; Atefi et al. 2014; Akbay et al. 2013; Shin et al. 2016). There is also a $CD8^+$ T cell-dependent accumulation of regulatory T cells expressing $FOXP3^+$ that exert an inhibitory influence over the immune response, which is mediated by chemokine release (Spranger et al. 2013). Regulatory T cells are immunosuppressive, downregulate the proliferation and induction of effector T cells and are critical to control autoimmunity or excess immunity. Additionally, tumour specific T cells mounting an immune response during the killing of cancer cells produce the major modulating inflammatory cytokine TNFα, which leads to a decrease in the expression of melanoma-specific genes, involved in melanocyte lineage differentiation, and an increase in the expression of genes that signal dedifferentiation and a biological profile more in keeping with neural crest-derived cells or immature melanocytes (Ribas and Tumeh 2012). In adoptive T cell transfer therapy, melanoma evolves to a less differentiated state that mediates resistance due to TNFα secretion (Landsberg et al. 2012). Thus, the loss of melanoma-specific antigens provides another mechanism of immunoevasion. Furthermore, a seminal recent study shows progressive selection of non-immunogenic clones, a process termed immunoediting, occurs during cancer progression via a T cell-dependent immunoselection process (Matsushita et al. 2012).

Taken together, research shows the fine regulatory relationships aimed to deliver accurate immune responses to infection and limit excess cytotoxicity in homeostasis are co-opted to the tumours' advantage in cancer.

10 Conclusions and Future Avenues

There is a rapid changing landscape of therapies for advanced melanoma patients. Current efforts aim to enhance the efficacy of existing targeted and immunotherapies, by understanding the biology driving response and resistance to guide therapeutic care. One of the challenges is identifying the first line therapies or combinations of therapies that will lead to more prolonged clinical responses, taking into consideration the genetic and immunological differences in each patient.

Moreover, in this rapidly changing landscape of novel therapies, new strategies to overcome resistances are currently underway. For example, promising new drugs block the reactivation of MAPK pathway in both BRAF and NRAS tumours, without driving the paradoxical activation of the MAPK pathway (Girotti et al. 2015). Another novel approach to overcome resistance includes the disruption of mitochondrial biogenesis using the small-molecule HSP90 inhibitor gamitrinib (Zhang et al. 2016). New drug discovery efforts have also found encouraging results using compounds triggering ER Stress in MAPK inhibitor resistant cells (Cerezo et al. 2016). For novel immunotherapies, understanding the activation levels of significant checkpoint molecules and the immunosuppressive context within each tumour sample will aid physicians select appropriate therapies or combinations of therapies to improve survival.

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Mechanisms of Resistance to Immune Checkpoint Antibodies

Rodrigo N. Ramos, Eliane Piaggio, and Emanuela Romano

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Abstract

Immunotherapy using checkpoint inhibitors has changed the way we treat several aggressive cancers such as melanoma, non-small cell lung and head & neck cancers, among others, with durable responses achieved in the metastatic setting. However, unfortunately, the vast majority of patients do not respond to checkpoint inhibition therapy and a minority of patients, who do respond to treatment, develop secondary resistance and experience relapse by mechanisms still inadequately understood. Emerging evidence shows that alterations in multiple signaling pathways are involved in primary and/or secondary resistance to checkpoint inhibition. In this review we discuss how selected cancer-cell autonomous cues may influence the outcome of cancer immunotherapy, particularly immune checkpoint inhibition.

Keywords

Acquired resistance • BRAF • EGFR pathway • HIPPO pathway • Immune checkpoint • Immunotherapy • Intrinsic resistance • *JAK1* • *JAK2* • PI3K/AKT/mTOR pathway • Wnt/β-Catenin pathway

1 Introduction

The goal of harnessing the immune system to fight cancer dates back over a century, when William Coley advocated that the bodys response to infection could have anti-tumoral effects (Brouckaert et al. 1992). However, decades of efforts using vaccines and other immune therapies to harness the immune system to fight tumors have had limited success and at times have been fraught with serious adverse effects. In the era of modern medicine, proof of the effectiveness of immune-based treatments against cancer was established with the development of high-dose IL-2 for patients with metastatic renal cell carcinoma and metastatic melanoma (Rosenberg et al. 1989). Inhibition of immune regulatory checkpoints, such as CTLA-4 and the PD-1/PD-L1 axis, is at the forefront of immunotherapy for cancers of various histological types. Several agents targeting two such negative checkpoints, the programmed death-1 (PD-1) pathway (pembrolizumab, nivolumab, and atezolizumab), and the cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (ipilimumab) are currently approved by Food and Drug Administration and European Medicines Agency. Both CTLA-4- and PD-1/L1-based therapies target pathways that negatively regulate T cell function, and recent studies have highlighted the ability of anti-CTLA-4 and anti-PD-1 antibodies to synergize and reverse T cell anergy within tumors by enhancing the local proliferation of effector tumor-infiltrating lymphocytes (TILs) (Curran et al. 2010). These negative checkpoint pathways play a role in immune tolerance in normal tissues and their activation is highly linked to the biological context. Immunotherapy using checkpoint inhibitors has changed the way we treat several aggressive cancers such as melanoma, non-small cell lung, and head and neck cancers, among others, with durable

responses achieved in the metastatic setting. However, unfortunately, the vast majority of patients do not respond to checkpoint inhibition therapy and a minority of patients, who do respond to treatment, develop secondary resistance and experience relapse by mechanisms still inadequately understood. Emerging evidence shows that alterations in multiple signaling pathways are involved in primary and/or secondary resistance to checkpoint inhibition. In this review we discuss how selected cancer-cell-autonomous cues may influence the outcome of cancer immunotherapy, particularly immune checkpoint inhibition. For instance, cancer-cell-autonomous cues might be responsible for TIL-negative tumors and cancer cell genetic evolution might predict the efficacy of checkpoint blockade. Activation of the oncogenic Wnt-β-catenin signaling pathway in melanoma cells has been shown to correlate with the absence of T cell and $CD103^+$ dendritic cells (DCs) infiltration into the tumor microenvironment (TME) due to β-catenin-mediated suppression of the chemokine CCL4, which in turn induces resistance to anti-PD-L1 and anti-CTLA-4 mAb-based therapies in experimental murine tumor models (Spranger et al. 2015). Similarly, loss of phosphatase and tensin homolog (PTEN) and activation of the PI3-kinase pathway in cancer cells can also promote resistance to checkpoint blockade (Peng et al. 2016) and has been linked to increased PD-L1 expression and immune resistance in human glioma (Parsa et al. 2007). Activating mutations in MAPK signaling pathway (i.e., oncogenic BRAF) has also been implicated in lower expression of tumor-associated antigens (Boni et al. 2010) and increased expression of immunosuppressive cytokines and VEGF (Frederick et al. 2013). Further evidence has shown that alterations in chromosomal region 9p24.1 in Hodgkin's lymphoma can induce the expression of PD-1 ligands through Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling (Ansell et al. 2015), and that defects in the pathways involved in interferon (IFN)-receptor signaling are associated with primary and secondary resistance to checkpoint inhibition (Zaretsky et al. 2016; Shin et al. 2016). Furthermore, Akbay et al. reported a correlation between activation of the epidermal growth factor receptor (EGFR) pathway and a signature of immunosuppression induced by the upregulation of PD-1, PD-L1, CTLA-4, and inflammatory cytokines (Akbay et al. 2013). This immune ablative fingerprint was associated with decreased TILs and increased markers of T cell exhaustion in mice bearing EGFR-driven adenocarcinoma of the lungs, albeit PD1 blockade could restore effector T (Teff) cell functions and prolong survival in this model. In addition, Moroishi and collaborators recently proposed that the activation of the Hippo pathway is responsible for the maintenance of low immunogenicity of tumors, through an impaired recognition by antigen presenting cells (APCs) and lack of an adaptive immune response. Strategies aimed at the ablation of Hippo pathway's components led to increased nucleic acid release by cancer cells, that in turn promoted immune activation and tumor control (Moroishi et al. 2016). We will also review multiple inhibitory feedback mechanisms in the TME that play a critical role in suppressing the anti-tumor immune response and are associated with resistance to checkpoint blockade.

2 "Hot" Versus "Cold" Tumors in the Context of Resistance to Checkpoint Inhibition

A series of recent studies have proposed a new paradigm of tumor classification based on the "temperature" measured in the TME, whereby "hot" tumors are characterized by the presence of a brisk immune cell infiltrate, while "cold" tumors lack such infiltrate (Fig. 1). This characterization has emerged thanks to in-depth studies of the TME and to advances in the use of big data from RNA sequencing technologies, as The Cancer Genome Atlas (TCGA), which together allow the classification of tumors based on the presence of myeloid or lymphoid infiltrates and the expression of immune-related genes. Indeed, recent studies have defined "cold tumors" as those displaying low or no T cell-inflammatory signatures; in contrast to "hot tumors" that display an increased presence of these genes, mainly by $CD8^+$ T cell-associated markers (Harlin et al. 2009; Sweis et al. 2016; Keck et al. 2015). Among the most investigated immune signatures, great emphasis has been given to T cell transcripts, mainly due to their implication on the clinical outcome following immunotherapy with checkpoint inhibitors. The most used transcripts to define "cold vs hot tumors" assess: CD3 and CD8 (including granzyme-B and perforin); the STING pathway (Stimulators of Interferon genes, including type I IFN) and IFN-gamma signaling; T cell-related chemokines (e.g., CXCL9, CXCL10, CCL2, CCL3, and CCL4); and MHC class I molecules. Also, other markers of myeloid-related signatures as CD68, CD14 for macrophages and Batf3 for DCs are used to assess the degree of immune infiltration of tumors (Corrales et al. 2016; Spranger et al. 2015).

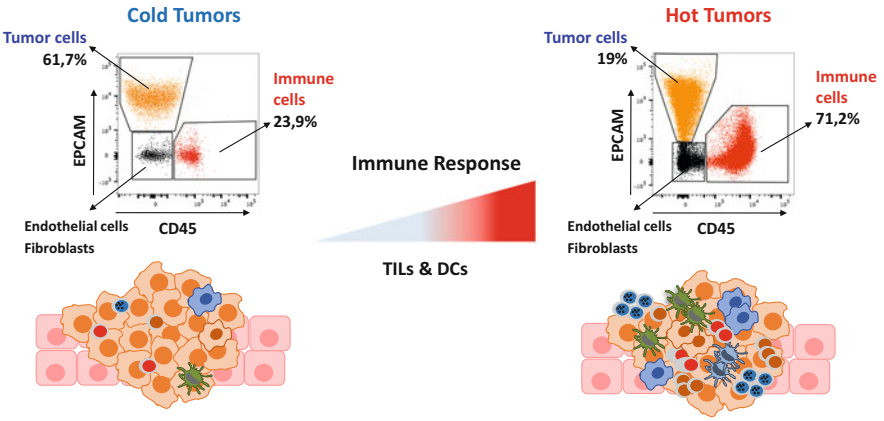

Fig. 1 "Cold" vs "Hot" tumors according to the immune contexture. Several tumor types have been recently defined based on immune signature features. Here, we show two representative flow cytometry plots from human breast tumor samples depicting two distinct phenotypes. "Cold tumors" (*left dot-plot*) are defined by the presence of low numbers of tumor-infiltrating lymphocytes (TILs) and dendritic cells (DCs), while "Hot tumors" (*right dot-plot*) present increased frequencies of both subsets

RNA-seq signatures are also emerging as an alternative approach to immunohistochemistry and/or flow cytometry to describe the qualities of the immune infiltrate of diverse tumor histologies, including breast, ovary, bladder, lung, kidney, and colon carcinomas, and melanoma (Pages et al. 2010). Accordingly, studies lead by Galon and colleagues also established the classification of colorectal carcinomas based on the analysis of immune-contexture in the TME (also called "Immunoscore") and defined gene signatures with prognostic value for cancer patients (Mlecnik et al. 2016).

Conversely, not only is the presence of T cell-inflammatory genes critical, but the presence of immune-inhibitory molecules as well as the tumor-intrinsic transcripts evaluated concomitantly, represent a great gain in the use of deep RNA sequencing technologies for tumor investigation. In this context, Danilova and colleagues (Danilova et al. 2016) recently found that PD-L1 and/or PD-L2 transcripts were positively correlated to host Th1/IFN-gamma signatures for various primary tumors, including renal cell carcinoma, bladder, lung adenocarcinoma, head and neck, breast, colon, and rectal cancers. In fact, PD−/L1 axis seems to be even better correlated to cytosolic pathways than the mutation load for some types of tumor. Additionally, for melanoma displaying a "hot" phenotype, increased presence of IDO, PD-L1, IL-10, LAG3, and Foxp3 molecules was also noted (Gajewski 2007; Taube et al. 2015). Some inhibitory molecules are induced upon combinatory events derived from the tumor microenvironment, probably due to the cross-talk of immune and cancer cells. In an elegant study, Spranger and collaborators (Spranger et al. 2013) suggested that the induction of the inhibitory signals PD-L1 and IDO, as well as regulatory T cell recruitment, in the melanoma microenvironment are mainly driven by $CD8^+$ T cell activity, operating as a homeostatic loop controlling the anti-tumor responses. This is consistent with previous studies showing that IFN-gamma can induce PD-L1 upregulation on macrophages (Loke and Allison 2003) and promotes IDO expression on human monocyte-derived DCs (Jurgens et al. 2009). Interestingly, two major evidences support the use of T cell inflammatory signatures as key elements for the design of immunotherapeutic strategies for cancer care: (1) CD8 infiltration is associated with better prognosis for several epithelial cancers (Fridman et al. 2012); (2) a pre-existing CD8 T cell infiltration is associated with responsiveness to immune checkpoint antibodies (Tumeh et al. 2014) and to neoadjuvant chemotherapy (Denkert et al. 2010).

Although recent efforts using inhibitory checkpoint antibodies have shown important benefit on the survival of cancer patients, in aggregate only about 20 to 40% of patients with metastatic solid cancers respond to anti-PD-1 therapy (Topalian et al. 2012) and only up to 20% of metastatic melanoma patients present long-term survival benefit under anti-CTLA4 treatment (Minn and Wherry 2016). One potential explanation for the failure in tumor elimination is the ability of tumor cells to escape the control of the adaptive immune system. Among these characteristics, the loss of tumor-antigen expression added to MHC down-regulation (Matsushita et al. 2012) and epigenetic tumor alterations (Schreiber et al. 2011) are some of the most prevalent mechanisms found when effective T

lymphocytes are present in the microenvironment. Thus, the increased accumulation of T lymphocytes within tumors is probably the most critical event triggering tumor cell mutation and escape. This concept has been lately strengthened by studies using big data analysis of gene signatures. Authors have lately clarified that tumors presenting a "hot" T cell-inflammatory phenotype are characterized by alterations in diverse tumor-intrinsic pathways. Most recent findings point out to molecular alterations in cellular pathways such as PI3K/AKT/mTOR, MAPK, Wnt/beta-catenin, EGFR, Jak, and Hippo, which are critically operating in different tumors and influence the degree of T cell infiltration and tumor inflammation. Alterations affecting these specific molecular pathways and their clinical significance will be reviewed and discussed in the following sections.

3 Alterations of Signaling Pathways Involved in Primary and/or Secondary Resistance to Checkpoint Inhibition

3.1 The PI3K/AKT/mTOR Pathway

This pathway is activated by receptor tyrosine kinases, downstream growth factor receptors, cytokine receptors, B and T cell receptors, and G-protein-coupled receptors. It regulates the cell cycle and different cellular functions such as cell growth, metabolism, proliferation, and survival, all key cellular processes participating to the initiation and the maintenance of cancer (Fig. 2).

The PI3K/AKT/mTOR pathway is constitutively activated in a high proportion of tumors as a result of mutations in several components of the pathway. One frequent finding is the loss of expression of the tumor suppressor PTEN, which inhibits downstream PI3K signaling (Song et al. 2012). Other common mechanisms leading to pathway activation include amplification or mutation of *PI3K* or *Akt*, or the activation of growth factor receptors.

The loss of *PTEN* has been associated with immunosuppressive mechanisms. In human glioma, it has been described that the expression of PD-L1 is increased posttranscriptionally after the deletion of *PTEN* and the activation of the PI3K pathway (Parsa et al. 2007). However, this effect was not observed across melanoma cell lines (Atefi et al. 2014), suggesting that the effect of altered PI3K signaling on PD-L1 expression may also depend on the presence of other oncogenic alterations unique to each tumor cell line. Illustrating this last point, in human melanoma, *PTEN* alterations are frequently observed with concomitant mutation of *BRAF*, contributing to the metastatic potential of the tumors (Dankort et al. 2009). Interestingly, Jiang X. et al. demonstrated that melanoma cells resistant to BRAF inhibition showed an increased expression of PD-L1, mediated by c-jun (under the control of Braf/MEK) and STAT3 (under the control of PI3K). Consequently, they showed that combination of therapies inhibiting MEK and PIK3 induced the suppression of PD-L1 expression and apoptosis in cancer cells (Jiang et al. 2013). These results pave the way for the rationalized combination of targeted inhibitors and immunomodulatory therapies for optimized cancer treatment.

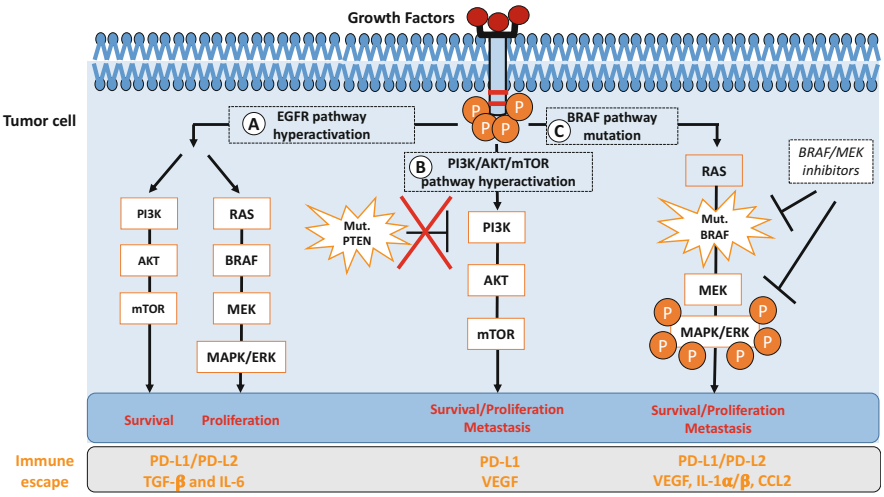

Fig. 2 Tumor immune resistance associated with genetic alterations in the EGFR, PI3K, and MAPK pathways. Representative scheme from collective studies describing tumor mutations found in the canonical EGFR, PI3K, and MAPK pathways. (**a**) Mutations responsible of EGFR pathway hyperactivation lead to increased proliferation and survival of tumor cells, while immune escape mechanisms are triggered by PD-L1/L2 upregulation, and the reselase of TGF-beta and IL-6. (**b**) PTEN loss of function induces hyper activation of the PI3K/AKT/mTOR pathway, resulting in enhanced tumor growth and spreading and immune escape mechanims via PD-L1 upregulation and VEGF secretion. (**c**) Activating BRAF mutations can lead to increased tumor growth and metastasis. Several strategies targeting activating BRAF mutations have been tested. However, a significant percentage of patients present acquired resistance to BRAF inhibitors, in which upregulation of PD-L1/L2 and increased production of VEGF, IL-1-alpha, and CCL2 emerge as acquired immune escape mechanisms

Sensitivity of tumors to immune checkpoint blockade seems to be dependent on the quality and quantity of the tumor immune infiltration. Consequently, it is of great importance to understand whether activation of the oncogenic PI3K/AKT/mTOR pathways in tumors hampers immune infiltration and resistance to immunotherapies. Along these lines, an interesting study by Peng and colleagues studied the impact of *Pten* loss and the consequent activation of the PI3K-AKT pathway on the anti-tumor T cell response (Peng et al. 2016). In experimental melanoma models they showed that *Pten*-silenced tumors were more resistant to T cell mediated killing in vitro and in vivo. In patients, they observed that tumors with lack of PTEN [defined as PTEN expression lower than 10% by immunohistochemistry (IHC)], had lower CD8+ T cell infiltration and TILs were more difficult to expand in vitro than patients with normal PTEN expression. Also, using TCGA data they observed that melanomas with low *PTEN* copy numbers had lower T cells infiltration by IHC, and lower transcripts of IFN-gamma and granzyme B, which are markers of cytolytic lymphocytes. They also showed that PTEN loss increased VEGF expression and that blocking VEGF in a mouse model increased infiltration of the tumor by T cells (Peng et al. 2016). Of note, VEGF has been involved in the

recruitment of suppressive myeloid and regulatory T cells, and several studies have also identified that active PI3K signaling was associated with an accumulation of immunosuppressive macrophages (Coussens et al. 2013). As inhibition of PI3K signaling in myeloid cells can block tumor progression in experimental tumor models (Schmid et al. 2011), it is tempting to speculate that pharmacological blocking of PI3K signaling could act at the same time on the tumor cells and inhibit myeloid cell suppression. Of translational importance also, Peng and colleagues showed that treatment of mice bearing melanomas with a PI3Kβ inhibitor improved the efficacy of anti-PD-1 and anti-CTLA4 antibodies (Peng et al. 2016), thus identifying a promising novel strategy to optimize immunotherapy in melanoma patients.

3.2 BRAF Activating Mutations as a Mechanism of Immune Resistance

Activating *BRAF* mutations are found in different types of cancer, including colorectal and thyroid carcinomas and it represents about half of all cases of human cutaneous melanomas (Davies et al. 2002). One of the major consequences of its genetic alteration results in the constitutive activation of the MAPK pathway, leading to an increased metastatic potential and to a reduced sensitivity to apoptosis of cancer cells (Lin et al. 2008) (Fig. 2). These features have an impact on the ability of the tumor cells to proliferate and on their interactions with the microenvironment and the immune infiltrating cells. Actually, approximately 40 different mutations were described in *BRAF* and, among them, about 70% of melanomas present the V600E modification, characterized by the substitution of valine for glutamic acid at the position 600 (Davies et al. 2002). In this scenario, multiple approaches for the inhibition of activating *BRAF* mutations have been tested in melanoma, leading to the registration of BRAF inhibitors in the mestastatic setting (Flaherty et al. 2010; Chapman et al. 2011). Furthermore, the advent of BRAF pathway inhibitors permitted a series of preclinical and clinical studies aiming to better understand the immune-related effects of *BRAF* mutations.

Reports in humans and mice have shown that activating BRAF mutations are associated with a weak or lack of T cell infiltration/activation on tumor sides (Boni et al. 2010; Ho et al. 2014; Tomei et al. 2015). Khalili and colleagues (Khalili et al. 2012) described that melanoma cells bearing *BRAF* mutations secreted elevated amounts of IL-1alpha and IL-1beta that potentially upregulates COX-2 and PD-L1/L2 expression on stromal fibroblasts, leading to T lymphocyte suppression. Another report using xenograft mouse models showed that *BRAF* V600E mutated cells produced high levels of VEGF, which is responsible for inhibiting the accumulation of T lymphocytes after adoptive cell transfer (ACT) (Liu et al. 2013). In agreement, Frederick and collaborators (Frederick et al. 2013) showed an increased CD8+ T cell infiltration and an upregulation of melanoma antigens in patients treated with BRAF and MEK inhibitors. Interestingly, TILs in these patients presented high expression of the exhaustion markers PD-1 and TIM-3. Other reports also described

the increase of intratumoral CD4+ T and cytotoxic CD8+ T cells following BRAF inhibition, which had an influence on tumor mass and metabolism (Wilmott et al. 2012) in addition to a noteworthy clonal change on the T cell repertoire after treatment (Cooper et al. 2013). Importantly, about 50% of melanoma-bearing patients develop resistance to BRAF inhibitors and may relapse early during treatment by evolving an array of compensatory mechanisms (Welsh et al. 2016). Actually, studies aimed at investigating the effects of secondary resistence to BRAF inhibitors demonstrated that both tumor cells and leukocytes upregulate checkpoints ligands as PD-L1 (Jiang et al. 2013; Kakavand et al. 2015). Thus, considering the importance of *BRAF* mutations on the suppression of anti-tumor immunity and the consequences on the TME following BRAF inhibition, some studies have recently tested the combination of BRAF pathway inhibition with several immunotherapeutic approaches, achieving encouraging results. Among them, the use of anti-CCL2 (Knight et al. 2013), adoptive cell therapy (Liu et al. 2013), and DC-based strategy (by using Flt3L + Poly-IC stimulation) (Salmon et al. 2016) have improved survival in tumor-bearing mice and induced adaptive immune activation. Using a Braf(V600E)/Pten(−/−) syngeneic tumor graft immunocompetent mouse model, Cooper and colleagues (Cooper et al. 2014) also showed prolonged anti-tumor effects when combining anti-PD1/PD-L1 with a BRAF inhibitor. Similarly, in a colon carcinoma mouse model, anti-PD-1 treatment showed advantages in tumor growth control and in increasing CD8+ T cell infiltration when administered with a MEK inhibitor (Liu et al. 2015). Collectively, rational strategies taking into account both the immune contexture of cancer patients and the intrinsic genetic alterations of cancer cells are further needed. Although clinical trials combining these targeted therapy and immunotherapy are ongoing, important questions still remain unanswered. Further studies using mouse models may provide therapeutic insights, including optimal timing and sequence of therapy. These experiments may also facilitate the prediction of potentially severe toxicities, as it has been observed when combining anti-CTLA4 therapy and BRAF inhibition (Ribas et al. 2013). The selection of patients based on T cell frequency/quality, checkpoint ligands expression, and *BRAF* status may provide a better understanding of the elements that ultimately influence the clinical outcome.

3.3 EGFR Pathway Activation and Its Suppressive Tumor Abilities

Mutations of the EGFR pathway were first reported in patients with non-small-cell lung cancer in 2004 (Lynch et al. 2004; Paez et al. 2004). Later, whole-genome sequencing in more than 180 lung cancer patients revealed *EGFR* as one of the most mutated genes in these individuals (Imielinski et al. 2012). Interestingly, the activation of EGFR pathway results not only in tumor growth/survival but also in the modulation of anti-tumor immune responses, as skin cancer-bearing mice treated with EGF-like growth factors showed an increased suppressive Treg activity (Zaiss et al. 2013) and loss of CCL27 expression, a chemokine involved in T cell attraction (Pivarcsi et al. 2007). In agreement with these observations, various

recent studies have reported that mutations affecting the EGFR pathway are associated with increased inhibitory immune signals as reviewed by Ji and collaborators (Ji et al. 2015) (Fig. 2). Also, Akbay and colleagues (Akbay et al. 2013) showed an upregulation of PD-L1, when mutant EGFR pathway was activated in murine lung tumors. Evaluating the TME, they also noted increased expression of PD-1 and CTLA-4 along with an increase in suppressive cytokines, such as TGF-β. The administration of anti-PD-1 antibody treatment led to enhanced T cell effector functions in those tumors, with an objective improvement in mice survival. In line with these findings, recent studies also showed PD-L1 upregulation on human tumor cell lines (Chen et al. 2015) and in primary tumor specimens from patients carrying EGFR mutations (Azuma et al. 2014).

3.4 HIPPO Pathway: Improving the Immunogenic Tumor Potential

The Hippo pathway is known as a tumor suppressor, acting on the survival and proliferation of normal cells, avoiding tumorigenesis (Harvey et al. 2013). Analysis of immune content in multiple human tumor types has revealed absent or weak presence/signature of TILs ("cold tumors"). This is probably due to poor immunogenicity of cancer cells, allowing silent growth and spread. Two kinases present in the Hippo pathway, LATS1/2 (large tumor suppressor 1 and 2), were recently described as important components controlling the immunogenic potential of malignant cells (Fig. 3). Moroishi and collaborators (Moroishi et al. 2016) demonstrated that LATS1/2 deletion abolishes tumor growth and diminishes metastasis in different mouse tumor models by an immune-mediated mechanism. In particular, tumor cells lacking LATS1/2 produced extracellular vesicles carrying concentrated amounts of nucleic acids that were able to stimulate APCs via the TLRs-MYD88/TRIF-IFN pathway. This generated an anti-tumor immune response leading to subsequent tumor elimination. In addition, analyzing human epidemiological data sets, the authors found significant correlation between both low LATS1 and LATS2 mRNA levels and favorable patient outcome. Additional studies using human specimens are needed to confirm these findings and to test combinations targeting the Hippo pathway on tumors with the aim of boosting immunity and reverting mechanisms associated with lack of immunogenicity.

3.5 Mutations in *JAK1* and *JAK2* Hamper IFN Signaling Pathway

A recent report from Ribas et al. (2016) demonstrated that about 25% of melanoma patients under anti-PD-1 treatment and having objective responses to therapy undergo disease progression within approximately 21 months. One way to explain these observations would be that the anti-tumor immune response restored by checkpoint antibodies may exert distinct "selective signals" triggering intrinsic genomic alterations in tumor cells, that, in turn, would alter its phenotype and

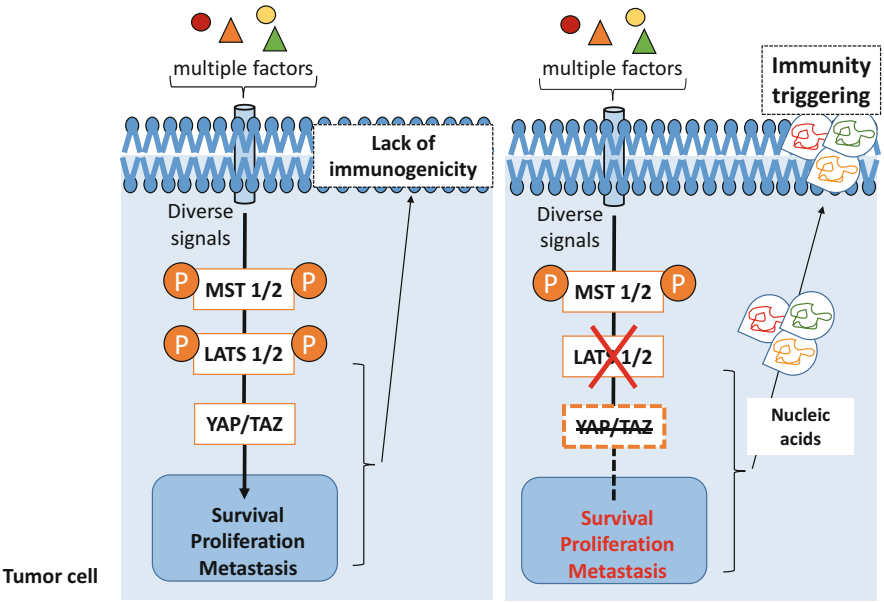

Fig. 3 The Hippo pathway as a tumor immunogenicity silencer. Activation of the Hippo pathway is responsible for sustained tumor survival and metastasis, in which no immunogenicity towards cancer cells is noted. Recent studies showed that the deletion of LATS1/2 components of the hippo pathway enables the release of nucleic acids, which, in turn, triggers APCs' activation and adaptive immunity and subsequent control of tumor growth

promote immune escape. In this scenario, Zaretsky and colleagues (Zaretsky et al. 2016) performed whole-exome sequencing in biopsies from 2 melanoma patients at baseline (before anti-PD-1 treatment) and after tumor relapse (post-anti-PD-1 treatment). Post-treatment changes in the TME were observed in both patients, which consisted of increased CD8+ T cells infiltration and PD-L1 expression in macrophages and stromal cells. Despite the fact that tumors at baseline and post-relapse showed more than 92% of similarities in non-synonymous mutations, the authors found two truncating mutations in the interferon-receptor–associated Janus kinase 1 and 2 (*JAK1*, *JAK2*), each one identified in one patient. As a consequence, *JAK1*- and *JAK2*-mutated tumor cells were insensitive to IFNgamma, including to its antiproliferative effects (Fig. 4). In agreement with these observations, another elegant study recently reported by Shin et al. (2016) suggested that loss-of-function mutations in the *JAK1* and *JAK2* genes could be also associated with primary resistance mechanisms to anti-PD-1 therapy. Evaluating the mutational load of melanoma and colon cancer patients that were refractory to anti-PD-1 treatment, the authors found pre-existing JAK1/2 inactivating mutations. Similarly, melanoma cell lines lacking functional IFN signaling pathway also failed to upregulate PD-L1 expression even in the presence of IFN-gamma. Importantly, using data from TCGA, similar mutations in *JAK1* and *JAK2* were found in other tumor histologies,

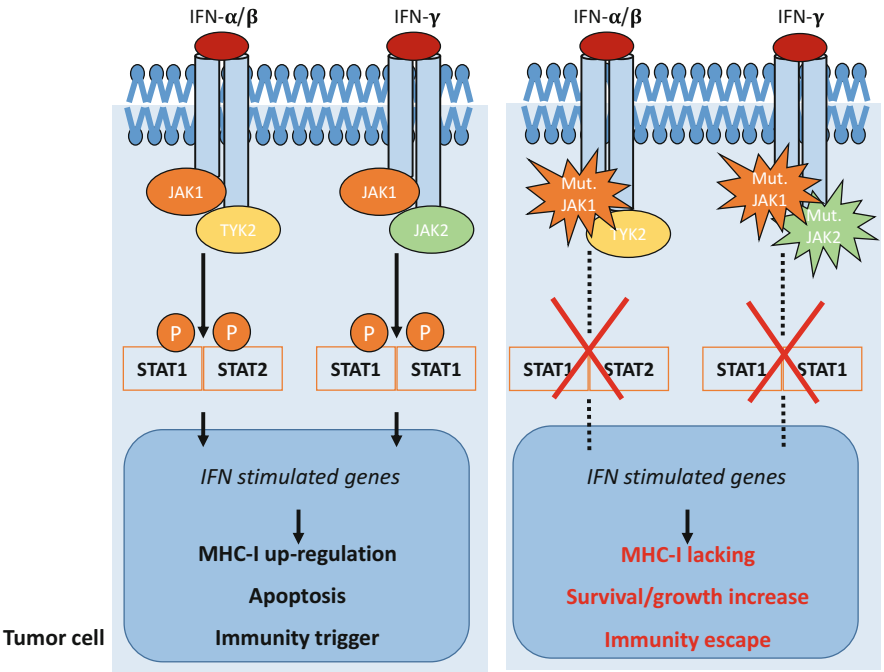

Fig. 4 JAK mutations and loss of IFN signaling are associated with resistance to checkpoint blockade. IFN signaling is dependent on the JAK/STAT pathway and is responsible for inducing immune-related effects on tumor cells, allowing their elimination. Recent data in mestastatic cancer patients showed the presence of primary and secondary resistence to checkpoint inhibition via loss-of-function mutations in JAK1 and/or JAK2 genes and subsequent lack of response to interferon-gamma, including insensitivity to its antiproliferative effects on cancer cells

including breast, prostate, lung, and colorectal adenocarcinoma, in which an impact on survival of patients was also noted. Thus, additional studies are needed to better define subgroups of patients exhibiting primary and/or secondary resistance via *JAK1/2* mutations.

3.6 The Wnt/β-Catenin Pathway and Immune Response

Tumors use multiple strategies to induce immunosuppression and evade immune recognition (Hanahan and Weinberg 2011) by directly inactivating effector T cells, altering DCs, promoting regulatory T cells and immune suppressive cell functions in the TME (Meyer et al. 2014; Romano et al. 2015). The Wnt/β-catenin pathway operates across many tumor types and it is critically involved in counteracting several steps of immune activation (Fig. 5). One of such underlying mechanisms has been demonstrated in DCs. The activation of β-catenin promotes DC-mediated CD4+ T cell tolerance and is associated with tumor-induced suppression of CD8+ T

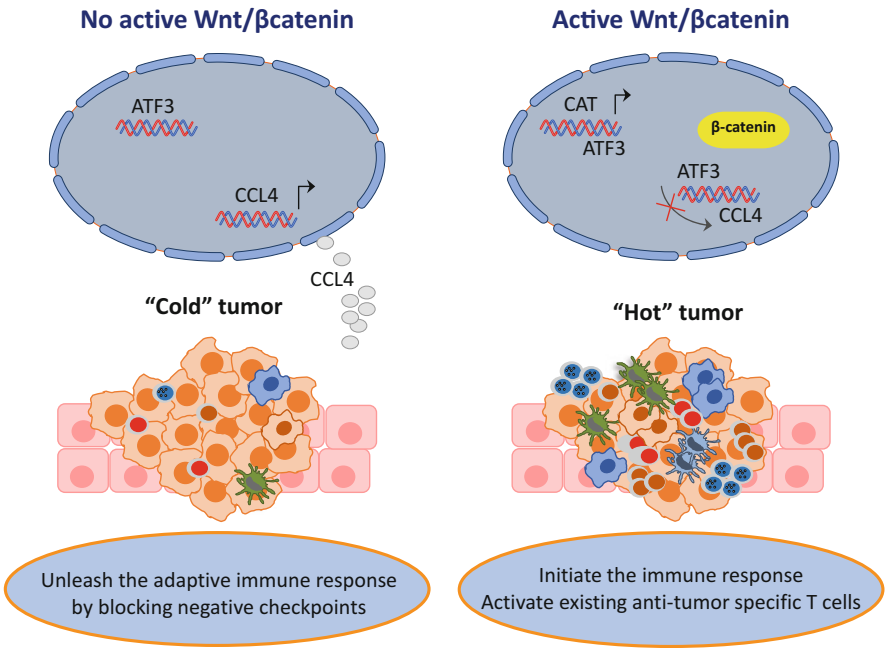

Fig. 5 Wnt/β-catenin signaling and the tumor immune infiltrate. In melanoma cells, β-catenin mediates immune ignorance in the TME through the induction of transcriptional repressor ATF3, which in turn inhibits CCL4 production via the transcriptional silencing of CCL4 gene. Lack of CCL4-secretion results in poor recruitment of CD103 dendritic cells, associated with an impaired cross-priming of antitumor T cells. In addition, the intratumoral T cells show predominantly a naïve phenotype with low PD-1, PD-L1, and LAG3 expression. Knock-down of β-catenin or ATF3 restores CCL4 production in these cells, which in turn leads to a T cell-inflamed melanoma, characterized by the presence of Batf3-lineage dendritic cells as well as transcripts encoding indoleamine-2, 3-dioxygenase (IDO), PD-L1, and FoxP3

cell immunity through the inhibition of the cross-priming capacity of DCs. This is consistent with the ability of β-catenin to negatively regulate T cell stimulatory functions in vitro (Jiang et al. 2007) and attenuate gut inflammation in vivo (Manicassamy et al. 2010). Liang and colleagues tested the hypothesis that tumor-induced suppression of CD8+ T cell immunity is mediated by β-catenin in DCs through the inhibition of the ability of DCs in cross-priming (Liang et al. 2014). When vaccinated with DC-targeting anti-DEC-205 mAb fused with tumor antigens, B16 melanoma-bearing mice exhibited dampened CD8+ immunity, similar to DC-β-catenin^{active} mice. DCs from DC-β-catenin^{active} and tumor-bearing mice were deficient in cross-priming, and antigen-specific CD8 + T cells primed in these mice resulted in dampened CD8+ lymphocytes-mediated memory responses. Importantly, DC-β-catenin deficient (DC$^{\beta\text{-catenin}-/-}$) mice completely abrogated tumor-mediated inhibition of cross-priming, suggesting that β-catenin is required for tumor-induced inhibition of cross-priming. Further mechanistic insight on the immune modulation of DC functions by β-catenin has been addressed with the

observation that activation of β-catenin signaling in DCs inhibited cross-priming of CD8+ T cells by the upregulation of IL-10 by mTOR (Fu et al. 2015).

Understanding DC biology is crucial in the design of vaccination strategies. Interestingly, vaccination of mice lacking β-catenin in CD11c + DC ($DC^{\beta\text{-catenin}-/-}$) failed to protect them against tumor challenge. In addition, $DC^{\beta\text{-catenin}-/-}$ mice were incapable of generating CD8+ T cell immunity despite normal clonal expansion, possibly a consequence of impaired IL-10 production in $DC^{\beta\text{-catenin}-/-}$. Deletion of β-catenin in DCs or blocking IL-10 post-clonal expansion resulted in comparable reduction of CD8+ T cells, suggesting that maintenance and post-clonal expansion of CD8+ T cells through IL-10 are fundamentally regulated by active β-catenin in DCs. These observations highlight mTOR/IL-10 as a mechanism for β-catenin-dependent inhibition of cross-priming and uncover a positive role of β-catenin in the maintenance of CD8+ T cells. Despite the opposite functions of β-catenin in regulating CD8+ T cell responses, selective blocking of β-catenin with a pharmacological inhibitor during the priming phase augmented DC vaccine-induced CD8+ T cell immunity and improved antitumor efficacy, suggesting that interfering with β-catenin signaling could represent a feasible therapeutic strategy to improve the efficacy of DC-based vaccines. Pharmacological targeting of the Wnt/β-catenin pathway remains, however, a challenge that requires further investigation.

In addition to its role in regulating the effector functions of DCs, active β-catenin signaling in tumor cells has been attributed to inflammation and impaired intratumoral T cell infiltration. Blocking antibodies targeting the receptor PD-1, or its ligand (PD-L1), have been identified as one of the breakthrough advances in cancer therapy that may induce a durable response rate of about 20–40% across several cancer types. Recent randomized phase II/III clinical trials with anti-PD-1 antibodies (nivolumab and pembrolizumab) have demonstrated an increased overall survival compared to previous standard treatments in melanoma, as well as in other tumor types including non-small cell lung cancer and renal cancer (Borghaei et al. 2015; Weber et al. 2015). These results lead to the accelerated approval of anti-PD-1 antibodies for metastatic melanoma, non-small cell lung cancer, and renal cell cancer. Full pipelines of immune modulatory agents are in different stages of clinical development for a wide variety of tumor types. It is still unclear, however, why only a limited number of patients harboring the same type of tumor respond to the therapy and why certain tumors do not respond at all. In the context of PD-1 blocking antibodies, preclinical and clinical data show that the tumor-specific CD8+ T cells express PD-1 in close proximity to PD-L1-expressing cells in the responding patients (Gandini et al. 2016; Tumeh et al. 2014). Gene expression analysis of metastatic human cutaneous melanoma indicated an inverse correlation of active β-catenin signaling with those genes associated with an endogenous immune response, such as APC2, SOX2, SOX11, and WNT7B (Spranger et al. 2015).

Using a genetically engineered murine tumor model driven by conditional BRAFV600E activation and *PTEN* deletion (BRAFV600E/ $PTEN^{-}/^{-}$) (Damsky et al. 2011), overexpression of a stabilized form of β-catenin almost completely

depleted T cells, and the few remaining intratumoral T cells showed predominantly a naïve phenotype with low PD-1, PD-L1, and LAG3 expression in the arisen melanomas. When a neo-antigen (SIY) was genetically engineered into the melanomas of these mice, adoptively transferred T cells with SIY–specific T cell receptor accumulated in the BRAFV600E/PTEN$^{-/-}$ tumors, but not in the BRAFV600E/PTEN$^{-/-}$ tumors expressing the non-degradable β-catenin (BRAFV600E/ PTEN$^{-/-}$ Bcat-STA), an indicative of defective homing of antigen-specific T cells in the tumors. In agreement with previous findings, these data suggest that tumor-intrinsic β-catenin signaling prevents early T cell priming. Following analysis of the Batf3-lineage DCs that are crucial in presenting tumor antigens to CD8+ T cells, it was found that CD8a + (skin-derived) and CD103+ (lymph node-derived) DCs were almost absent, with reduced IFN-β cytokine expression in BRAFV600E/ PTEN$^{-/-}$ Bcat-STA tumors but not in its counterpart BRAFV600E/ PTEN$^{-/-}$. Interestingly, BRAFV600E/PTEN$^{-/-}$ Batf3$^{-/-}$ bone marrow chimeras showed comparable decrease of T cell infiltration; however, intratumoral injection of DCs activated by poly I:C could restore T cell infiltration in BRAFV600E/ PTEN$^{-/-}$ Bcat-STA tumors, suggesting that defective recruitment of CD103+ DCs is a major immunologic defect in melanomas with high intrinsic β-catenin signaling. Finally, treatment of both mouse models with a combination of anti-CTLA-4 and anti-PD-L1 antibodies induced a significant delay in tumor growth in the BRAFV600E/ PTEN$^{-/-}$ model, but not in the BRAFV600E/PTEN$^{-/-}$ Bcat-STA model; while intratumoral injection of activated DCs into the BRAFV600E/PTEN$^{-/-}$ Bcat-STA tumors could partially restore the therapeutic effect of anti-CTLA-4 and anti-PD-L1 antibodies. Overall, these findings highlight the role of β-catenin in mediating inhibition of an adaptive immune response to tumors and could represent a new and potentially generalized mechanism that tumors employ to achieve immunosuppression. The understanding of β-catenin signaling in human tumors and its potential involvement in shaping the response to targeted as well as immune therapies is of great interest. It is important to underline that current studies have not been able to show evidence of a correlation between the β-catenin signaling with a differential responsiveness with immune modulatory therapies in human. Furthermore, since patients with "hot" melanomas seem to derive higher benefit from PD-1 therapy, the future challenge is to characterize immune signatures in patients lacking intratumoral T CD8+ infiltration.

4 Concluding Remarks and Future Prospects

Recent clinical trials using immune checkpoint inhibition therapy have demonstrated its potential to control cancer by disinhibiting the immune system. Immune checkpoint blocking antibodies against CTLA-4 or PD-1/L1 have reported durable clinical responses in various types of cancers and have been approved in the USA and EU in selected clinical settings; however, multiple mechanisms of resistance exist. In particular, tumor- and host-related factors have been established as common denominators responsible for the heterogeneity in the clinical outcome

to therapy with immune checkpoint inhibitors. Additional patient cases will need to be studied to assess how common these mechanisms of resistance might be; however, collectively the findings discussed in this chapter enable us to better understand immune features of the TME as well as tumor-intrinsic genetic alterations associated with primary and secondary resistance to immune checkpoint inhibitors and the potential to revert multiple resistance routes to improve immunotherapy.

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Immunotolerance as a Mechanism of Resistance to Targeted Therapies in Melanoma

Mario Mandalà and Daniela Massi

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Abstract

The therapy of metastatic melanoma (MM) was radically changed by the introduction of inhibitors of BRAF, an oncogene mutated in ≈40–50% of patients. Oncogenic BRAF promotes an immune-compromised tumour microenvironment (TME). Inhibition of MAPK pathway signaling with BRAF (BRAFi) and MEK inhibitors (MEKi) attenuates immune escape and increases the melanoma immunogenicity through multiple mechanisms, including elevation of melanoma antigen expression and improved T cell infiltration and function. These changes sustain the TME for response to immunotherapy. In this chapter we discuss preclinical and clinical data supporting the immunomodulating activities of targeted therapies, the

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immunotolerance as a mechanisms of resistance and highlight the rationale for novel combinations of targeted therapies and immunotherapies with the potential to significantly improve the future treatment of MM patients.

Keywords
BRAF • BRAF inhibitors • Immunotolerance • MEK inhibitors • Resistance

1 Introduction

Over the past 5 years, the FDA approval of the targeted therapy with inhibitors of the BRAF/MAPK pathway and novel immunotherapy with immune checkpoint inhibitors has significantly advanced the treatment of metastatic melanoma (MM) patients.

Approximately 50% of melanomas harbor activating (V600E) mutations in the serine-threonine protein kinase B-RAF (BRAFV600E) and the BRAF inhibitors vemurafenib and dabrafenib have shown to improve tumour regressions in patients with BRAFV600E mutant MM (Flaherty et al. 2010; Sosman et al. 2012; Falchook et al. 2012; Hauschild et al. 2012). Vemurafenib, the first type I BRAF inhibitor to enter clinical development, has shown more than 50% confirmed response rates in phase 1, 2 and 3 clinical trials (Flaherty et al. 2010; Sosman et al. 2012; Chapman et al. 2011), with an improved median overall survival (14–16 months) (Sosman et al. 2012; Chapman et al. 2011) in patients with melanoma harbouring the BRAF V600E mutation when compared with conventional chemotherapy. Unfortunately, after an initial improvement, most patients experience progression, with a median progression-free survival of 5–7 months. Like vemurafenib, dabrafenib, a second type I BRAF inhibitor, induces confirmed responses in more than 50% of MM patients (Falchook et al. 2012).

Recently, several MEK inhibitors have entered clinical development and trametinib has shown confirmed response rates of more than 20% in BRAF mutant melanoma, a phase 3 trial illustrating significant progression-free and overall survival benefit compared with dacarbazine or paclitaxel (Flaherty et al. 2012). Emerging data showed that the combination of BRAF and MEK inhibitors reduces single-agent toxicity of each agent thus delaying or even preventing the onset of resistance. Notably, no increase in the risk of developing secondary cancers was observed. Therefore, this combination approach is now regarded the new standard for the treatment of MM patients (Robert et al. 2015a; Long et al. 2015; Larkin et al. 2014).

Following these important advancements, a further major breakthrough in the treatment of melanoma has occurred by the introduction of immune checkpoint inhibition. This novel strategy has proved an exciting opportunity of long-term responses in clinically significant proportion of patients. Monoclonal antibodies targeting immunomodulatory molecules such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death receptor-1 (PD-1) have shown objective response rates ranging between 15% and 40%, respectively, when used as

monotherapy, and up to 60% when combined in large randomized clinical trials (Schadendorf et al. 2015; Ribas et al. 2016; Robert et al. 2015b).

Of importance, oncogenic BRAF can promote an immune-compromised tumour microenvironment (TME) (Sumimoto et al. 2006; Khalili et al. 2012) and inhibition of MAPK pathway signaling with BRAF (BRAFi) and MEK inhibitors (MEKi) attenuate mechanisms of immune escape and increase the melanoma immunogenicity via multiple mechanisms, including elevation of melanoma antigen expression and improved T cell infiltration and function (Frederick et al. 2013; Wilmott et al. 2012). These changes may serve to prime the TME for response to immunotherapy (Figs. 1 and 2).

In this chapter we discuss preclinical and clinical data supporting the immunomodulating activities of targeted therapies, the immunotolerance as a mechanisms of resistance and highlight the rationale for novel combinations of targeted therapies and immunotherapies with the potential to significantly improve the future treatments of MM patients.

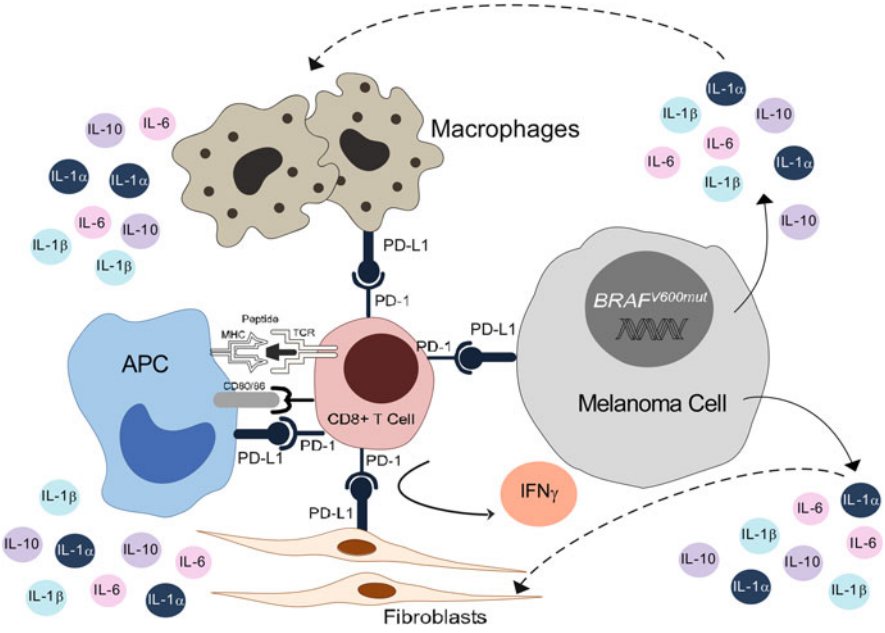

Fig. 1 BRAFV600 melanoma and tumour microenvironment

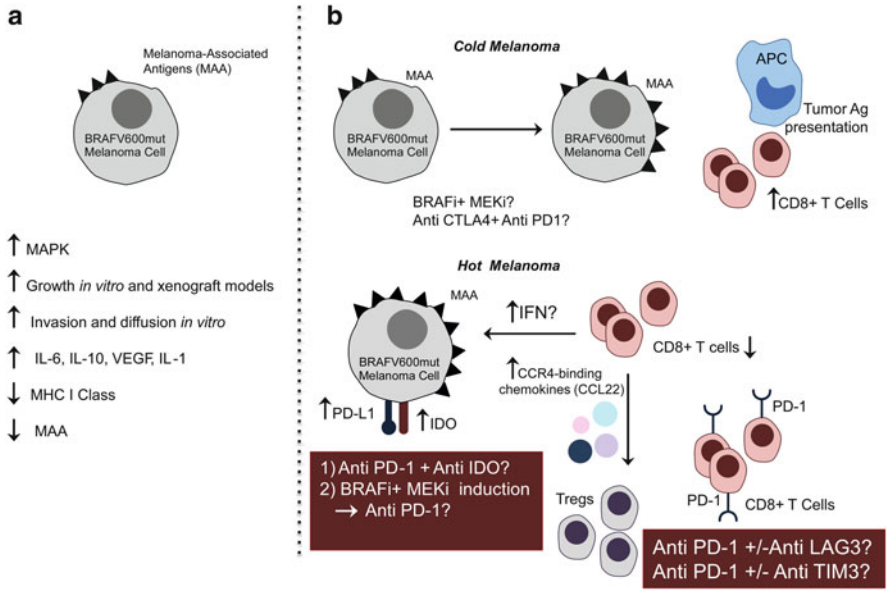

Fig. 2 (**a**) BRAFV600 melanoma and immunotolerant TME. (**b**) Biology-driven treatment strategy

2 BRAF and Immune Suppression Are Two Key Hallmarks in Melanoma

In 2000, Hanahan and Weinberg described six biological hallmarks, acquired during the multistep cancer development: (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) resisting cell death, (4) enabling replicative immortality, (5) inducing angiogenesis, and (6) activating invasion and metastasis (Hanahan and Weinberg 2000). In the last two decades, based on new experimental data, two further hallmarks have been added: (7) reprogramming of energy metabolism and (8) evading immune destruction. Two hallmarks are of paramount importance in melanoma: (1) the MAPK pathway-activating oncogenic mutations and (2) immune suppression in the TME (Hanahan and Weinberg 2011).

BRAF mutation is present in 40–50% of cutaneous melanomas and results in the constitutive activation of the MAPK pathway, responsible for controlling cellular proliferation, apoptosis, and migration. A valine-to-glutamate substitution in the glycine-rich loop is the most frequent BRAF mutation (V600E). This gain-of-function BRAF mutation accounts for nearly 70–80% of the BRAF alterations described in melanoma, with alternative point mutations at the same position (V600D, V600K, V600R) contributing in the remaining cases, specifically, 15% are V600K, while V600R mutations constitute approximately 3–5% of all BRAF mutations. Immunosuppression involves active evasion by cancer cells from attack

and elimination by immune cells; this capability reflects the dichotomous roles of an immune system that both antagonizes and facilitates tumour development and progression.

Several studies support the notion that these two hallmarks are strictly linked, with MAPK signaling regulating the transcription of genes that downregulate the antitumour immune response (Sumimoto et al. 2006; Khalili et al. 2012; Hugo et al. 2015). For instance, oncogenic BRAF induces T cell suppression directly through the secretion of inhibitory cytokines such as IL-10, TGF-β, or VEGF, IL-1 or through membrane expression of co-inhibitory molecules such as the PD-1 and its ligands: PD-L1 and PD-L2. Furthermore, the presence of oncogenic BRAF leads to an immune suppressive TME characterized by the presence of inhibitory immune cells such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumour-associated macrophages, which can in turn inhibit the function of tumour-infiltrating T cells. It has been reported that melanoma cell lines with constitutively activated MAPK, due to BRAFV600 mutation, produce various immunosuppressive factors (IL-10, VEGF, or IL-6, IL-1), which promote recruitment of Tregs, MDSCs (categorized by CD11bþ/Gr-1þ surface staining), alternative activated macrophages (often referred to as M2-like macrophages), and immature dendritic cells, which also accumulate within the TME (Mandalà et al. 2016). Furthermore, IL-1 up-regulates the transcription of several genes known to promote immune suppression, such as COX-2, PD-L1 and PD-L2, which may also contribute to the induction of a functional T-cell inhibition (Mandalà et al. 2016).

Another immunosuppressive effect of mutant BRAF derives from its ability to down-regulate MHC class I (MHC-I) molecules by melanoma cells. MHC-I expression is reduced in melanoma cells overexpressing mutant BRAFV600. BRAFV600 mutation drives rapid and constitutive internalization of MHC-I from the cell surface and subsequent sequestration within endocytic compartments (Johansen et al. 2016). As a corollary of the immunosuppressive microenvironment driven by oncogenic BRAF, melanomas treated with BRAFi show increased T lymphocyte infiltration and expression of melanoma derived antigens (MDA). Related to T cell infiltration, increased MDA expression in melanoma samples as well as decreased VEGF and the immunosuppressive cytokines (IL-6 and IL-8), associated with an enriched granzyme B and perforin + T-cell infiltrate within 14 days of the initiation of BRAFi therapy has been described (Mandalà et al. 2016).

More importantly, in paired biopsies, the addition of MEKi to BRAFi did not reduce immune infiltration of CD4+ and CD8+ lymphocytes in the early phase of treatment (Hu-Lieskovan et al. 2015).

However, three major issues remain to be clarified: (1) there is remarkable heterogeneity and variability in the MDAs induced by BRAFi and or MEKi across different melanoma cell lines. For instance, although MDAs are proportionally up-regulated in different cell lines, the level of induction varies significantly between different melanoma cell lines; (2) although MAPK blockade by BRAFi or MEKi leads to increased MDAs expression, MDAs are not neoantigens, which are primarily recognized by the immune system. Upregulation of generic MDAs does not warrant a better identification of the immune system as only neoantigens

are a major factors for immune recognition thus promoting and optimization of immunotherapies; (3) functional studies to investigate specificity and clonality of T cell infiltration are strongly needed to better characterize the TME upon treatment with BRAFi and/or MEKi.

3 Immune-Mediated Resistance to BRAFI and MEKI

Approximately 80% and 95% of MM patients treated, respectively, with BRAFi and MEKi or BRAFi alone, respectively, progress at 3 years (Menzies and Long 2014). Furthermore, 20% of patients does not respond to therapy due to intrinsic or acquired resistance mediated by hyperactivation of receptor serine/threonine kinases, MAP kinase and alternative PI3K/Akt signaling, and interactions with TME. In the context of MAPKi treatment, there is an intrinsic inter-patient variability in the degree and duration of response. Therefore, identification of biomarkers that can allow accurate selection of the individual patient tailored treatment in BRAF-mutated melanoma is required.

Clinically, acquired resistance to MAPKi therapies for melanoma cannot be fully explained by genomic mechanisms and may be accompanied by co-evolution of intra-tumoural immunity. There is now enough evidence that TME plays a role in the developing of resistance during treatment with BRAFi or BRAFi and MEKi. Although BRAFi and MEKi enhance MDAs expression and promote the immune response against tumour cells, this more favourable microenvironment during BRAFi treatment seems to be counterbalanced by an increased expression of PD-1 and PD-L1 within 2 weeks upon starting BRAFi, a finding that suggests a potential immune-mediated resistance mechanism to BRAF inhibition (Wargo et al. 2014; Frederick et al. 2013).

Regulation of PD-L1 expression by melanoma is currently an area of intense translational and clinical investigation. PD-L1 expression can be induced by microenvironmental signals, including interferon-gamma, which is produced by activated CD8+ T lymphocyte. On the other side, PD-L1 expression may be enhanced through an oncogenic signalling pathway (Massi et al. 2014, 2015; Merelli et al. 2014).

Four different types of TME can be distinguished based on the presence or absence of tumour-infiltrating lymphocytes and PD-L1 expression has been described. These include type I (PD-L1 positive with TILs driving adaptive immune resistance), type II (PD-L1 negative with no TIL indicating immune ignorance), type III (PD-L1 positive with no TIL indicating intrinsic induction), and type IV (PD-L1 negative with TIL indicating the role of other suppressor(s) in promoting immune tolerance) (Teng et al. 2015).

We recently demonstrated that in BRAFi-treated MM patients, the presence of PD-L1 immunohistochemical expression in melanoma cells in the absence of tumour-infiltrating mononuclear cells (TIMC) is significantly associated with shorter progression-free survival and melanoma-specific survival (Massi et al. 2015). Furthermore, the absence of tumoural PD-L1 staining and the presence of

TIMC are associated with a better response to treatment. These results highlight the importance of the pre-existing immunological status in determining the response and outcome of MM patients treated with MAPKi.

Additional findings provide further information on the immune-mediated resistance to BRAFi and MEKi. Hugo et al. showed that LEF1 down-expression and ß-catenin modulation cause acquired MAPKi-resistance (Hugo et al. 2015). Similarly, Biechele et al. showed that β-catenin activation could modulate innate BRAFi sensitivity in melanoma cell lines (Biechele et al. 2012). Tumour-intrinsic ß-catenin pathway activation is mechanistically involved in the exclusion of T cells as well as CD103 dendritic cells via inhibition of CCL4 secretion (Spranger et al. 2015). The inverse relationship of active ß-catenin signaling and T cell infiltration in both human melanoma samples and transgenic melanoma mouse models by Spranger and colleagues provided a first insight into a potential new mechanism of immune resistance. No correlation was found between these findings and MAPKi treatment. Recently, our group extended these observations showing that the presence of CD8+ T cell infiltration as well as the subset of CD8+CD103+ T cells in melanoma samples obtained before starting treatment with MAPKi correlated with the therapeutic response (Massi unpublished observations). Furthermore, responding patients showed a significantly increased expression of mRNA transcripts associated with adaptive immunity and antigen presentation. In a multivariate analysis, the presence of CD8+ T cells was found to predict prognosis.

About 50% of MM patients with acquired MAPKi-resistance exhibit a profound CD8 T-cell deficiency and/or an exhaustion of the phenotype (Hugo et al. 2015). There is evidence that *CD8A* expression in MM patients progressing during BRAFi treatment decreases not only with respect to patient-matched baseline expression level but also to the general expression range at baseline. Thus, distinctive expression patterns of *CD8A,* in both relative and absolute terms, suggest both CD8 T-cell depletion and exhaustion. As overexpression of PD-L1 during the process of the immune adaptive resistance correlates with a better response to anti PD-1, the anti-PD-1 has been proposed as the first line treatment. However, it must be underlined that definition of best first line treatment (immune checkpoint inhibitors or MAPKi) remains uncertain and should only derive from appropriate clinical trials. Finally, the high intra-tumoral CD8+ T cell infiltration upon response disappears at disease progression. Thus, understanding the functional contributions of immune evasion to acquired MAPKi-resistance may contribute to develop combined treatments and improve the anti-tumor response of BRAFi alone.

In addition to the lymphocytes' orchestrated mechanisms of resistance, also secreted, soluble factors originating from the stroma can induce resistance. These include: (1) the stromal fibroblast-derived hepatocyte growth factor that activates receptor tyrosine kinases resulting in the reactivation of the pathway by signalling through RAS (Straussman et al. 2012); and (2) the TNFα, which has been described to block apoptosis in BRAF-depleted melanoma cells (Gray-Schopfer et al. 2007). TNFα promotes tumour growth, angiogenesis, and invasion. Recently, it has been reported that MAPK pathway inhibition directly influences the TME by increasing

the number of macrophages. Macrophages release TNFα, which, in turn, increases resistance due to its ability to enhance the expression of the melanoma survival factor MITF (Smith et al. 2014).

MITF-dependent resistance is probably due to its crucial role in regulating multiple survival and antiapoptotic genes, including BCL2A1 (Haq et al. 2013). Furthermore, components of the differentiation program that stimulates up-regulation of MITF are also involved in MAPK pathway inhibitor resistance (Johannessen et al. 2010).

Overall, insights from these studies provide a framework for considering the immune microenvironment as a target to be exploited in combination strategies between targeted therapies and immunotherapy.

4 Biology-Driven Strategy to Combine Immunotherapy and Target Therapy and Its Impact on the Design of Clinical Studies

The systemic treatment of MM has evolved with the introduction of BRAFi and MEKi and immunotherapy to the complementary therapeutic strategy, with the aim to restore immune functions, boosting T cell specific responses against the tumour. Elucidating mechanisms of response and resistance to each of these therapeutical strategies is key to better understand how to combine these medicines. Translational research studies conducted in parallel and in sequence in in vitro, in murine models as well as in patient tissue samples are of paramount instrumental value. Table 1 reports ongoing studies, which evaluate the best combination or sequencing strategy with immunotherapy and targeted therapy. All these trials have been planned following the classical clinical design of oncological studies. Nevertheless, several questions still remain unanswered. The acute and late toxicity of new combinations, the best schedule indicate the need of new clinical trails strictly based on the biology of the disease.

4.1 New Combinations: Opportunity and Challenges

Several interferon-inducible genes that are part of the negative immune regulatory loops could limit T cell responses to cancer and could provide novel targets for immunotherapy. Among them T-cell immunoglobulin domain and mucin domain-3 (TIM-3), the lymphocyte activation gene 3 (LAG-3) as well as the carcinoembryonic antigen cell adhesion molecule-1 (CEACAM1), which has been reported to be a partner of TIM-3, can be blocked therapeutically using antibodies resulting in antitumour activity (Smyth et al. 2016).

Furthermore, cancer treatment with agents that inhibit immunosuppressive metabolites is another promising strategy. These targets include adenosine, indoleamine 2,3-dioxygenase (IDO), which is expressed by both tumour cells and infiltrating myeloid cells, and arginase, which is produced by MDSCs. IDO and

Table 1 Ongoing clinical trials with targeted therapy combined (combination or sequential) to immunotherapy in advanced melanoma patients (from www.clinicaltrials.gov, updated at December 31, 2016)

Targeted therapy + immune checkpoint inhibitors				
Drugs (*clinical trial*)	Phase	Pathology	Primary endpoint(s)	Secondary endpoint(s)
Phase III Sequential strategy of immunotherapy and targeted therapy in melanoma				
SECOMBIT STUDY (*NCT02631447*) *Arm A* LGX 818 + Mek 162 until PD followed by Ipi and Nivo *Arm B* Ipi and Nivo until PD followed by LGX 818 + Mek 162 *Arm C* LGX 818 + Mek 162 for 8 weeks followed by Ipi and Nivo until PD and then LGX 818 + Mek 162	**3**	Unresectable stage IIIc/IV melanoma with BRAF V600E/K mutation	**OS**	**PFS** **ORR**
Arm A Dabrafenib + Trametinib until PD followed by Ipi and Nivo *Arm B* Ipi and Nivo until PD followed by Dabrafenib + Trametinib	**3**	Unresectable stage IIIc/IV melanoma with BRAF V600E/K mutation	**OS**	**PFS** **ORR**
Combination strategy of immunotherapy and targeted therapy in melanoma				
Dabrafenib +/− trametinib +/− ipilimumab (*NCT01767454*)	1	Unresectable stage IIIc/IV melanoma with BRAF V600E/K mutation	● AEs ● MTD	● ORR ● PK
Atezolizumab + vemurafenib +/− cobimetinib (*NCT01656642*)	1b	Unresectable stage IIIc/IV melanoma with BRAF V600E/K mutation	● AEs ● MTD	● PK ● BOR ● OR ● OS ● PFS

(continued)

Table 1 (continued)

Targeted therapy + immune checkpoint inhibitors				
Drugs (*clinical trial*)	Phase	Pathology	Primary endpoint(s)	Secondary endpoint(s)
				● **Ancillary endpoints** (mean Atezolizumab dose, presence of anti-Atezolizumab antibodies)
Dabrafenib +/− trametinib +/− pembrolizumab (*NCT02130466*)	1/2	Unresectable stage IIIc/IV melanoma with BRAF V600E/K mutation	● AEs ● MTD ● PFS	● ORR
Trametinib +/− Dabrafenib +/− MEDI4736 (*NCT02027961*)	1	Unresectable stage IIIc/IV melanoma with BRAF V600E/K mutation	● AEs ● MTD	● Antitumour activity (OR, DR, PFS, OS) **Ancillary endpoints** ● PK of MEDI4736 presence of anti-MEDI4736 antibodies
Ipilimumab + imatinib mesylate (*NCT01738139*)	1	C-KIT positive metastatic or unresectable GIST, melanoma, other tumour histotype	● MTD	–
Vemurafenib + ipilimumab (*NCT01400451*)	1	Stage IV melanoma with BRAF V600E/K mutation	● AEs ● Hepatic DLT ● MTD	–
Vemurafenib + Pembrolizumab (*NCT02818023*)	1	Unresectable stage III/IV melanoma with BRAF V600E/K mutation	● DLT ● ORR*	● PFS ● OS
Ipilimumab +/− Dabrafenib +/− Trametinib +/− Nivolumab (*NCT01940809*)	1	Unresectable stage III/IV melanoma with BRAF V600E/K mutation	● G3 or higher irAEs	● Disease control-rate ● RR

Ipilimumab + dabrafenib (*NCT02200562*)	1/2	Unresectable stage III/IV melanoma with BRAF V600E/K mutation	● AEs ● MTD	–
Dabrafenib + trametinib + pembrolizumab (*NCT02625337*)	2	Stage IV melanoma with BRAF V600E/K mutation	● AEs ● MTD ● Feasibility	● RR ● PFS ● Long-term toxicities
Imatinib + pembrolizumab (*NCT02812693*)	1/2	Unresectable stage III/ IV melanoma with c-KIT mutation/ amplification	● BORR	● Change in PD-1 and PDL-1 expression levels ● Incidence of AEs ● OS ● PFS ● TTP
Targeted therapy + cytokines				
Vemurafenib + IL-2 (*NCT01754376*)	2	Stage IIIc/IV melanoma with BRAF V600E mutation	● PFS	● ORR ● OS ● Toxicity and Safety of IL-2 and Vemurafenib **Ancillary endpoints** ● Biomarkers
Vemurafenib + high dose IL-2 (*NCT01683188*)	4	Stage IV melanoma with BRAF V600E/K mutation	● CR rate	–
Vemurafenib + IL-2 (infusional 96 h) + INF alfa-2b (*NCT01603212*)	1/2	Unresectable stage III/IV melanoma with BRAF V600E/K mutation	● AEs ● MTD ● PFS	–
Vemurafenib + pegylated IFN (*NCT01959633*)	1/2	Unresectable stage III/IV melanoma with BRAF V600E/K mutation	● AEs	● ORR
Vemurafenib + high-dose INF alfa-2b (*NCT01943422*)	1/2	Unresectable stage III/IV melanoma with BRAF V600E/K mutation	● AEs	● PFS ● OS

(continued)

Table 1 (continued)

Targeted therapy + immune checkpoint inhibitors				
Drugs (*clinical trial*)	Phase	Pathology	Primary endpoint(s)	Secondary endpoint(s)
Targeted therapy + T-cell				
Vemurafenib + cyclophosphamide and fludarabine + TIL + high dose IL-2 (*NCT 01585415*)	1	Stage IV melanoma with BRAF V600E/K mutation	● AEs ● MTD	/
Vemurafenib + ACT with TIL infusion + High Dose IL-2 (*NCT01659151*)	2	Unresectable stage III/IV melanoma with BRAF V600E/K/D mutation	● ORR ● Drop Out Rate	● PFS
Vemurafenib + ACT + TIL infusion (*NCT02354690*)	1/2	Unresectable stage III/ IV melanoma with BRAF V600E/K mutation	● AEs MTD	● Immune-related responses ● ORR ● OS ● PFS

DLT dose-limiting toxicity, *ORR* overall response rate, *AEs* adverse events, *SAEs* serious adverse events, *BOR* best overall response, *OS* overall survival, *PFS* progression free survival, *MTD* maximum tolerated dose, *DR* duration of response, *PK* pharmacokinetic, *CR* complete response, *IL-2* interleukin-2, *IFN* interferon, *irAEs* immune-related adverse events, *TTP* time to progression, *OR** overall response, *TIL* tumour infiltrating lymphocytes, *ACT* adoptive cell transfer

arginase are able to inhibit immune responses through the local depletion of amino acids functionally important in lymphocytes, or through the generation of specific ligands for cytosolic receptors that can alter lymphocyte functions. Overall, these new molecules introduce new challenges and difficulties. For example, acute and late toxicity as well as long-term benefit with these new agents is poorly known and combination may be tested without precise and predictive estimates, that are key elements for the design of clinical trials.

4.2 Are We Using the Best Schedule?

Another issue is debate regards treatment schedule. It has been suggested that an intermittent dosing may both delay the development of acquired resistance and postpone clinical disease progression once resistant clones have emerged (Das Thakur et al. 2013). Exploring the role of intermittent schedule of BRAFi and MEKi with immunotherapy introduced during target therapy holidays will allow shedding the light on the best integration between targeted therapy and immunotherapy.

4.3 Study Design According to the Tumour Biology

Biology based investigation has recognized that the adaptive immune resistance is a process through which cancer reactively expresses molecules that actively turn off a potential effective antitumour immune response. The antitumour activity of PD-1 blockade therapy is particularly effective in the subset of T-cell inflamed melanomas that show high expression of PD-L1, IDO, and FOXP3+ Tregs (Tumeh et al. 2014). However, how to target "cold" melanomas with lack of detectable immune reaction (without TILs and with no PD-L1 expression), which represent approximately 41% of patients, is still unknown. In this group of patients, single-agent checkpoint blockade would most likely not to be successful given the lack of pre-existing T cell infiltrates. In this poorly predictable clinical scenario, BRAFi and MEKi could increase MDAs expression and elicit T cell activation, bringing T cells into tumours and then avoiding them being turned off. Recognizing adaptive immune resistance in baseline biopsies may lead to precision immunotherapy.

5 Conclusions

The presence of oncogenic BRAF protein recapitulates several well-known immune suppressive mechanisms, including the inhibition of T cell function, which are common to multiple cancers, with their presence frequently associated with poor patient prognosis. In addition to these established molecular mechanisms of resistance, there is growing evidence that the therapeutic efficacy of BRAFi relies on additional factors involved in tumour–host interactions, including the enhancement of melanoma antigen expression and the increase in immune response against tumour cells,

following antigen release. Based on preclinical and clinical data supporting the immunomodulating activities of targeted therapies, novel combinations of targeted therapies and immunotherapies with the potential to significantly ameliorate the future treatment of MM patients are ongoing and will tell us if the survival bar can be further raised in MM patients.

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Plasticity of Resistance and Sensitivity to Anti-Epidermal Growth Factor Receptor Inhibitors in Metastatic Colorectal Cancer

Andrea Sartore-Bianchi and Salvatore Siena

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Abstract

Colorectal cancer (CRC) is one of the most prevalent cancers and the second leading cause of cancer mortality worldwide. Survival in the metastatic setting has been gradually improved by the addition to cytotoxic chemotherapy of agents targeting the vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR). Considerable heterogeneity exists within CRC due to the varied genetic and epigenetic mechanisms involved in differing pathways of carcinogenesis. The knowledge of molecular abnormalities underlying colorectal tumourigenesis and the progression of dysplastic precursors to invasive and ultimately metastatic lesions has advanced in recent years by comprehensive sequencing studies. From these genome-scale analyses, we know that a handful of genes are commonly affected by somatic mutations,

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whereas recurrent copy-number alterations and chromosomal translocations are rarer in this disease. Even though some of these molecular abnormalities make genes acting as drivers of cancer progression, translation of this recognition for therapeutic purposes is still limited, encompassing only as standard of care the exclusion of *RAS*-mutated cancers for better selecting patients to candidate to EGFR-targeted therapy with monoclonal antibodies. However, the effort of ameliorating molecular selection should not be considered exhausted by demonstration of *RAS* and *BRAF*-induced resistance, as the genomic landscape of response to EGFR blockade has been demonstrated to be wider and dynamically multifaceted. In this chapter we will review main molecular biomarkers of de novo (primary) and acquired (secondary) resistance to EGFR-targeted monoclonal antibodies in metastatic CRC and discuss therapeutic implications.

Keywords
Cetuximab • Colorectal cancer • EGFR • Liquid biopsy • Panitumumab • RAS

1 Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers and the second leading cause of cancer mortality worldwide (Siegel et al. 2017). Survival in the metastatic setting has been gradually improved by the use of fluorouracil/leucovorin in doublet or triplet combinations with oxaliplatin (FOLFOX) and/or irinotecan (FOLFIRI) together with agents targeting the vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) (Ciombor et al. 2015). Nowadays, precision oncology significantly influences current and emerging therapies for metastatic CRC patients by the demonstration that the molecular refinement has led to substantial improvements in clinical outcomes (overall survival, OS and progression-free survival, PFS) (Douillard et al. 2013). This approach, together with advancement in surgical resection for selected patients with limited liver and/or lung involvement, has indeed significantly improved median overall survival to over 40 months from diagnosis (Heinemann et al. 2014; Schwartzberg et al. 2014; Van Cutsem et al. 2011; Venook et al. 2014).

Considerable heterogeneity exists within colorectal tumours due to the varied genetic and epigenetic mechanisms involved in differing pathways of carcinogenesis. The knowledge of molecular abnormalities underlying colorectal tumourigenesis and the progression of dysplastic precursors to invasive and ultimately metastatic lesions has advanced in recent years by comprehensive sequencing studies (Cancer Genome Atlas Network 2012). From these genome-scale analyses, we know that a handful of genes are commonly affected by somatic mutations, whereas recurrent copy-number alterations and chromosomal translocations are rarer in this disease (Vogelstein et al. 2013). Even though some of these molecular abnormalities make genes acting as drivers of cancer progression, translation of this recognition for therapeutic purposes is still limited, encompassing only as standard of care the exclusion of *RAS*-mutated cancers for better selecting patients to candidate to EGFR-targeted therapy with monoclonal antibodies. It should be acknowledged

that the process of refining molecular selection for these therapeutics has paralleled, and in some instances enhanced the quest for targets actionable at the clinical level. In particular, well-known oncogenes such as *BRAF* and *ERBB2*, that are now among most promising targets in this tumour (Corcoran et al. 2014; Sartore-Bianchi et al. 2016b), have been studied as biomarkers of resistance to anti-EGFR therapies. On the other hand, the effort of ameliorating molecular selection should not be considered exhausted by demonstration of *RAS* and *BRAF*-induced resistance, as the genomic landscape of response to EGFR blockade has been demonstrated to be wider (Bertotti et al. 2015) and dynamically multifaceted (Siravegna et al. 2015). In this chapter we will review main molecular biomarkers of de novo (primary) and acquired (secondary) resistance to EGFR-targeted monoclonal antibodies in metastatic CRC and discuss therapeutic implications.

2 Primary Resistance

Primary resistance is defined as ab initio refractoriness to anticancer treatment. It could be explained by resistance-conferring factors pre-existing in the bulk of tumour cells (Leto and Trusolino 2014) that we may not recognize due to tumour heterogeneity (Tannock and Hickman 2016). Intratumour heterogeneity is present early in cancer development and cancer treatment selects for resistant subclones. The main therapeutic implication is that a single drug may not be adequate enough to treat a genetically heterogeneous tumour, since a pre-treatment cancer cell population harbouring resistance genetic alteration, even if present at a low frequency, can contribute to therapeutic failure and poor outcome in a Darwinian fashion (Fisher et al. 2013; Tannock and Hickman 2016; Misale et al. 2014; Sartore-Bianchi et al. 2016a). Among molecular biomarkers of resistance to EGFR-targeted therapies in CRC, alterations in the *RAS/RAF/MAPK* pathway have been the most consistently shown to predict resistance, and *RAS* mutations have been the only reaching clinical grade. The landscape of molecular alterations impacting on sensitivity or resistance to these therapeutics is still interspersed with many other biomarkers, however they should be considered only in the context of translational research.

RAS In the EGFR signalling pathway a dominant downstream direction involves the activation of the G-protein intermediate *RAS*, and subsequent signalling through *BRAF*, *MEK*, and *ERK* (the *MAP* kinase pathway). Mutations in the *RAS* family of proto-oncogenes (*KRAS*, *NRAS*, *HRAS*) result in constitutive activation of *MAP* kinase pathway signalling that is independent of activation of receptor tyrosine kinases such as EGFR. In CRC *KRAS* is the predominantly mutated isoform, whereas *NRAS* mutations are found more rarely and the *HRAS* mutated isoform is extremely uncommon and therefore not tested by routine (Prior et al. 2012). As a consequence, the upstream pharmacological blockade of the receptor can be circumvented for cancer progression by constitutive signalling of activated GTP-bound *RAS* forms (Bardelli and Siena 2010). This discovery by our group and others has been acknowledged as a landmark step for the evolution of precision

medicine in the field of CRC (Benvenuti et al. 2007; Lièvre et al. 2006; Ushijima and Yoshino 2016). The most common *RAS* mutations in colon cancer occur at exon-2 (codons 12 and 13) of *KRAS*, and are present in about 42% of cases (Peeters et al. 2015). It is estimated that among tumours classified as *KRAS* exon 2 wild type, about one out of five carries other mutations in *KRAS* exon 3 (3.8–4.3%), *KRAS* exon 4 (6.2–6.7%), *NRAS* exon 2 (2.9–3.8%), *NRAS* exon 3 (4.2–4.8%) or *NRAS* exon 4 (0.3–0.5%), overall accounting for an additional total 11% of the so-called extended *RAS* or pan-*RAS* mutated CRCs (Peeters et al. 2015; Sorich et al. 2015). Initially, resistance to anti-EGFR mAbs was reported as associated with mutations confined to those occurring in codons 12 and 13 of exon 2 of the *KRAS* gene (Benvenuti et al. 2007; Lièvre et al. 2006). These findings were subsequently confirmed in retrospective analyses of large clinical trials (Amado et al. 2008; Van Cutsem et al. 2011) and reached clinical grade (Schmoll et al. 2012). However, retrospective analyses of multiple trials demonstrated that also additional mutations in exons 2, 3 and 4 of *KRAS*/*NRAS* exerted a similar predictive negative effect (De Roock et al. 2010), and this has been confirmed by analyses of phase III pivotal studies for development of anti-EGFR moAbs (Douillard et al. 2013; Van Cutsem et al. 2015). A meta-analysis of nine randomized controlled trials also confirmed that the treatment with both cetuximab and panitumumab had superior efficacy in terms of PFS and OS for extended *RAS* WT (i.e. *KRAS* exons 3 and 4 and *NRAS* exons 2, 3 and 4) compared with the expanded *RAS* mutant subgroup, and the efficacy was not significantly different between the expanded *RAS* mutant and *KRAS* exon 2 mutant subgroups (Sorich et al. 2015). Based on these studies, the screening of expanded *RAS* mutations for patients with metastatic CRC is currently recommended by principal treatment guidelines and included in the license for panitumumab and cetuximab for metastatic CRC (Van Cutsem et al. 2016) (Fig. 1). However, even though the presence of *RAS* mutations is a prerequisite for anti-EGFR moAbs, efficacy, its absence does not warrant response, as only 40–50% of patients with *RAS* WT tumours achieve objective response to treatment (Misale et al. 2014). Finally, the *KRAS* gene has been found not only to be mutated but also amplified, although in a very small percentage of CRC patients (0.7%), and this amplification has been observed as a mechanism in both primary and acquired resistance to EGFR inhibitors (Valtorta et al. 2013).

BRAF *BRAF* is an oncogene that encodes a downstream effector of *KRAS* in the *MAPK* pathway. In CRC tumours, mutations leading to constitutive *BRAF* activation have been reported in 47% of hyper-mutated tumours and 3% of non-hypermutated tumours (Cancer Genome Atlas Network 2012), and approximately 5–10% of CRC tumours overall (De Roock et al. 2010). Of note, *KRAS* and *BRAF* mutations are mainly mutually exclusive in CRC (Richman et al. 2009). The presence of an activating mutation in *BRAF* conveys a strong prognostic significance, with mutated tumours conferring a poor prognosis with aggressive tumour biology and shorter OS, regardless of the treatment regimen (Safaee Ardekani et al. 2012). It should be noted that this prognostic impact should be regarded as confined to the *BRAF* V600E mutation, whereas *BRAF* mutations affecting codons 594 and

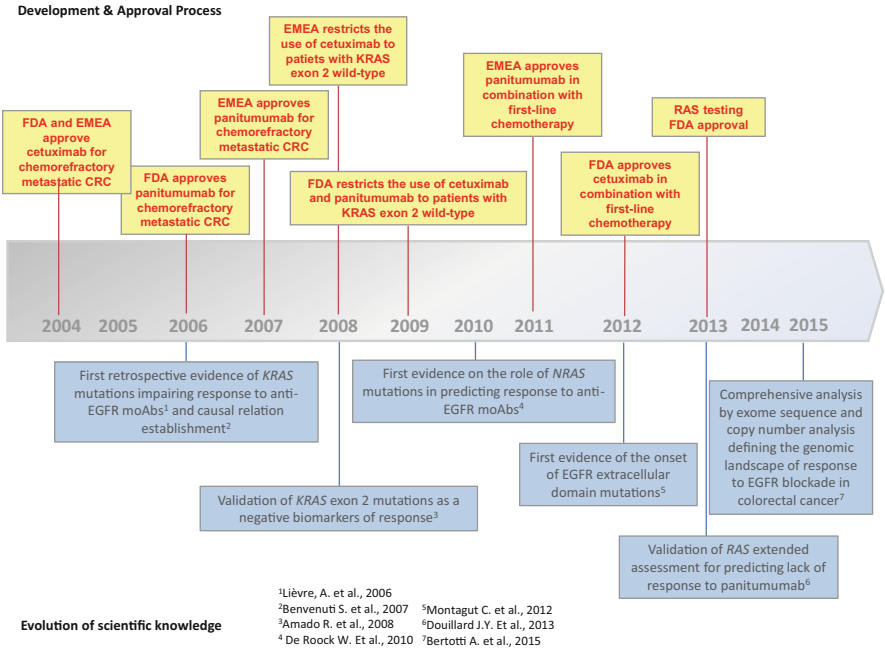

Fig. 1 Parallel development of approval processes and evolution of the knowledge of molecular mechanisms of resistance to EGFR-targeted monoclonal antibodies in metastatic colorectal cancer

596, occurring in <1% of CRCs, display differences in terms of clinicopathologic features and less adverse clinical outcome (Cremolini et al. 2015).

Many different retrospective studies and meta-analyses by our group and others have suggested that the presence of a *BRAF* mutation also confers a weaker benefit from anti-EGFR monoclonal antibodies, negatively interfering with EGFR blockade (Benvenuti et al. 2007; Bertotti et al. 2015; Di Nicolantonio et al. 2008; Yuan et al. 2013). For these patients, even though there is not a formal demonstration by first-line combination trials that cetuximab or panitumumab are not effective (Douillard et al. 2013; Van Cutsem et al. 2015; Rowland et al. 2015), a general consensus exists for using an ab initio more intense chemotherapy regimens or enrolling in clinical studies with *BRAF*-directed strategies (Sartore-Bianchi et al. 2016a) to counteract the poor prognosis, making anti-EGFR treatment a less preferable option.

Family of HER *HER2* – the human epidermal growth factor receptor 2 (*HER2*/neu) is a well-established oncogenic driver in breast and gastric cancer, and recent data are highlighting a renewed role for this molecular target in CRC (Sartore-Bianchi et al. 2016a, c). Expression rates in historical series for CRC range widely from 1.6% (Ingold Heppner et al. 2014) to 47.4% (Park et al. 2007), but the sample

size inclusion of distinct subgroups and the use of different diagnostic methods and scoring systems may account for this variability. In three of the most recent series, the rate of *HER2* positivity (immunohistochemistry [IHC] score of 2+/3+, or *HER2* gene amplification by in-situ hybridization) ranged from 1.6 to 6.3% (Ingold Heppner et al. 2014; Seo et al. 2014; Richman et al. 2016). In a consensus study aimed at defining CRC-specific criteria for *HER2* positivity (Valtorta et al. 2015), we demonstrated that there is a clinically sizeable 5% fraction of *KRAS* wild-type CRC patients harbouring *HER2*-positive tumours, and this knowledge paralleled translational research with demonstration of *HER2* as a therapeutic target (Sartore-Bianchi et al. 2016c).

On the other hand, *HER2* amplification has been proposed as a biomarker of resistance to anti-EGFR antibodies. In 2011, we recognized *HER2* amplification as a potential mechanism of primary resistance to cetuximab within a "quadruple wild type" population (*KRAS*, *NRAS*, *BRAF* and *PIK3CA* wild type) of immune-compromised mice harbouring CRC xenograft (patient-derived xenografts, PDX, a.k.a. "xenopatients") (Bertotti et al. 2011). The same adverse effect was shown by retrospective analyses of patients treated with cetuximab (Yonesaka et al. 2011) or panitumumab (Martin et al. 2013; Sartore-Bianchi et al. 2016c). Interestingly, this effect has been demonstrated to be dependent on the level of amplification (Martin et al. 2013) and to contribute to both de novo and acquired drug resistance (Yonesaka et al. 2011). These results altogether suggest that patients with *HER2*-amplified CRC should be enrolled in clinical trials with *HER2*-targeted therapies regardless of having received a previous anti-EGFR inhibitor (Sartore-Bianchi et al. 2016c).

Together with amplification, also *HER2* somatic mutations have been reported to occur in CRC at a frequency of about 3%, independently or concomitantly with amplification (Cancer Genome Atlas Network 2012). *HER2* activating mutations tend to fall in several hotspots (residues 309–310 in the extracellular domain and residues 755–781 and 842 in the kinase domain), and cause oncogenic transformation of colon epithelial cells (Kavuri et al. 2015). It has been shown that these mutations produce resistance to cetuximab and panitumumab in preclinical models of CRC including PDXs and that dual *HER2*-targeted therapy with either trastuzumab plus neratinib or trastuzumab plus lapatinib can induce tumour regression (Kavuri et al. 2015).

HER3 also has been described to have a role as a potential biomarker of resistance to anti-EGFR treatments. In a cohort of metastatic CRC patients treated in second- or third-line therapy with irinotecan and cetuximab, *HER3* overexpression was associated with shorter PFS and OS (Scartozzi et al. 2011). *HER3* has been found to be also mutated in approximately 11% of CRC patients (Jaiswal et al. 2013), even though further studies are needed to elucidate the predictive role in conferring primary or acquired resistance to EGFR inhibitors.

cMET The mesenchymal-epithelial transition (*MET*) protooncogene encodes for *c-MET*, a receptor with tyrosine kinase activity targeting hepatocyte growth factor (HGF) (Trusolino et al. 2010). Activation of this pathway by gene amplification has

been implicated in metastatic progression of CRC, since *MET* has been historically reported to be overexpressed in 50% and amplified in 2–10% of primary CRCs, with the rate of amplification increasing up to 18–89% in metastases (Di Renzo et al. 1995; Zeng et al. 2008). However, more recent studies adopting ISH technologies together with newer NGS approaches clearly indicate that *MET* amplification ranges between 0.4 and 2.2% of cases (Cancer Genome Atlas Network 2012; Raghav et al. 2016), in both primary tumours and metastatic deposits (Raghav et al. 2016). As far as resistance to anti-EGFR therapies is concerned, in a recent complete exome sequence and copy number analyses of 129 PDX and targeted genomic analyses of 55 patient tumours, *MET* amplification was identified as a cause of primary resistance to cetuximab in 2.3% of cases (Bertotti et al. 2015). All in all, *MET* amplification rarely occurs and accounts for primary resistance in CRC, even though it is among alterations predominantly involved in acquired resistance (see below section on acquired resistance).

3 Biomarkers of Sensitivity

EGFR Gene Copy Number Gain During the initial development of EGFR antibodies in metastatic CRC, it was predicted that EGFR protein expression would be required for therapeutic efficacy. Therefore, initially only EGFR-expressing CRCs were allowed into clinical trials (Saltz et al. 2004; Cunningham et al. 2004). However, subsequent analyses demonstrated a lack of association between EGFR expression and response (Chung et al. 2005; Moroni et al. 2008), eventually leading to decline of this restriction in label. On the other hand, an association was found with an *EGFR* gene copy number increase (Moroni et al. 2005) and this was originally postulated by our group as a biomarker of sensitivity to anti-EGFR therapy (Moroni et al. 2005). This finding was confirmed in preclinical experiments (Bertotti et al. 2015) as well as clinical cohorts (Personeni et al. 2008; Sartore-Bianchi et al. 2007; Scartozzi et al. 2009), however overt EGFR gene amplification is rarely observed in CRC, and correlation with response has been mainly based on balanced chromosome 7 polysomy rather than amplification, even though it is unknown whether the former could have an equivalent biologic effect in driving cancer progression and predicting response to EGFR-targeted agents. Also, detection by ISH methods of copy number gain suffers from issues of standardization, as it was shown by an interlaboratory reproducibility ring study conducted by our group (Sartore-Bianchi et al. 2012). In the end, even though an association between an increase in EGFR gene dosage and response has been established and confirmed especially in case of amplification (Bertotti et al. 2015), still this is not a validated biomarker for EGFR-targeted agents.

IRS2 The insulin receptor substrate (IRS) family of proteins are cytoplasmic adaptor that mediate through phosphorylation signalling between receptor tyrosine kinases of IGF1R and downstream effectors with roles in normal growth, metabolism and differentiation such as PI3K activation. IRS2 over-expression has been

reported in 6–7% of MSS CRCs (Cancer Genome Atlas Network 2012; Nunes et al. 2015). A preclinical study has shown that IRS2 over-expression in the absence of an upstream activator leads to AKT phosphorylation and also increases CRC cell adhesion (Day et al. 2013). Amplifications and sequence alterations in the tyrosine kinase receptor adaptor gene IRS2 have been identified also in tumours with increased sensitivity to anti-EGFR therapy. Expression analyses of 100 CRC PDXs with wild-type *KRAS*, *NRAS*, *BRAF* and *PIK3CA* showed increased IRS2 levels as a significant predictor of cetuximab sensitivity in cases without other mechanisms of resistance to EGFR therapy (Bertotti et al. 2015).

4 Acquired (Secondary) Resistance

Acquired (or secondary) resistance refers to disease progression during an ongoing treatment that was initially effective. This occurs eventually in all metastatic CRCs and can be caused by gene mutations of the molecular target arising during treatment, expansion of resistant subclones in the context of intratumour heterogeneity selected under the pressure of cancer treatment, upregulation of a partially inhibited pathway or activation of alternative pathways. Even in patients with a refined *RAS* extended wild-type status, the tumour becomes refractory after a median of 5.2 months by developing secondary resistance (Kim et al. 2016). Liquid biopsy for monitoring of circulating tumour DNA (ctDNA) has been demonstrated by our group and others to be a powerful diagnostic tool for understanding dynamic mechanisms of tumour evolution in CRC (Misale et al. 2014; Van Emburgh et al. 2014). Molecular analysis performed on a tissue biopsy from a tumour is indeed a single snapshot in time subjected to selection bias due to spatial tumour heterogeneity, whereas analyses performed by liquid biopsy overcome this limitation and unveiled main mechanisms of acquired resistance to EGFR inhibitors.

RAS and BRAF Various studies from our group and others have already demonstrated concordance between liquid biopsy and tumour-tissue biopsy for molecular characterization of clinically validated biomarkers for CRC such as *KRAS* and *BRAF* mutations (Thierry et al. 2014; Siravegna et al. 2015). At the same time, experiments in preclinical models (Misale et al. 2012) and translational studies with longitudinal monitoring by liquid biopsy have revealed clonal evolution during therapies with anti-EGFR antibodies showing that mutant *RAS* clones rise in blood during EGFR blockade (Misale et al. 2012) and decline upon withdrawal of treatment (Siravegna et al. 2015). It is conceivable that these subclones are less fit in the untreated tumour and acquire fitness as a consequence of adaptation to the perturbation induced by the treatment itself. Further, anti-EGFR pressure gives rise to multiple emergent circulating mutations of *MAPK* pathway within the same patient, with and individual average of almost three mutations (Bettegowda et al. 2014), in what has been called a “war of clones”. Interestingly, the relative frequency of individual *KRAS* alleles is similar but not identical in primary and acquired resistance: we firstly reported the secondary occurrence of codon

61 mutations (Misale et al. 2012) that rarely occur in anti-EGFR naïve patients, and now it is established that these mutations in either the *KRAS* or *NRAS* genes are more prevalent in the acquired than in the primary resistance setting (Bettegowda et al. 2014).

Family of HER *HER2* amplification has been associated also to acquired resistance to anti-EGFR moAbs. Yonesaka et al. showed that patients with acquired resistance to cetuximab had an increased percentage of *HER2* amplification in post-treatment samples compared to the proportion present in pretreatment tumour cells (Yonesaka et al. 2011). These authors reported that in this context hyper-activation of *HER2* signalling is triggered not only by *HER2* amplification but also by overproduction of heregulin, a *HER3* ligand.

MET HGF-induced *MET* activation has been proposed as a mechanism of cetuximab resistance in CRC (Liska et al. 2011). We firstly reported by tissue analysis and longitudinal ctDNA monitoring that *MET* amplification is also associated with secondary resistance to anti-EGFR monoclonal antibodies (Bardelli et al. 2013). This observation was paralleled by functional analysis in CRC preclinical models indicating that HGF plays an important role in driving *MET*-mediated resistance to anti-EGFR monoclonal antibodies. HGF stimulation was demonstrated to be sufficient to confer cetuximab and panitumumab resistance both in vitro and in vivo, thus supporting the possibility that HGF overexpression by cancer cells or the surrounding stroma might be an independent mechanism of acquired (or primary) resistance to cetuximab (Bardelli et al. 2013). Further, we recently showed that *MET* amplification can simultaneously arise together with *KRAS* amplification within the same patient after initial response to EGFR inhibition in a context of substantial intrapatient heterogeneity (Sartore-Bianchi et al. 2016d).

EGFR External Domain Mutations Mutations affecting the extracellular domain of the EGFR have not been reported in the absence of treatment with EGFR inhibitors in metastatic CRC (Esposito et al. 2013; Montagut et al. 2012). In 2012 it was firstly demonstrated that that cell lines with acquired resistance to cetuximab showed a mutation of the extracellular domain of the EGFR, 1476C>A, leading to a substitution of serine to arginine at amino acid 492 (S492R) (Montagut et al. 2012). This mutation interferes with binding to cetuximab but not to panitumumab in preclinical models and parallel observations in patients with acquired resistance to cetuximab. These findings have been confirmed subsequently in retrospective cohorts (Arena et al. 2015) and at the clinical level in the ASPECCT trial of cetuximab versus panitumumab for chemorefractory metastatic CRC, where the EGFR S492R was detected in 1% of patients in the panitumumab arm and 16% in the cetuximab arm in post-treatment plasma ctDNA samples (Newhall et al. 2014). It has been subsequently discovered that several other mutations in the EGFR extracellular domain can occur in preclinical models of cetuximab resistance (S464L, G465R and I491M) and in patients (R451C and K467T), mainly located in the cetuximab-binding region, except for the R451C mutant (Arena et al. 2015).

From a clinical standpoint there are important therapeutic implications, since *EGFR* ectodomain mutations prevent binding to cetuximab but a subset is permissive for interaction with panitumumab (Arena et al. 2015), and it has been shown that new generation EGFR inhibitors such as the anti-EGFR antibody mixture Sym004 that bound and abrogated ligand-induced phosphorylation of EGFR mutants can overcome cetuximab/panitumumab resistance mediated by EGFR mutations (Sánchez-Martín et al. 2016). Interestingly, emerging knowledge is also indicating that there is a differential kinetic in the appearance of *EGFR* versus *RAS* mutated alleles during EGFR-targeted treatment, since *RAS* mutations emerge earlier than *EGFR* ECD variants (Van Emburgh et al. 2014). Subclonal *RAS*, but not *EGFR* extracellular domain mutations, have been shown indeed to be present in CRC samples obtained before exposure to EGFR blockade. Finally, retrospective analysis in a clinical cohort indicates that patients who experience greater and longer responses to EGFR blockade preferentially develop EGFR extracellular domain mutations, while *RAS* mutations emerge more frequently in patients with smaller tumour shrinkage and shorter progression-free survival (Van Emburgh et al. 2016).

5 Conclusions

Approved anti-EGFR antibodies cetuximab and panitumumab provide significant clinical benefit for the treatment of CRC, with patients in the metastatic setting now reaching an OS of more than 30 months. These advances have been made thanks to evolution of surgical techniques for metastasectomy, introduction of new agents, but to an important extent also to a refinement of molecular selection based on newer pharmacogenomics strategies. In this regard, the discovery of mechanisms of resistance to anti-EGFR monoclonal antibodies has enhanced clinical results, paving the way for a precision medicine approach in this disease. This effort should not be considered exhausted, as the genomic landscape of response to EGFR blockade has been demonstrated to be starred by a myriad of molecular abnormalities and, thanks to application of liquid biopsy for longitudinal analysis of the instable tumour genome, dynamically multifaceted (Fig. 2). Next challenges in this field will include the translation of current knowledge of tumour evolution mechanisms in the clinic for preventing or overcoming resistance at the individual patient level.

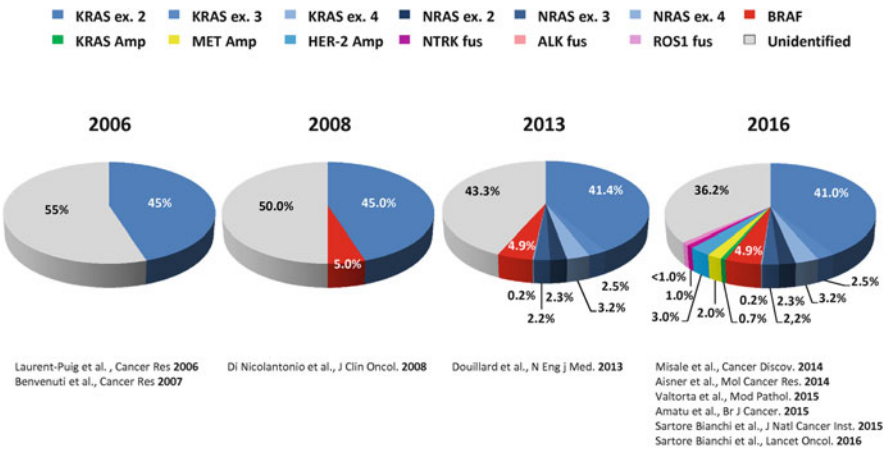

Fig. 2 Biomarkers of resistance to EGFR-targeted monoclonal antibodies and known actionable targets for therapy in metastatic colorectal cancer. Evolution from 2006 until today

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Targeting DNA Repair

Giuseppe Curigliano

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Abstract

Genomic instability is a characteristic of most human cancers and plays critical roles in both cancer development and progression. There are various forms of genomic instability arising from many different pathways, such as DNA damage from endogenous and exogenous sources, centrosome amplification, telomere damage, and epigenetic modifications. DNA-repair pathways can enable tumor cells to survive DNA damage. The failure to respond to DNA damage is a characteristic associated with genomic instability. Understanding of genomic instability in cancer is still very limited, but the further understanding of the molecular mechanisms through which the DNA damage response (DDR) operates, in combination with the elucidation of the genetic interactions between DDR pathways

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and other cell pathways, will provide therapeutic opportunities for the personalized medicine of cancer.

Keywords
Cancer • DNA damage • Instability • Resistance

1 Introduction

Genomic instability is a characteristic of most human cancers and plays critical roles in both cancer development and progression.

Genomic stability is dependent on faithful DNA repair and chromosome segregation during cell division (Ferguson et al. 2015).

To maintain genomic integrity, eukaryotes have evolved a system called the DNA damage response (DDR). DDR is a complex signal transduction pathway that allows cells to sense DNA damage and transduce this information to the cell to arrange the appropriate cellular responses to DNA damage (Lee et al. 2015; Ciccia and Elledge 2010). The failure to respond to DNA damage is a characteristic associated with genomic instability. This instability can manifest itself genetically on several different levels, ranging from simple DNA sequence changes to structural and numerical abnormalities at the chromosomal level. During S phase, the centrosome and genomic material are replicated concurrently, and replication errors are repaired prior to mitotic entry. During mitosis, equal segregation of chromosomes requires a bipolar mitotic spindle, telomeric preservation, and completion of the spindle assembly checkpoint. Ectopic amplification of centrosomes, telomerase dysfunction, and failure of the spindle assembly checkpoint may result in aborted mitosis. The majority of cancers exhibits chromosomal instability (CIN), which refers to the high rate by which chromosome structure and number changes over time in cancer cells compared with normal cells (Negrini et al. 2010). Although CIN is the major form of genomic instability in human cancers, other forms of genomic instability have also been described. These include accumulation of DNA base mutations and microsatellite instability (MSI), a form of genomic instability that is characterized by the expansion or contraction of the number of oligonucleotide repeats present in microsatellite sequences (Negrini et al. 2010; Lengauer et al. 1997; Fishel et al. 1993), and forms of genomic instability that are characterized by increased frequencies of base pair mutations (Leach et al. 1993).

2 Hereditary Versus Sporadic Cancers

Familial breast cancer (BC) accounts for approximately 5%–10% of BC cases. The most prevalent mutations leading to hereditary breast and ovarian cancer affect the homologous recombination (HR) genes BRCA1 and BRCA2. Heterozygous individuals carrying mutations of the BRCA1 or BRCA2 genes have a 40%–80% risk of developing BC (Fackenthal and Olopade 2007).

Patients (pts) with BRCA2 mutations have increased incidence of male breast, pancreas, and prostate cancer (Ciccia and Elledge 2010). Tumors with BRCA1 or BRCA2 mutations are significantly associated with low level of 53BP1, indicating that 53BP1 mutation might confer a survival advantage in the absence of BRCA1 and BRCA2 (Bouwman et al. 2010). Moreover, mutations in three additional HR genes, BACH1, PALB2, and RAD51C, have been identified in approximately 3% of familial BC pts and have been associated with a twofold increased risk of BC (Levy-Lahad 2010). Mutations of CHK2, ATM, NBS1, and RAD50 have also been associated with a doubled risk of BC, indicating the importance of the ATM pathway, together with HR, in preventing BC formation. In hereditary cancers that are characterized by the presence of CIN, the genomic instability can also be attributed to mutations in DNA-repair genes. The identification of mutations in DNA-repair genes in hereditary cancers provides strong support for the *mutator hypothesis*, which states that genomic instability is present in precancerous lesions and drives tumor initiation by increasing the spontaneous mutation rate (Negrini et al. 2010; Nowell 1976; Loeb 1991). According to mutator hypothesis, the genomic instability in precancerous lesions results from mutations in caretaker genes; that is, genes that primarily function to maintain genomic stability (Negrini et al. 2010; Nowell 1976; Loeb 1991). Indeed, in inherited cancers, germline mutations targeting DNA-repair genes are present in every cell of the patient's body. Thus, a single event – loss of the remaining wild-type allele – would lead to genomic instability and drive tumor development, as predicted by the mutator hypothesis. The classical caretaker genes are DNA-repair genes and mitotic checkpoint genes (Negrini et al. 2010). Germline mutations in caretaker genes can explain the presence of genomic instability in inherited cancers. However, efforts to identify caretaker genes, the inactivation of which leads to genomic instability in sporadic (nonhereditary) cancers, have met with limited success (Negrini et al. 2010; Rajagopalan and Lengauer 2004). Thus, unlike hereditary cancers, the molecular basis of genomic instability in sporadic cancers remains unclear. A second hypothesis could explain the presence of CIN in sporadic cancers. That is, *the oncogene induced DNA replication stress model for cancer development* (Halazonetis et al. 2008; Gorgoulis et al. 2005; Bartkova et al. 2005, 2006; Di Micco et al. 2006). According to the second model, CIN in sporadic cancers results from the oncogene induced collapse of DNA replication forks, which in turn leads to DNA double-strand breaks (DSBs) and genomic instability (Negrini et al. 2010).

3 Cellular Mechanisms that Prevent or Promote Genomic Instability

3.1 Telomere Damage

Telomeres, which are located at the ends of each chromosome, consist of approximately 5–10 kbp of specialized, tandem repeat, noncoding DNA complexed with a variety of telomere associated proteins (Ferguson et al. 2015; Blackburn 2000; Greider 1991). These elements create a protective cap that prevents the recognition

of the chromosomal termini as DSBs and their consequent aberrant repair via non-homologous end joining (NHEJ) or HR (Ferguson et al. 2015; Konishi and de Lange 2008; Karlseder et al. 2004; Hockemeyer et al. 2005; de Lange 2010). Due to the inability of DNA polymerase to fully replicate the ends of linear DNA molecules, in the absence of compensatory mechanisms, telomeric DNA is lost at the rate of approximately 100 base pairs (bp) per telomere per cell division (Ferguson et al. 2015; Harley 1991; Levy et al. 1992; Aubert and Lansdorp 2008). In normal somatic cells, this telomere erosion is used by the cell to monitor its division history, with moderate telomere shortening triggering either irreversible cell-cycle arrest, termed replicative senescence, or apoptosis (Ferguson et al. 2015). This block to continued proliferation is thought to have evolved to prevent the development of cancer in long-lived organisms by restricting the uncontrolled outgrowth of transformed cell clones, and also by preventing further telomere erosion which would accompany such abnormal growth and eventually destabilize the telomeres leading to CIN (Ferguson et al. 2015; Harley 1991; Harley and Sherwood 1997).

3.2 Centrosomes

Centrosome amplification, the presence of greater than two centrosomes during mitosis, is a common characteristic of most solid and hematological tumors that may induce multipolar mitoses, chromosome missegregation, and subsequent genetic imbalances that promote tumorigenesis (Ferguson et al. 2015; Nigg 2002).

The centrosome is the primary microtubule organizing center in dividing mammalian cells (Ferguson et al. 2015). The centrosome is duplicated in a semiconservative fashion with one daughter centriole formed next to a preexisting mother centriole, and this process only occurs once in every cell cycle (Ferguson et al. 2015; Nigg and Stearns 2011; Doxsey 2001).

Centrosome amplification arises from many different mechanisms, including centrosome over duplication (Ferguson et al. 2015; Doxsey 2001; Ko et al. 2005), de novo assembly (Ferguson et al. 2015; Khodjakov 2002), and mitotic failure downstream from mono- (Glover et al. 1995) or multipolar division (Maxwell et al. 2005). Given that centrosome clustering may be advantageous for cancer cell survival, this process may be an attractive and specific therapeutic target (Ogden et al. 2012; Gergely and Basto 2008; Marthien et al. 2012). Bipolar chromosome attachment during mitosis is ensured by a quality control mechanism known as the spindle assembly checkpoint (Ferguson et al. 2015). The assembly checkpoint relies upon kinase signaling to delay cell-cycle progression and correct attachment errors. Aurora kinase B, for example, detects misattached chromosomes (Ferguson et al. 2015) and overexpression of the kinase is sufficient to disrupt the checkpoint and promote tetraploidy (Ferguson et al. 2015). Moreover, mutations or expression changes in other checkpoint gene products may compromise the checkpoint and favor tumorigenesis (Fang and Zhang 2011).

3.3 DNA Methylation and Chromatin Remodeling

A vast array of epigenetic mechanisms contribute to the genomic instability in cancer cells (Sharma et al. 2010). One of them is the DNA methylation, which consists of the addition of a methyl group at the carbon 5 position of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring (Cedar and Bergman 2009). Most cytosine methylation occurs in the context of cytosine-phosphate-guanine (CpG) dinucleotides, and occurs via a group of DNA methyl-transferase enzymes resulting in silencing of gene transcription (Ferguson et al. 2015). A prominent example is the aberrant methylation of CpG islands in the promoter regions of DNA mismatch repair (MMR) genes that result in cancer cells with a "mutator phenotype" (Ferguson et al. 2015; Hitchins 2010). In addition to DNA methylation, histone molecules that form the primary protein component of chromatin also regulate genome stability as well as gene transcription (Sproul et al. 2005). A number of posttranslational modifications such as acetylation, deacetylation, methylation, phosphorylation, and ubiquitination have been identified that alter the function of histones (Ferguson et al. 2015). Various combinations of these posttranslational histone modifications have been hypothesized to form a "histone code" that dictates distinct chromatin structures that can affect genome stability pathways and transcription (Ferguson et al. 2015). Therefore, in most cases, histone acetylation enhances transcription while histone deacetylation represses transcription. In addition, histone acetylation can affect DNA repair. Similarly, histone ubiquitination can also modify DNA-repair capacity (Ferguson et al. 2015; Mailand et al. 2007). Finally, histone phosphorylation is an early event following DNA damage and required for efficient DNA repair (Ferguson et al. 2015).

3.4 Mitochondrial DNA Alteration in Human Cancers

Mitochondria are the key component of the oxidative phosphorylation system to generate cellular adenosine triphosphate. Mitochondrial genetic reprogramming and energy balance within cancer cells play a pivotal role in tumorigenesis (Ferguson et al. 2015). Most human cells contain hundreds of nearly identical copies of mt-DNA, which are maternally inherited. A substantial number of studies identified somatic mt-DNA mutations involving coding and noncoding mt-DNA regions in various cancers (Ferguson et al. 2015).

4 DNA-Repair Pathways

Repeated exposure to both exogenous and endogenous insults challenges the integrity of cellular genomic material. To maintain genomic integrity, DNA must be protected from damage induced by environmental agents or generated spontaneously during DNA metabolism.

Environmental DNA damage can be produced by physical or chemical sources. For example, the ultraviolet (UV) component of sunlight can cause up to 1×105 DNA lesions per cell per day, many of which are pyrimidine dimers. If left unrepaired, dimers that contain cytosine residues are prone to deamination, which can ultimately result in cytosine being replaced with thymine in the DNA sequence. Likewise, ionizing radiation (for example, from sunlight or cosmic radiation) can cause single-strand breaks (SSBs) and DSBs in the DNA double helix backbone. If misrepaired – for example, the inaccurate rejoining of broken DNA ends at DSBs, these breaks can induce mutations and lead to widespread structural rearrangement of the genome (Lord and Ashworth 2012). Table 1 (Lindahl and Barnes 2000; Hoeijmakers 2009) showed environmental agents that cause DNA damage and mutations.

Spontaneous DNA alterations can be due to dNTP misincorporation during DNA replication, interconversion between DNA bases caused by deamination, loss of DNA bases following DNA depurination, and modification of DNA bases by alkylation. Additionally, DNA breaks and oxidized DNA bases can be generated by reactive oxygen species (ROS) derived from normal cellular metabolism.

Organisms respond to chromosomal insults by activating a complex damage response pathway. This pathway regulates known responses such as cell-cycle arrest and apoptosis (programmed cell death), and has been shown to control additional processes including direct activation of DNA-repair mechanisms. Most of the subtle changes to DNA, such as oxidative lesions, alkylation products, and SSBs, are repaired through a series of mechanisms that are termed base excision repair (BER). In BER, damaged bases are first removed from the double helix, and the "injured" section of the DNA backbone is then excised and replaced with newly synthesized

Table 1 DNA lesions generated by endogenous and exogenous DNA damage (Ciccia and Elledge 2010)

Exogenous DNA damage	Dose exposure (mSV)	DNA lesions generated
Peak hour sunlight	–	Pyrimidine dimers (6–4) photoproducts
Cigarette smoke	–	DSBs
Chest X-ray	0.02	DSBs
Mammography	0.4	DSBs
Body CT scan	7	DSBs
Tumor PET scan	10	DSBs
Airline travel	0.005/h	DSBs
Endogenous DNA damage	**Dose lesions generated**	**Number of lesions/cell/day**
Depurination	AP site	10,000
Cytosine deamination	Base transition	100–500 s
SAM-induced methylation	3meA	600
	7meA	4,000
	O^6 meG	10–30
Oxidation	8oxoG	400–1,500

DNA (David et al. 2007). Key to this process are members of the poly(ADP-ribose) polymerase (PARP) family. The PARP family has 16 members, but only PARP1 and PARP2 have been implicated in the DDR (Schreiber et al. 2006). PARP1 and PARP2 are activated by SSBs and DSBs and catalyze the addition of poly (ADP-ribose) chains on proteins to recruit DDR factors to chromatin at breaks (Ciccia and Elledge 2010). Mispaired DNA bases are replaced with correct bases by MMR (Jirincy 2006). In addition to BER, the pool of deoxynucleotides (deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and deoxycytidine triphosphate (dCTP)) that provide the building blocks of DNA can be chemically modified before they are incorporated into the double helix. The nucleotide pool is, therefore, continually "sanitized" by enzymes such as nudix-type motif 5 (NUDT5). Whereas small base adducts are repaired by BER, some of the bulkier single-strand lesions that distort the DNA helical structure, such as those caused by ultraviolet light, are processed by nucleotide excision repair (NER) through the removal of an oligonucleotide of approximately 30 bp containing the damaged bases. NER is often subclassified into transcription-coupled NER, which occurs where the lesion blocks, and is detected by elongating RNA polymerase, and global-genome NER, in which the lesion is detected not as part of a blocked transcription process but because it disrupts base pairing and distorts the DNA helix. Although these processes detect lesions using different mechanisms, they repair them in a similar way: DNA surrounding the lesion is excised and then replaced using the normal DNA replication machinery. Excision repair cross-complementing protein 1 (ERCC1) is the key to this excision step. The major mechanisms that cope with DSBs are HR (Moynahan and Jasin 2010) and NHEJ (Lieber 2010). HR acts mainly in the S and G2 phases of the cell cycle and is a conservative process in that it tends to restore the original DNA sequence to the site of damage. Part of the DNA sequence around the DSB is removed (known as resection) and the DNA sequence on a homologous sister chromatid is used as a template for the synthesis of new DNA at the DSB site. Crucial proteins involved in mediating HR include those encoded by the BRCA1, BRCA2, RAD51, and PALB2 genes. In contrast to HR, NHEJ occurs throughout the cell cycle. Rather than using a homologous DNA sequence to guide DNA repair, NHEJ mediates repair by directly ligating the ends of a DSB together. Sometimes this process can cause the deletion or mutation of DNA sequences at or around the DSB site. Therefore, compared with HR, NHEJ, although mechanistically simpler, can often be mutagenic.

SSBs are repaired by single-strand break repair (SSBR), whereas DSBs are processed either by NHEJ or by HR (Ciccia and Elledge 2010). DNA repair is carried out by a plethora of enzymatic activities that chemically modify DNA to repair DNA damage, including nucleases, helicases, polymerases, topoisomerases, recombinases, ligases, glycosylases, demethylases, kinases, and phosphatases.

In summary, DDR can be divided into a series of distinct, but functionally interwoven, pathways, which are defined largely by the type of DNA lesion they process (Fig. 1). DDR pathways encompass a similar set of tightly coordinated processes: namely, the detection of DNA damage, the accumulation of DNA-repair factors at the site of damage, and finally the physical repair of the lesion.

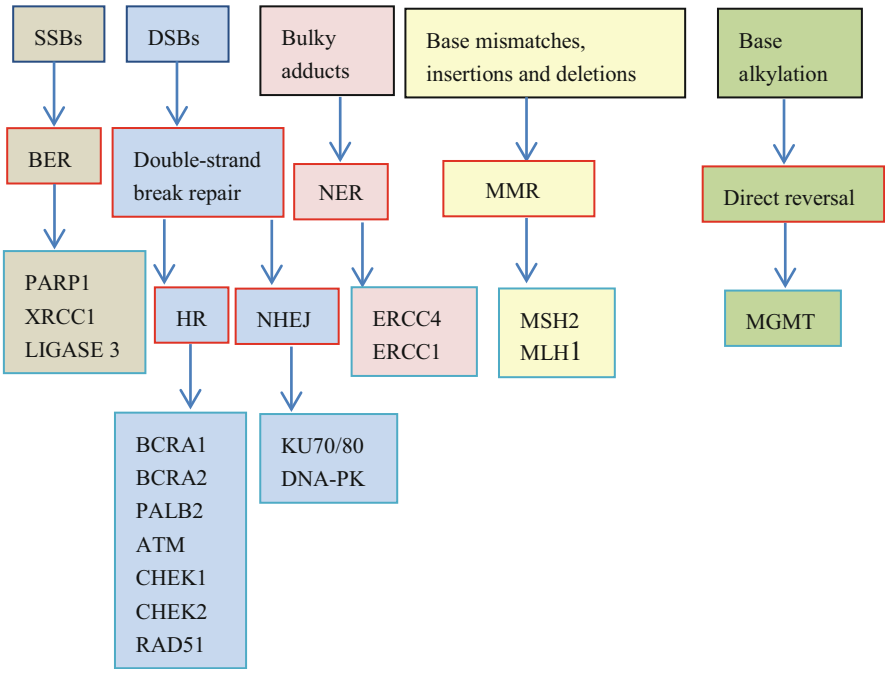

Fig. 1 DNA-repair mechanisms maintain genomic stability. *SSBs* single-strand breaks, *DSBs* double-strand breaks, *HR* homologous recombination, *NHEJ* nonhomologous and joining, *MMR* mismatch repair

MMR (Jirincy 2006) is crucial to the DDR. Key to the process of MMR are proteins encoded by the mutS and mutL homologue genes, such as MSH2 and MLH1.

Finally, translesion synthesis and template switching allow DNA to continue to replicate in the presence of DNA lesions that would otherwise halt the process. Translesion synthesis and template switching are therefore usually considered to be part of the DDR. In translesion synthesis, relatively high-fidelity DNA replication polymerases are transiently replaced with low-fidelity "translesion" polymerases that are able to synthesize DNA using a template strand encompassing a DNA lesion. Once the replication fork passes the site of the lesion, the low-fidelity DNA polymerases are normally replaced with the usual high-fidelity enzyme, which allows DNA synthesis to continue as normal. In template switching, the DNA lesion is bypassed at the replication fork by simply leaving a gap in DNA synthesis opposite the lesion. After the lesion has passed the replication fork, the single-strand gap is repaired using template DNA on a sister chromatid, similar to the process used during HR.

Although sometimes considered distinct from the DDR, the mechanisms that control the integrity of telomeric DNA at the end of each human chromosome also act as a barrier against genomic instability and mutation (Artandi and DePinho 2010).

The core DDR machinery does not work alone but is coordinated with a set of complementary mechanisms that are also crucial to maintaining the integrity of the genome. For example, chromatin-remodeling proteins allow the DNA-repair apparatus to gain access to the damaged DNA (Bell et al. 2011). DDR core components interact with the cell-cycle checkpoint and chromosome-segregation machinery. These interactions allow DNA repair to occur before mitosis takes place and ensure that the correct complement of genetic material is passed on to daughter cells (Warmerdam and Kanaar 2010).

5 Therapeutic Targeting of Genomic Instability in BC

When as CIN, and as changes to the structure of DNA, such as nucleotide substitutions, insertions, and deletions they occur in crucial "driver" genes (of which there are probably fewer than ten per tumor), these mutations can alter cell behavior, confer a selective advantage, and drive the development of the disease. Importantly, these mutations can also influence how the tumor will respond to therapy. Alongside key driver mutations, emerging data from cancer genome sequencing suggests that a typical tumor may contain many thousands of other genetic changes. These "passenger" mutations do not contribute directly to the disease but are probably collateral damage from exposure to various environmental factors or defects in the molecular mechanisms that maintain the integrity of the genome. DNA damage causes cell-cycle arrest and cell death either directly or following DNA replication during the S phase of the cell cycle. Cellular attempts to replicate damaged DNA can cause increased cell killing, thus making DNA-damaging treatments more toxic to replicating cells than to nonreplicating cells. However, the toxicity of DNA-damaging drugs can be reduced by the activities of several DNA-repair pathways that remove lesions before they become toxic. The efficacy of DNA damage-based cancer therapy can thus be modulated by DNA-repair pathways. In addition, some of these pathways are inactivated in some cancer types. These two features make DNA-repair mechanisms a promising target for novel cancer treatments. Increasing knowledge of DNA repair permits rational combination of cytotoxic agents and inhibitors of DNA repair to enhance tumor-cell killing. Thus, DNA-repair inhibitors can be used in combination with a DNA-damaging anticancer agent. This will increase the efficiency of the cancer treatment by inhibiting DNA repair-mediated removal of toxic DNA lesions.

Moreover, DNA-repair inhibitors can be used as monotherapy to selectively kill cancer cells with a defect in the DDR or DNA repair. Synthetic lethal interactions between a tumor defect and DNA-repair pathway can be used to identify novel treatment strategies.

High levels of DNA damage cause cell-cycle arrest and cell death. Furthermore, DNA lesions that persist into the S phase of the cell cycle can obstruct replication fork progression, resulting in the formation of replication-associated DSBs. Evidence is also building that the DDR is not only invoked but also dysfunctional at an early stage in the development of neoplasia. Markers of DSBs, such as nuclear γH2AX foci (a histone phosphorylation event that occurs on chromatin surrounding

a DSB), are markedly elevated in some precancerous lesions (Halazonetis et al. 2008; Bartkova et al. 2006). The activation of oncogenes such as MYC and RAS stimulates the firing of multiple replication forks as part of a proliferative program. These forks rapidly stall, collapse, and form DSBs because they exhaust the available dNTP pool or because multiple forks collide on the same chromosome. Regardless of the mechanism, stalled and collapsed forks normally invoke the DDR and cell-cycle checkpoints that enable DNA lesions to be repaired before mitosis takes place. For precancerous lesions to progress to mature tumors, it is thought that critical DSB signal transduction and cell-cycle checkpoint proteins, such as ataxia telangiectasia (ATM) and ATM-Rad3 related (ATR), and the master "gatekeeper" protein p53 become inactivated. With these DDR components rendered dysfunctional, collapsed forks are not effectively repaired, and cells proceed through the cell cycle with DNA lesions intact, increasing the chance of mutagenesis (Halazonetis et al. 2008; Bartkova et al. 2006).

Common types of DNA damage that interfere with replication fork progression are chemical modifications (adducts) of DNA bases, which are created by reactive drugs that covalently bind DNA either directly or after being metabolized in the body. These ***alkylating agents*** are grouped into two categories: *monofunctional alkylating agents* with one active moiety that modifies single bases and *bifunctional alkylating agents* that have two reactive sites and crosslink DNA with proteins or, alternatively, crosslink two DNA bases within the same DNA strand (intrastrand crosslinks) or on opposite DNA strands (interstrand crosslinks). Interstrand crosslinks pose a severe block to replication forks.

Despite the adverse side effects caused by alkylating agents on bone marrow and other normal tissues, drugs such as cyclophosphamide, ifosfamide, chlorambucil, melphalan, and dacarbazine remain some of the most commonly prescribed chemotherapies in adults and children with various solid and hematological malignancies, particularly in combination with anthracyclines and steroids in multi-agent regimens. The repair of alkylated lesions is thought to be quick, with the majority of lesions probably being repaired within 1 h. If the lesions are removed before the initiation of replication, the efficiency of alkylating agents in killing the tumor is significantly reduced. Thus, modulation of DNA repair that clearly influences the efficacy of alkylating agents is often explained by increased expression and/or activity of DNA-repair proteins.

Antimetabolites, such as 5-fluorouracil (5FU) and thiopurines, resemble nucleotides, nucleotide precursors, or cofactors required for nucleotide biosynthesis and act by inhibiting nucleotide metabolism pathways, thus depleting cells of dNTPs. They can also impair replication fork progression by becoming incorporated into the DNA (Swann et al. 1996).

An alternative approach of interfering with replication is to target specific DDR components. Topoisomerase inhibitors, such as irinotecan (a topoisomerase I inhibitor) and etoposide (a topoisomerase II inhibitor), could be considered as the first generation of DDR targeted agents (Lord and Ashworth 2012). Topoisomerases are a group of enzymes that resolve torsional strains imposed on the double helix during DNA transcription and replication. They induce transient DNA breaks to relax supercoiled DNA

or allow DNA strands to pass through each other (Helleday et al. 2008). Etoposide and Irinotecan that inhibit this function leave DNA breaks across the genome. Topoisomerase II poisons cause DSBs, and topoisomerase I poisons cause positive supercoils in advance of replication forks and replication-associated DSBs (Helleday et al. 2008).

PARP inhibitors as targeted therapy: PARP inhibitors are the next generation of DDR inhibitors.

It has been reported that the expression levels of DNA-repair genes are frequently associated with chemotherapy sensitivity and prognosis in BC subtypes. The poly (ADP-ribose) polymerase-1 (PARP1), one of the best characterized nuclear enzymes of the 17-member PARP family, participates in the repair of DNA SSB via the BER pathway.

PARP1 and PARP2 catalyze the polymerization of ADP-ribose moieties onto target proteins (PARsylation) using NAD^+ as a substrate, releasing nicotinamide in the process. This modification often modulates the conformation, stability, or activity of the target protein (Lord and Ashworth 2012). The best understood role of PARP1 is in SSBR, a form of BER. PARP1 initiates this process by detecting and binding SSBs through a zinc finger in the PARP protein. Catalytic activity of PARP1 results in the PARsylation of PARP1 itself and the PARsylation of a series of additional proteins, such as XRCC1 and the histone H1 and H2B; when PARP activity is inhibited, SSBR is compromised (Lord and Ashworth 2012).

The PARP inhibitors have been shown a substantial efficacy for hereditary BRCA1/2-related and triple-negative BC (TNBC) therapy (Bryant et al. 2005; O'Shaughnessy et al. 2011; Zhai et al. 2015). Meanwhile, there are reports demonstrating that PARP inhibitors might be also active in nonhereditary BC cells lacking mutations in BRCA1 or BRCA2 (Zhai et al. 2015; Frizzell and Kraus 2009). From a historical perspective, PARP-1 inhibitors entered the arena as promising co-adjuvant components of standard chemo- and radiotherapy regimens. Later, the discovery that tumor-cell lines bearing deficiencies or mutation in DNA-repair genes (e.g., BRCA1 or BRCA2) do not tolerate PARP-1 inhibition fuelled the application of PARP inhibitors as single agent therapies in breast and ovarian BRCA-mutated cancer settings. More recently, the discovery of new potential combinative synergisms (e.g., PI3K, NAMPT, and EFR inhibitors) as well as the broadening of "synthetic lethality" context (e.g., PTEN and ATM mutations, MSI colorectal cancer phenotypes, and Ewing's sarcomas) in which the inhibition of PARP-1 can be therapeutically valuable has further raised interest in this target.

PARP inhibitors were designed to imitate the nicotinamide portion of NAD^+ with which they compete for the corresponding PARP-1 binding site. PARP inhibition probably works by allowing the persistence of spontaneously occurring SSBs, or by inhibiting PARP release from a DNA lesion. Whichever is the case, both of these DNA lesion types could credibly stall and collapse replication forks, potentially creating lethal DSBs (Lord and Ashworth 2012). Recent data propose an indirect mechanism, according to which PARP1 activity would be dispensable for BER sheer execution, and would be rather engaged to seize potentially detrimental SSB intermediates and to promote their resolution. Recently, PARP1 contribution to SSB repair has also been

extended to MMR and NER. In normal cells, the effects of PARP inhibition are protected by HR, which repairs the resultant DSB. However, effective HR is reliant on functioning BRCA1 and BRCA2, so when these genes are defective – as they are in tumors of germline BRCA-mutant carriers – DSBs are left unrepaired, and potent PARP inhibitors can cause cell death. BRCA1 plays a role in both the G1/S and G2/M cell-cycle checkpoint regulation in response to DNA damage, again preserving genomic integrity. Moreover, the sensitivity to PARP inhibitors seems to be defined more by the BRCA genotype of a cancer cell than by its tissue of origin. Breast, ovarian, and prostate cancers with BRCA mutations all seem to be profoundly sensitive to these drugs.

As early as in 1980s, Durkacz and colleagues used the still immature, low-potency PARP inhibitor 3-aminobenzamide (3-AB) to derail DNA damage repair and enhance the cytotoxicity of dimethyl sulfate, a DNA alkylating agent (Durkacz et al. 1980).

The first clinical trial in pts was initiated in 2003 and allowed safety, pharmacokinetic, and pharmacodynamic evaluation of the PARP inhibitor AG014699 (*rucaparib* (Rouleau et al. 2010)) in combination with temozolomide (TMZ), a DNA alkylator and methylator, in advanced solid tumors (Plummer et al. 2008). However, the subsequent phase II study in melanoma (Plummer et al. 2013), as well as additional independent clinical trials, featured a common (albeit not universal) shortcoming of combinatorial strategies with PARP inhibitors, namely, enhanced toxicity. Myelotoxicity was the main dose-limiting concern, in the face of variable response rates. The need to reduce the dosage of either chemotherapy or PARP inhibitor (or both) to overcome excessive toxicity raises obvious questions about the real contribution of PARP inactivation to combinatorial regimens.

Currently, almost eight PARP inhibitors are at different stages of clinical investigation, targeting several tumor types either as single agents or in combination (Table 2).

Veliparib (Veli, ABT-888) is a potent, oral inhibitor of PARP-1 and PARP-2 (Penning et al. 2009). It is orally bioavailable and crosses the blood–brain barrier. Veli potentiated the cytotoxic effect of TMZ in several human tumor models. ABT-888 was investigated in an innovative phase 0 trial, the first such study in oncology (Kummar et al. 2009). The primary study endpoint was target modulation by the PARPi. There is an extensive clinical trial program associated with this agent with 32 ongoing clinical trials of Veli in combination with cytotoxics in ovarian, breast, colorectal, prostate, liver cancers, neurologic malignancies, and leukemias. In a phase 2 study (Isakoff et al. 2010) combined ABT-888 and TMZ is active in metastatic BC (MBC). Exploratory correlative studies including BRCA mutation analysis are underway to determine predictors of response. The dose and schedule of Veli suggest that the clinical activity seen is not likely due to Veli alone but rather to the combination. Promising antitumor activity was observed in pts with BRCA mutations.

Olaparib (Ola, AZD2281) also inhibits PARP-1 and PARP-2 at nanomolar concentrations. Preclinical studies have largely concentrated on investigations of synthetic lethality in BRCA1 or BRCA2 defective models or combinations with platinum in these models. The first clinical study of PARP inhibition in BRCA-mutant cancers

Table 2 PARP inhibitors under investigation

PARP inhibitor	Cancer type
Veliparib	Ovarian, breast, gastric, colorectal and pancreatic tumors and a range of other solid tumors
Niraparib (Nira, MK4827)	Ovarian cancer and BRCA+ breast cancer
Olaparib (Ola, AZD2281)	Ovarian, breast, gastric, colorectal and pancreatic tumors and a range of other solid tumors
Iniparib (BSI-201)	Breast cancer, ovarian cancer, lung cancer, glioma, glioblastoma
Rucaparib (AG014699)	Breast and other solid tumors
BMN-673	Ovarian, breast, gastric, colorectal and pancreatic tumors and a range of other solid tumors
CEP9722	Lymphoma, breast, ovarian cancer
E7016	Melanoma
AZD-2641	Solid tumors
INO-1001	Melanoma, breast cancer
E7449	Melanoma, breast cancer, ovarian, B-cell malignancies

was with this agent. In this phase I study which enrolled 60 pts, Ola doses were escalated from 10 mg daily for 2 of every 3 weeks to 600 mg twice daily (Fong et al. 2009). Olaparib is one of the most investigated PARP inhibitors through clinical trials either as monotherapy (Yamamoto et al. 2012; Bundred et al. 2013) or in combination with other anticancer drugs (Samol et al. 2012; Rajan et al. 2012; Dean et al. 2012; Liu et al. 2013; Dent et al. 2013; Del Conte et al. 2014). There is general agreement that 400 mg b.i.d. is the maximum tolerable dose of Ola. At this dose, Ola exhibited an acceptable safety profile. Most common adverse effects reported are of Grade 1/2 type, such as procedural pain, nausea, and other gastrointestinal symptoms of mild to moderate intensity, and thus are manageable. An important outcome of combination phase I trials results is the general tolerance of Ola when given in combination with bevacizumab (Dean et al. 2012), cediranib (Liu et al. 2013), and liposomal doxorubicin (Del Conte et al. 2014). Ola-paclitaxel combination against TNBC (Dent et al. 2013) and the Ola–CDDP combination against breast or ovarian cancer in pts carrying germline BRCA1/BRCA2 also report partial efficacy. In both studies, dose-limiting hematological toxicities were neutropenia and thrombocytopenia.

Five phase II trials were conducted with Ola alone. As with the phase I clinical trials for Ola, despite inherent differences in the study design, cancer types, patient variability, and evaluation protocols, important similarities are evident in the outcomes of these phase II clinical trials. A study in pts with confirmed BRCA1 or BRCA2 mutations and recurrent ovarian cancer (Audeh et al. 2010) yielded the objective response rate (ORR) of 33% for Ola 400 mg b.i.d. In pts with BRCA1 or BRCA2 mutations and advanced BC, ORRs were significantly higher (41%) for the 400 mg dose (Tutt et al. 2010). In another study conducted at this dose level (Gelmon et al. 2011), TNBC pts with or without BRCA mutations failed to show any objective

response (OR). Interestingly, in the same study, a very strong ORR of 41% was obtained for ovarian cancer pts with BRCA1 or BRCA2 mutations; pts without the BRCA1 or BRCA2 mutations also responded at a robust ORR of 11% (Gelmon et al. 2011). In summary in phase II clinical studies, 40% of pts with breast or ovarian cancer with germline BRCA mutations had a favorable response to the drug. This is a particularly high response given that the pts in these trials had been heavily pretreated and had become resistant to a range of chemotherapies (Lord and Ashworth 2012; Plummer et al. 2008).

INO-1001 is an isoindolinone derivative and is being developed for both oncological and cardiovascular indications. Preclinical studies demonstrate its protective effect in models of cardiac dysfunction and reversal of TMZ resistance in MMR-defective xenografts. This agent is being developed in oncology in melanoma and glioma and as a single agent in cancer for BRCA1- and BRCA2-deficient tumors. In phase I trials, INO-001 was tested alone or in combination with TMZ (Bedikian et al. 2009). Pharmacokinetic analyses indicate lack of interactions between TMZ with INO1001 and establish a "safe to administer" dose of the combination for further evaluation of the efficacy of INO1001 against advanced melanoma. However, outcomes of some clinical trials are less encouraging.

CEP9722 in phase I trials was tested alone or in combination with TMZ (Plummer et al. 2014). These dose escalation phase I trials established what the authors call an "adequately tolerated" dose for these compounds. Thus, while no neutropenia and other hematological toxicities were noticed, dose-dependent PARP inhibition was also not observed, with only limited clinical activity.

Niraparib (Nira, MK4827) is a potent inhibitor of PARP-1 and PARP-2 that is currently in phase III clinical trials for ovarian cancer and BRCA+ BC. In a phase III, randomized, open label, multicenter, controlled trial, Nira has compared versus physician's choice in previously treated, HER2 negative, germline BRCA mutation-positive BC pts. MK4827 (in a 2:1 ratio) is administered once daily continuously during a 21-day cycle. Physician's choice will be administered on a 21-day cycle. Health-related quality of life will be measured. The safety and tolerability will be assessed by clinical review of adverse events (AEs), physical examinations, electrocardiograms (ECGs), and safety laboratory values.

Iniparib (BSI-201) is an anticancer agent with PARP inhibitory activity in preclinical models. Although the full mechanism of its antitumor activity is still under investigation, iniparib enhances the antiproliferative and cytotoxic effects of carboplatin and gemcitabine in vitro models of TNBC. Phase 1–1b studies of iniparib alone and iniparib in combination with chemotherapy in pts with advanced solid tumors have shown iniparib to have mild toxicity, with no maximal dose reached in terms of side effects. O'Shaughnessy et al. (2011), in a phase II trial, evaluate whether iniparib could potentiate the antitumor effects of gemcitabine and carboplatin with acceptable toxicity levels. A total of 123 pts were randomly assigned to receive gemcitabine (1,000 mg per square meter of body-surface area) and carboplatin (at a dose equivalent to an area under the concentration–time curve of 2) on days 1 and 8 – with or without iniparib (at a dose of 5.6 mg per kilogram of body weight) on days 1, 4, 8, and 11 – every 21 days. Primary end points were the rate of clinical benefit (CB) (i.e.,

the rate of OR [complete or partial response] plus the rate of stable disease (SD) for ≥6 months) and safety. Additional end points included the ORR, progression-free survival (PFS), and overall survival (OS). The addition of iniparib to chemotherapy improved the CB and OS of pts with metastatic TNBC without significantly increased toxic effects. On the basis of these results, a phase 3 trial adequately powered to evaluate overall survival and progression-free survival is being conducted.

In summary, there are many differences in the studies evaluating anticancer activity of PARP inhibitors used alone or in combination with one or more anticancer agents. While there are many differences in the studies, some common observations should be noted with particular emphasis on various enzymatic activities associated with this multi-domain group of proteins as it applies to developing new anticancer agents and/or regimens. Specifically, the discovery of activation of PARP-2 and PARP-3 by phosphorylated DNA ends mimicking substrates or intermediates in various DNA-repair pathways is quite important. These observations shed new light on the molecular functions of different PARPs. Additionally, better understanding of the substrate specificity of individual members of the PARP family will allow researchers to further refine inhibitor chemistry and minimize adverse effects of drugs currently under evaluation. Another area of considerable potential for research and development of PARP inhibitors as first-line anticancer drugs is their application to personalized medicine. Targeted therapy is rapidly becoming a hallmark of a number of anticancer drugs.

Platinum chemotherapies: cisplatin, carboplatin, and oxaliplatin have become three of the most commonly prescribed chemotherapeutic drugs used to treat solid cancers in pts (Helleday et al. 2008). Platinum resistance, either intrinsic or acquired during cyclical treatment, is a major clinical problem as additional agents that can be added to therapy in order to circumvent tumor resistance do not currently exist. Platinum chemotherapy is now being tested with PARP inhibition clinical trials. The rationale for combining PARP inhibition with platinum chemotherapy is based on preclinical observations that PARP inhibitors preferentially kill neoplastic cells and induce complete or partial regression of a wide variety of human tumor xenografts in nude mice treated with platinum chemotherapy (Helleday et al. 2008). For example, Veli has been shown to potentiate the regression of established tumors induced by cisplatin, carboplatin therapy in rodent orthotopic and xenografts models (Helleday et al. 2008). However, the biological mechanisms of chemo-sensitization of cancer cells to platinum chemotherapy by PARP inhibition remain to be resolved.

Ionizing radiation and radiomimetic agents such as bleomycin cause replication-independent DSBs that can kill nonreplicating cells. In addition, such treatments can also rapidly prevent DNA replication by activation of cell-cycle checkpoints to avoid formation of toxic DNA replication lesions (Helleday et al. 2008).

Targeting microsatellite instability (MSI). MSI is a marker of defective MMR. The predictive value of MMR status as a marker of response to 5FU, irinotecan, and other drugs is still controversial. Two large retrospective analyses from several randomized trials confirmed the detrimental effect of a 5FU-based adjuvant therapy in stage II colorectal patients (Bedikian et al. 2009), not applicable to stage III patients (Plummer et al. 2014). These latter authors, however, reported that MSI stage III

tumors harboring genetic mutation in the MMR genes seem to benefit from the 5FU adjuvant therapy. These data imply that molecular differences within the MSI subgroup influence the response to 5FU. Combination therapy with methotrexate (MTX) and PARP inhibitors may be effective against tumors with MMR mutations. MTX elevates ROS and DSBs and the combination of MMR mutation and PARP inhibition may attenuate repair and induce growth arrest or apoptosis (McCabe et al. 2006; Vilar et al. 2011; Miquel et al. 2007).

Targeting gene expression of cell cycle and DNA-repair components: Resveratrol, a phytoalexin produced by plants such as the Japanese knotweed, prevents hypermethylation of the BRCA1 promoter (Papoutsis et al. 2012), and maybe effective for TNBC or basal subtype BC. Other natural compounds, like genistein and lycopene, can alter DNA methylation of the glutathione S transferase p1 (GSTP1) tumor suppressor gene.

Targeting centrosome abnormalities: griseofulvin, an antifungal drug that suppresses proliferation in tumor cells without affecting non-transformed cells, declusters centrosome, although the precise mechanisms behind the drug's action remain unknown (Ogden et al. 2012). In a similar fashion, depletion of a kinesin-like motor protein can selectively kill tumor cells with supernumerary centrosomes (Ogden et al. 2012). Finally, the PARP inhibitor PJ34 also declusters super numerary centrosomes without deleterious effects on spindle morphology, centrosome integrity, mitosis, or cell viability in normal cells (Kwon et al. 2008).

6 Conclusion

Genomic instability plays a critical role in cancer initiation and progression. The fidelity of the genome is protected at every stage of the cell cycle. In cancer, the presence of aneuploid or tetraploid cells indicates the failure of one or many of these safety nets. The resultant genomic heterogeneity may offer the cancer "tissue" a selection advantage against standard of care and emerging therapies. Understanding these safety nets, and how they are bypassed in cancer cells, may highlight new and more specific mechanisms for cancer prevention or therapeutic attack. The therapeutic targeting of genomic instability may check and inhibit other enabling characteristic of tumors cells, such as replicative immortality, evasion of antigrowth signaling, and tumor promoting inflammation. To this end, vitamins, minerals, and antioxidants, such as vitamin B, vitamin D, carotenoids, and selenium, as well as nutraceuticals, such as resveratrol, have shown remarkable plasticity in elucidating antitumor responses. In addition to alleviating genomic instability, these compounds are known to inhibit proliferative signaling, attenuate oncogenic metabolism, and block inflammation.

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Resistance to Hormonal Therapy in Prostate Cancer

Alfredo Berruti and Alberto Dalla Volta

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Abstract

Several therapeutic strategies are actually available in the management of prostate cancer: Targeting the androgen receptor (AR) is the goal both for initial androgen deprivation therapy (ADT) and second-generation androgen ablative agents (abiraterone and enzalutamide). Chemotherapy with taxanes, administered upon progression or as first line approach in association with ADT, is another therapeutic option. Unfortunately, none of these therapies is curative and patients are destined to develop a resistant phenotype.

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Progression to ADT leads to the attainment of a castration resistant disease whose mechanisms remain incompletely understood. Reactivation of AR has been shown to occur and second-generation of AR targeting drugs are usually prescribed. Upon progression to these agents AR signaling still remains the primary driver although it often becomes ligand independent, since it can be either restored through mutations on the ligand binding domain and/or formation of AR splicing variants or by passed through a cross talk with other oncogenic signaling pathways.

AR-independent signaling pathways may represent additional mechanisms underlying castration resistant progression. It is clear that castration resistant prostate cancer is a group of diverse diseases and new treatment paradigms need to be developed.

Keywords
Androgen receptor splice variant • Castration resistant prostate cancer • Endocrine therapy resistance

1 Introduction

Prostate cancer (PC) is a hormone-dependent disease that is treated with a variety of hormonal therapies targeting the androgen receptor (AR) pathway.

Androgen deprivation therapy (ADT) with luteinizing hormone releasing hormone agonists (LHRH-A) plus/minus antiandrogens is the first line management of metastatic prostate cancer, resulting in a delay in disease progression (Loblaw et al. 2007).

However, after an initial response, acquired castrate resistant disease inevitably occurs. Castration resistant PC (CRPC) still remains AR-driven since this phenotype is due in most cases to genomic alterations of the AR axis (Cornford et al. 2016).

In this setting, second-generation of AR targeting drugs, such as enzalutamide and abiraterone acetate (AA), have improved overall survival and quality of life.

AA is a CYP17A1 inhibitor blocking the production of androgens in the testes, adrenal glands, and tumor microenvironment by inhibiting both 17a-hydroxylase and 17,20 lyase activities of the CYP17A1 enzyme (Mostaghel 2014). In addition, the drug in is converted by the 3β-hydroxysteroid dehydrogenase (3βHSD) to the more active Δ4-abiraterone (D4A) that blocks multiple steroidogenic enzymes and antagonizes the AR (Li et al. 2015). Enzalutamide is a novel antagonist of AR that inhibits nuclear translocation, chromatin binding, and interactions with AR coregulators (Schalken and Fitzpatrick 2016).

Both of AA and enzalutamide treatments are not curative and are associated with the emergence of resistance. This paper is focused on the major mechanisms of androgen pathway resistance.

2 Serum Testosterone and Intracrine and Paracrine Androgen Production

Testosterone (T) is the natural growth factor for PC. The attainment of serum T levels as low as possible (i.e. below 20 ng/mL) during ADT is associated with a better outcome (Bertaglia et al. 2013). However, in untreated PC patients lower serum concentrations have been associated with worrisome features such as high grade and disease stage (Khera et al. 2014), suggesting that PC is stimulated to dedifferentiate in a T-deficient environment. More importantly, serum T levels measured in CRPC patients revealed longer survival in patients with higher levels (Ryan et al. 2013), suggesting that testosterone may maintain a differentiating role also when the tumor progresses to ADT. These data, however, do not take into account the intraprostatic hormone levels that are more important than circulating levels. Significant amounts of intraprostatic androgens still remain following ADT in localized PC (Nishiyama et al. 2004) and metastatic CRPC (Montgomery et al. 2008). Intraprostatic dihydrotestosterone (DHT) level, which is more potent than T, was shown to decrease only by 40% after ADT (Labrie 2011) and tissue biopsy studies have demonstrated higher levels of androgens in tumor tissue of CRPC patients than patients with androgen-sensitive disease (Montgomery et al. 2008).

Tissue androgen levels may be responsible of both primary and acquired resistance to ADT in PC. Dehydroepiandrosterone-sulphate DHEA-S is the major source of adrenal androgens that stimulates intratumoral androgen biosynthesis. DHEA-S is actively transported into the cells by membrane transporting peptides such as organic anion-transporting polypeptides (OATPs). The members of this family involved in steroid uptake in the prostate include *SLCO1A2* and *SLCO2B1* (Cho et al. 2014). The active form of *SLCO2B1* was found to be associated with prostate cancer progression under ADT (Yang et al. 2011). The HSD3B1 is the enzyme involved in either the conversion of DHEA to Δ4-androstene-3,17-dione (Δ4-AD) or the conversion of Δ5-androstene-3β17β-diol (Δ5-Adiol) to T in prostate cancer. The 1245C allele results in a coding change (N367T) leading to a gain-of-function 3βHSD1 without altering the enzymatic activity but leading to reduced ubiquitination and degradation of the enzyme. This results in increased enzyme abundance. In a recent retrospective study a strong correlation was found between the presence of the HSD3B1 (1245C) polymorphism and poorer outcome in 443 men who underwent ADT (Hearn et al. 2016). Both inherited SNPs of *SLCO* and HSD3B1 gene expression provide mechanisms of primary ADT resistance.

In prostate cells, Androstenedione is ultimately converted through the classic route to DHT by aldo-keto reductase family 1 member C3 (AKR1C3, also known as 17-β-hydroxysteroid dehydrogenase type 5) (Koh et al. 2002). Overexpression of AKR1C3 in CPRC would provide a mechanism to divert residual androgens after ADT to potent androgens via different pathways within the tumor. AKR1C3 upregulation is therefore an adaptive response to ADT, and could contribute to drug resistance observed with AA or enzalutamide (Penning 2015).

A neoadjuvant clinical trial evaluated the effect of Leuprolide alone (12 weeks) followed by Leuprolide plus AA (12 weeks) versus Leuprolide plus AA (for all

24 weeks) (Taplin et al. 2014). Serum hormones, measured at baseline and at 12 and 24 weeks reveled that once AA was administered the adrenal androgens were decreased by >90%. Also, levels of DHEA-S were significantly reduced, however a persistent pool of DHEA-S may stimulate intratumoral androgen biosynthesis, providing a potential mechanism for the clinical failure of AA.

3 AR Splice Variants and Point Mutations

The Androgen Receptor (AR) is a transmembrane protein whose structure includes four main regions:

1. N-terminal Domain (NTD),
2. DNA binding Domain (DBD),
3. Hinge region,
4. Ligand binding Domain (LBD)

Upon binding of DHT to the LBD, AR is switched to the active conformation and forms a homodimer, that is transferred to the nucleus where it binds to androgen response elements (AREs).

AR splice variants and point mutations are two mechanisms through which AR can be activated without the direct intervention of DHT (and testosterone).

3.1 Androgen Receptor Splice Variants (ARVs)

LBD-lacking ARVs were described in 2008, when nucleotide sequences transcribed from intronic regions of the DNA, causing alternatively spliced mRNAs, were identified (Dehm et al. 2008), The result of their transcription is a set of mRNAs presenting with a premature stop codon, that are translated into truncated proteins lacking the LBD. These ARV proteins are able to bind DNA in the absence of androgens, thus promoting constitutively active signals to the nucleus (Fig. 1).

The extensive search for AR intronic regions led to identify seven distinct ARVs, named ARV1-7 (Hu et al. 2009).

Other variants were subsequently found, one of them is the result of exon-skipping during mRNA splicing, leading to transcripts lacking exons 5, 6 and 7. Consequently this variant was named AR^{v567es} (Sun et al. 2010).

The ARV8-14 includes the variants 8–11 lacking the whole LBD, while ARV12-14 retains part of the LBD and shows functional inactivity (ARV13,14) (Watson et al. 2010; Hu et al. 2011).

Despite the abundance of AR variants, only ARV7 and AR^{v567es} have been extensively studied. Recent whole transcriptome analysis confirmed that ARV7 is the most abundant ARV expressed in human cell lines and clinical samples (Robinson et al. 2015).

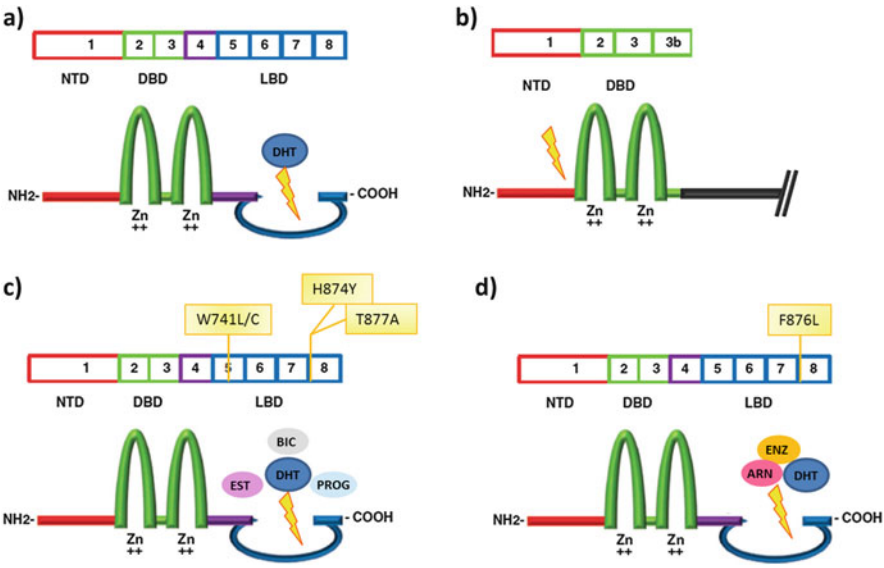

Fig. 1 (**a**) Wild-type AR activated by di-hydro-testosterone (DHT); (**b**) AP splice variant 7, activated regardless to the presence of ligands; (**c**, **d**) AR with point mutations, promiscuously activated by alternative ligands such as estradiol (EST), progesterone (PROG), bicalutamide (BIC), enzalutamide (ENZ), apalutamide (ARN)

ARV7 relies on full-length AR signaling to enhance its own transcription: in fact, ligand-dependent AR signaling reduces transcription of AR-FL, thereby decreasing ARV7 transcription as well. This observation is consistent with the selective advantage provided to ARV7^{+} cells by ADT, and with the report of negative correlation between ARV expression and testosterone serum levels.

ARVs are involved in oncogenesis and resistance to primary treatments. A study carried out on matched prostate specimens revealed progressive up-regulation of ARV7 in benign tissue, hormone-naïve and castrate-resistant adenocarcinoma (Hu et al. 2009).

Whether ARVs expression can assume the role of predictive and/or prognostic biomarker in the CRPC setting is a matter of debate.

The prognostic role of ARVs was demonstrated in a clinical study, in which these variants were evaluated on bone metastatic samples from 30 CRPC patients. ARV7 expression in the upper quartile and/or detectable expression of AR^{v567es} were associated with a decreased cancer-specific survival (Hornberg et al. 2011).

To assess the predictive role of ARV7 expression for response to enzalutamide, a prospective phase II trial was conducted in patients with bone metastatic CRPC who underwent transilial bone marrow biopsies prior to and after 8 weeks of enzalutamide treatment. Baseline expression of ARV7 by immunohistochemistry did not predict responsiveness to enzalutamide, however the samples collected after 8 weeks of enzalutamide showed a significant increase of ARV7 positive staining in

the resistance group, compared to the responsive group. In addition, none of the enzalutamide long-responders had ARV7 positive staining, either at baseline or after 8 weeks of treatment (Efstathiou et al. 2015).

In a subsequent paper, ARV7 expression was prospectively assessed on pooled epithelial cell adhesion molecule (EpCAM)-positive circulating tumor cells (CTCs) from peripheral venous blood of metastatic CRPC patients. Patients treated with enzalutamide or abiraterone whose CTCs at baseline proved to be ARV7-positive had significantly lower PSA response rate and shorter median overall survival compared with ARV7-negative patients (Antonarakis et al. 2014).

Another prospective study conducted on patients at multiple stages of disease confirmed that ARV7 expression on CTCs could identify responders to hormonal therapeutic strategies (including ADT, enzalutamide and abiraterone) (Steinestel et al. 2015).

The role of ARV7 expression in predicting the efficacy of taxane-based chemotherapy was also explored. Thirty-seven patients, stratified at baseline on the basis of ARV7 expression on CTCs, were treated with either docetaxel or cabazitaxel, $ARV7^-$ patients had a greater PSA response rate (41 vs 65%, $p = 0.19$) and a longer median PFS than $ARV7^+$ ones, although the differences failed to attain the statistical significance (Antonarakis et al. 2015).

Another prospective trial was conducted stratifying for ARV7 expression mCRPC patients that underwent systemic therapy with either taxanes or AR-directed treatment. $ARV7^+$ patients had better outcomes when treated with taxanes rather than AR-directed treatments (median OS 8.9 vs 4.6 months, HR 0.24 for risk of death), even adjusting for baseline known prognostic factors in a multivariable model (Scher et al. 2016).

3.2 Androgen Receptor Point Mutations

Similar to splice variants, point mutations of the AR coding sequence increase in prevalence and become detectable in the setting of advanced CRPC. It was shown that none of early stage prostate cancer patients had AR mutations, in front of 21% of patients with CRPC (Marcelli et al. 2000).

Two are the main mechanisms through which somatic mutations can uncouple AR activation from its ligand (Liu et al. 2015):

1. change in amino acids that have a stronger affinity for helix 12. This mechanism pulls helix 12 closer to the active position, thus increasing the AR sensitivity and making it less reliant on DHT for activation;
2. amino acids substitutions in the LBD, resulting in a larger pocket that can accommodate more ligands.

Although several mutations were identified, only a few of them are described to be significant in driving prostate cancer progression and drug resistance (Gottlieb et al. 2004) (Fig. 1).

The H874Y mutation applies to the first group, as it involves the substitution of a histidine with a tyrosine between helix 11 and 12, pushing the last one closer to the LBD and conferring transactivation from other ligands (such as estradiol or progesterone) (Duff and Mcewan 2005).

H874Y also enhances the association between the AR and p160 co-activators.

The F876L missense mutation (phenylalanine for leucine) also favors helix 12 movement closer to the LBD and enhance AR activation. In this case the AR is made sensitive to antagonists like enzalutamide (Korpal et al. 2013).

The T877A mutation is the primary AR mutation in LNCaP cell line, frequently detected in CRPC (Veldscholte et al. 1992); it lays in the second group, as above described, in that the incorporation of alanine in the helix 11 results in a larger ligand-binding pocket thereby accommodating more ligands with different shapes (particularly preferring estradiol) (Steketee et al. 2002).

Finally, W741L/C mutation alters the tertiary structure of helix 12 in a way that lowers the affinity of the LBD for androgens, but conversely causes bicalutamide to act as an AR agonist (Taplin et al. 1999).

Available data suggest that AR point mutations may have a major role in resistance to anti-androgens rather than to ADT. In a clinical study, in fact, only one out of 17 patients treated with ADT had a somatic point mutation in their AR coding sequence (Carreira et al. 2014). Conversely, 5 of the 16 patients treated with the androgen antagonist flutamide had the T877A missense mutation (Urushibara et al. 2007).

Enzalutamide resistance could be driven by the expression of F876L, which switches the potent AR-antagonist to an agonist. The expression of this AR mutation was detected in all enzalutamide resistant cell lines, but not in weakly resistant or control lines (Korpal et al. 2013).

Furthermore, F876L mutation has also been correlated with PSA rising after chronic exposure to the new anti-androgen ARN-509 (Joseph et al. 2013). As to the involvement of AR point mutations in the resistance to Abiraterone, a clinical study detected both H874Y and T877A mutations in circulating cell-free DNA of abiraterone-resistant patients (Azad et al. 2015).

4 Glucocorticoid and Progesterone Receptor Upregulation

Glucocorticoid treatment has resulted in both subjective and objective responses in patients with advanced prostate cancer (Venkitaraman et al. 2008). Glucocorticoids inhibit adrenocorticotropic hormone (ACTH) production by the pituitary and this mechanism results in reduced androgen levels, providing explanation of the efficacy of glucocorticoid in PC patients. With the use of the new therapeutic agents, however, glucocorticoid-related mechanisms can cause iatrogenic stimulation of prostate cancer growth, which might contribute to drug resistance and disease progression. The glucocorticoid receptor (GR) and AR belong to class I nuclear steroid receptors and therefore share several transcriptional targets, including the antiapoptotic genes serum and glucocorticoid-regulated kinase 1 (SGK1) and Map

kinase phosphatase 1 (MKP1)/dual specificity phosphatase 1 (DUSP1). In men whose prostate cancers express high GR levels as a consequence of iatrogenic stimulation, GR activation in tumor PC cells prevails (Fig. 2) (Watson et al. 2015). GR bypass may be an alternative strategy adopted by the tumor as a consequence of the increased selection pressure conferred by second-generation antiandrogens.

In a pre-clinical and clinical study (Arora et al. 2013), many common gene that are targets of AR and GR were upregulated in LNCaP xenografts expressing wild-type AR that became resistant enzalutamide and ARN-509. In addition, knockdown of GR in cells derived from resistant tumors restored the sensitivity to enzalutamide when administrated in VCaP cells. Moreover, analysis of bone marrow biopsies from patients treated with enzalutamide confirmed the role for GR induction in the clinical resistance to enzalutamide (Arora et al. 2013). In a biological study performed on tissue samples of the previously mentioned neoadjuvant study of AA + Leuprolide versus Leuprolide (Taplin et al. 2014), AA + prednisone administration was associated with greater upregulation of GR than Leuprolide alone. These data notwithstanding, whether increased GR expression plays a role in abiraterone resistance remains to be determined and needs to be studied, however this study shows for the first time that acquired GR induction after ADT + AA may occur early.

In addition to GR, the progesterone receptor (PGR) also belong to the steroid hormone nuclear receptor family structurally related to AR, sharing substantial homology within the DNA binding domain. As with GR, PGR could transcriptionally regulate a subset of AR target genes in PCa, and thereby bypass AR. PGR expression has been demonstrated in prostate tumor cells in some studies.

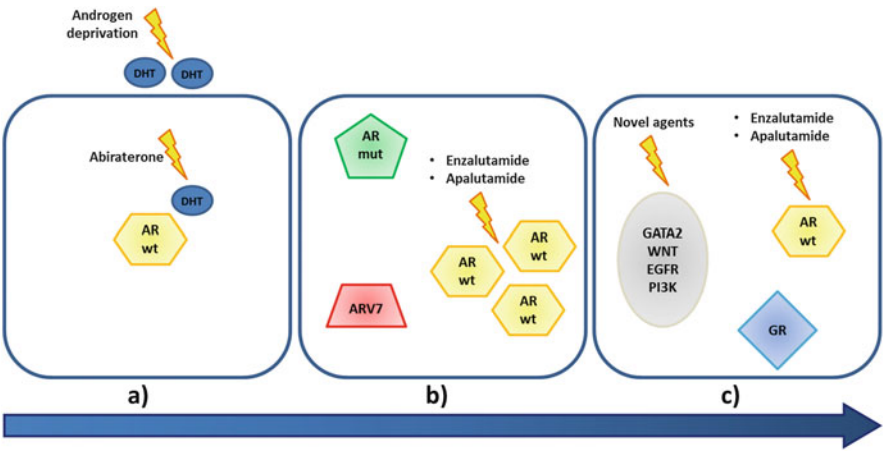

Fig. 2 Prostatic adenocarcinoma could escape from androgen deprivation and anti-androgenic therapies, through different mechanisms: (**a**) androgen biosynthesis, (**b**) AR amplification, AR point mutations (AR mut), AR truncated variants (es. ARV7), (**c**) expression of glucocorticoid receptor (GR) and other alternative oncogenic pathways (GATA-2, WNT/Bcatenin, EGFR, PI3K/Akt)

Interestingly, high PGR staining in primary PCa was associated with clinical recurrence in a recent, large retrospective analysis (Grindstad et al. 2015).

5 Other Oncogenic Signaling Pathways

Complementary signaling pathways may also drive prostate cancer growth in conjunction with the AR, therefore numerous alternative oncogenic pathways are activated and promote the transcriptional activities of AR after androgen deprivation and AR inhibition.

The loss of the tumor suppressor gene phosphatase and tensin homolog (PTEN) and activation of PI3K signaling was found in almost 70% of metastatic PC (Taylor et al. 2010). PI3K is an intracellular kinase that is activated by G-protein coupled receptors or receptor tyrosine kinases. Activation of PI3K leads to phosphorylation of AKT and mTOR. This leads to downstream effects including cellular proliferation, survival and angiogenesis. Akt-mediated AR phosphorylation increases the interaction of AR with the transcriptional factor p300, inhibiting AR ubiquitination and degradation (Debes et al. 2002).

P300 is an essential co-activator in gene transcription control. This protein bridges DNA binding factors and transcription factors; catalyzes histone acetylation via its intrinsic histone acetyltransferase activity; and acetylates transcriptional factors to further facilitate their activity. P300 is involved in the regulation of expression and function of a large number of tumor-associated proteins, including AR. Therefore, it is a major promoter of PC and high expression of this protein is critical for the androgen-dependent and androgen-independent transactivation of AR (Karantanos et al. 2015).

The epidermal growth factor receptor (EGFR) and HER2 have been implicated in activation of AR and promotion of PC growth (Karantanos et al. 2015). HER2 and HER3 in vitro stabilize AR and increase its binding to AREs, while knockdown of HER2 inhibits the AR transcriptional activity. A recent paper also showed that EGFR promotes survival of prostate cancer cells that metastasize to bone (Day et al. 2017).

The Wnt/B-catenin is an additional pathway involved in AR signaling that has a role in PCa progression to an androgen independent phenotype. AR has been shown to signal through Wnt/B-catenin in a ligand-independent manner as an adaptation to androgen deprivation therapy (Schweizer et al. 2008). Increased levels and nuclear co-localization in vivo of AR and Beta-catenin occur in CRPC, suggesting an aberrant B-catenin-dependent AR activation in the progression to CRPC (Wang et al. 2008). Moreover, simultaneous inhibition of both Wnt and AR pathways have shown antitumor activities in xenograft PCa models (Lee et al. 2013). However, B-catenin might also have an AR-independent oncogenic role in CRPC. High levels of nuclear B-catenin in association to low or no AR expression have been identified in a subgroup of bone metastatic PCa patients (Wan et al. 2012).

Forkhead Box A1 (FOXA1) is not a classic transcriptional coactivator but it rather serves to open sites of condensed chromatin to facilitate AR binding,

resulting in enhanced transcriptional activity (Wang et al. 2008). Amplification and overexpression of FOXA1 have been detected in primary tumors but is more common in metastatic CRPC, highlighting its role in persistent AR signaling in the castrate state (Grasso et al. 2012).

GATA2 is another AR regulating factor with multiple roles in the AR signaling axis: it is required for AR transcriptional activity, enhances AR:chromatin associations and promotes AR (and AR-V) expression. GATA2 is overexpressed in CRPC and its expression is associated with poor outcome (Chiang et al. 2014). GATA2 also has an intimate, bidirectional relationship with FOXA1, with important implications for development and progression of CRPC (Zhao et al. 2016).

6 AR Pathway Independent Mechanisms and Neuroendocrine Phenotype

While most research to date has focused on the continued importance of AR in CRPC, alternative signaling pathways supporting proliferation and survival of CRPC cells have been shown to be capable of completely bypassing AR.

DNA repair pathways have emerged recently as a clinically relevant way for therapeutic manipulation. The identification of a subgroup of metastatic CRPC with DNA repair defects provides a strong rationale for developing specific therapeutic strategies (Mateo et al. 2017).

Poly (ADP-ribose) polymerases (PARP) are a family of proteins involved in a number of cellular processes involving mainly DNA repair and programmed cell death. Activation of PARP1 and PARP2 triggers the damage response and recruits key effectors of repair. A recent study showed that patients with defects in certain DNA repair enzymes (including BRCA1/2, ATM, Fanconi's anemia genes, and CHEK2) had an 88% response rate to the PARP inhibitor olaparib (Mateo et al. 2015).

Noteworthy, BRCA1 and BRCA2 mutations are associated with only a small fraction of prostate cancer cases, however recent genomic analysis has revealed that germline or somatic inactivating mutations in BRCA1 or BRCA2, or other genes involved in the homologous recombination pathway of DNA repair, collectively occur in as much as 20–25% of advanced CRPCs. This provides an opportunity for the use of PARP inhibitors as a therapeutic strategy for the treatment of metastatic CRPC with either germline or somatic defects in BRCA2, ATM, PALB2, and other DNA repair genes (the so called "BRCAness"). The finding that PARP enzymes also have a role in AR transcriptional regulation (Mateo et al. 2017) further reinforce the rationale for their use in this setting that is being studied in multiple clinical trials.

A subset of patients with advanced prostate cancer may eventually evolve into an androgen receptor (AR)–independent neuroendocrine phenotype. Although aggressive NE phenotype can arise de novo, it usually occurs as recurrent tumor in men who have received hormonal therapy for prostatic adenocarcinoma. The prevalence of neuroendocrine aggressive phenotype is estimated to be

approximately 1% of primary prostate cancers and up to 25–30% in metastatic castrate-resistant prostate cancers (Terry and Beltran 2014). Recent genomic profiling studies have demonstrated that prostate cancers with an aggressive neuroendocrine phenotype are enriched for loss of RB, loss or mutation of TP53, loss of AR and AR target gene expression, and overexpression of MYCN and AURKA (Beltran et al. 2016) Thus, neuroendocrine differentiation represents a viable option for prostate cancer to escape hormone therapy and progress to a condition of true hormone refractoriness. The typical clinical picture of a patient bearing treatment induced neuroendocrine prostate cancer (NEPC) is characterized by rapidly progressive disease involving visceral, bone (with typical lytic lesions) often in the setting of a low or modestly rising serum prostate-specific antigen level. Traditionally, NEPC are managed clinically with cisplatin-based chemotherapy regimens, but the prognosis is poor. A systematic review and pooled analysis of published cases revealed a median time to NEPC of 20 months and a median overall survival after NEPC diagnosis of 7 months (Wang et al. 2014).

7 Conclusions

The androgen receptor signaling axis remains a crucial driver of prostate cancer progression and treatment resistance. However, AR often become ligand independent, since it can be either restored through mutations on the ligand binding domain and/or formation of AR splicing variants or by passed through a cross talk with other oncogenic signaling pathways. AR-independent signaling pathways may represent additional mechanisms underlying castration resistant progression. It is clear that prostate cancer that has become resistant to conventional and newer therapeutic agents is a group of diverse diseases and new treatment paradigms need to be developed.

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Mechanism of Resistance in Gastrointestinal Stromal Tumors

E. Tamborini

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Abstract

Imatinib has revolutionized the treatment of GIST since this drug is able to inhibit tumoral growth by blocking the activity of receptor tyrosine kinases, KIT or PDGFRA, that in these tumors are constitutively activated because of the presence of mutations that alters their catalytic activity. However, despite this enormous improvement in the RFS and OS and in the quality of life of GIST patients, imatinib is not able to eradicate the disease: recurrences occur and acquired resistance is a common event which develops during targeted treatments. Several mechanisms have been demonstrated to be responsible for tumoral growth reactivation which is due to the reactivation of the altered KIT/PDGFRA receptors, no more blocked by the drug. Secondary point mutations are generally observed in the regrowing tumors, and it has been demonstrated that they alter the architectural structure of the site in which the interaction between the drug and the receptor happens. Other mechanisms causing drug resistance have been investigated, indicating that many aspects need to be still explicated and fully understood in order to define a strategy able to fight definitively GIST growth.

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Acquired resistance · Gastrointestinal stromal tumor · KIT · PDGFRA · TK inhibitors

1 Introduction

Gastrointestinal stromal tumors represent an exquisite paradigm of target therapy related to genotype in solid tumors: oncogenic driver mutations affecting the receptor tyrosine kinase KIT (or alternatively PDGFRA) are blocked by imatinib, thus leading to the stop of tumor growth.

Imatinib changed totally GIST history, making this tumor histotype, in the past a nonresponding tumor for which no efficacious chemotherapeutic option existed, a tumor in which target therapy alone is able to prolonged survival for many years.

GISTs parallel the same revolution observed in CML with the same drug. These successes led to an accelerated approval process by the FDA, and imatinib was approved for the treatment of CML in 2001 and, 1 year later, of GIST.

In 2012 imatinib was approved by the FDA and EMEA as adjuvant therapy following surgical removal of KIT-positive tumors, since data from the multicenter international, phase III trial demonstrated that 36 months of imatinib prolonged recurrence-free survival (RFS) compared to 12 months, improving also the overall survival (OS). In addition, 36 months of imatinib treatment resulted in a 55% reduction in the risk of death compared to 12 months of treatment ($P = 0.0187$). However, the risk of recurrence remained almost unchanged ($P < 0.0001$), indicating that imatinib secondary resistance is still a unresolved problem (Joensuu et al. 2012).

Despite this enormous success, represented by more than 80% of patients showing clinical benefit from imatinib monotherapy, more than half will develop progressive disease in 2 years (Kee and Zalcberg 2012).

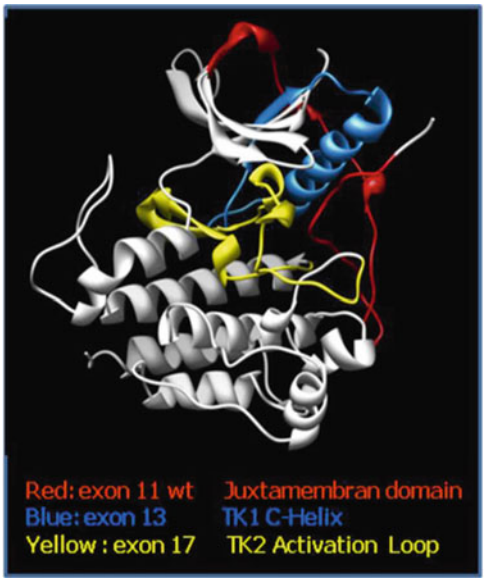
2 History of GIST

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal malignancy of the gastrointestinal tract and are characterized by a proliferation of spindle-shaped (70% of the cases), rarely epithelioid (20%), cells, and 10% have mixed histology. They commonly express the KIT protein (CD117) as well as DOG1 (Miettinen et al. 2009), and approximately 60–70% of GISTs are $CD34^+$, while 30–40% are positive for SMA. Only rare GISTs are positive for desmin and about 5% of GISTs are $S\text{-}100^+$ (Miettinen and Lasota 2006). GIST carrying mutation in PDGFRA resulted immunohistochemically positive for this protein, often with a dot-like staining (Tamborini et al. 2012).

GISTs are characterized by the presence of constitutively activated receptor tyrosine kinases (RTKs) KIT (CD117) and PDGFRA in the 80–85% and 10–5% of the cases, respectively (Hirota et al. 1998; Heinrich et al. 2003). The mechanism responsible for the constitutive activation is the presence of gain-of-function mutations in the corresponding genes. In the heterogeneous group of wild-type GISTs with no mutations in both KIT and PDGFRA genes, cases carrying loss of function alterations in SDH genes represent the bigger fraction (Janeway et al. 2011).

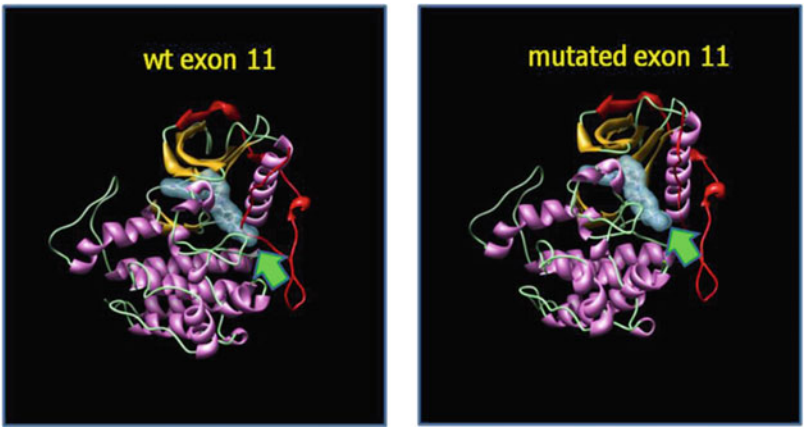
Evidence derived from the clinical experience points out that generally all *c-Kit* exon 11 mutation types, with the exception of L576P substitution, demonstrated to be refractory to imatinib inhibition (Conca et al. 2009) and correlate with a good response rate to imatinib at 400 mg/day (Heinrich et al. 2008). Other *c-Kit* mutations instead need a higher dose to achieve a clinical response. This is observed with KIT exon 13 or 17 or 9 alterations.

This behavior can be ascribed to the conformation of the ATP pocket of the two receptors (where imatinib is positioned), and in which way, all the common *c-Kit* or *PDGFRA* mutations can alter the ATP pocket shape. The explanation lies on the tridimensional conformation of these two receptors, derived from both the kinase domain crystallography of KIT (ref) and recently of PDGFRA (Liang et al. 2016) and molecular modeling techniques (Pierotti et al. 2011). This double approach shows how exons, site of mutation in GISTs, are shaped into the space. Exons 13 and 17 (corresponding to TK1 and TK2, respectively) and their respective exons in PDGFRA delimit a cave in which ATP is located. In particular, the activation loop (exon 17), whose position controls the opening of the cave itself, plays a fundamental role for correct RTK behavior, governing the receptor in the active or inactive form.



A major role is played by exon 11, conformed as a hairpin (juxtamembrane domain) that is able to modulate the wideness of the opening of the cave (see below).

Mutations affecting all these domains, thus destroying the peculiar architecture of this "perfect world," lead to a deregulation of the physiological KIT/PDGFRA functions. The final result is a constitutive activation of these receptors, irrespectively to the physiological activation caused by their specific ligand binding. In particular exon 11 mutations of KIT, by deforming the position of the hairpin, permit a bigger opening of the cave, and as consequence, imatinib itself has a major possibility of entering, and this provides the first tridimensional explanation of imatinib's successful inhibition in tumors carrying KIT exon 11 mutations.



Courtesy of S. Pricl, University of Trieste

3 Mechanisms of Acquired Resistance

3.1 Secondary Point Mutations

These mechanisms are complex, often heterogeneous, and not fully understood.

It is undoubtedly clear that about 70–85% of the patients carrying exon 11 mutations showing acquired resistance to imatinib develop secondary mutations affecting KIT receptor (Antonescu et al. 2005). Intriguingly, a lower percentage of secondary mutations is detected in patients showing exon 9 primary mutations (Heinrich et al. 2008; Antonescu et al. 2005). The secondary acquired point mutations are always on the same allele, thus corroborating the idea that the destruction of a suitable ATP pocket in which imatinib can stay is an actual explanation. They generally affect the A loop (secondary mutations affecting

exon 17 of KIT or exon 18 of PDGFRA), stabilizing the receptor in the active conformation: i.e., always unlocked and open to accept ATP. Alternatively, they affect the ATP pocket itself (secondary mutations in exon 14 or 13) preventing the perfect imatinib allocation in the kinase domain itself. It has been reported that more than one secondary point mutation can arise during the treatment, thus revealing a polyclonal nature of this phenomenon.

No published studies have been done with new and very sensitive techniques (such as NGS or digital PCR), demonstrating the presence of these secondary mutations before treatment and definitively demonstrating the heterogeneous genotype of GISTs. However, the clonal evolution under selective drug selection of cells carrying secondary and insensitive imatinib mutations (resistant sub-clones) seems the most reliable explanation of this phenomenon.

Even though this mechanism of drug resistance is perfectly understandable, it is observed only in a fraction of patient undergoing imatinib resistance.

In an even more restricted set of patients, secondary mutations affecting the downstream pathway can be observed, affecting BRAF (even if this alteration is principally present in wild-type GISTs) (Agaram et al. 2008) and in KRAS genes (Miranda et al. 2012). Recently, Lasota and collaborators demonstrated also the presence of PIK3CA mutations, suggesting a proliferative advantage during disease progression, as well as a possible role in the reactivation of PI3K/AKT/mTOR pathway (Lasota et al. 2016).

3.2 Gene Amplification

An alternative explanation can be found in KIT gene amplification, observed both in treated and nontreated GIST tumors (Miselli et al. 2007). This mechanism causes a reactivation of the oncogene with or without secondary mutations. However, the analysis of phosphorylation levels of KIT does not correlate with amplification status of the gene, nor with clinical response, indicating a heterogeneity linked to tumor diversity (Wardelmann et al. 2006).

3.3 Autophagy and Apoptosis

Other more complex mechanisms involving several cellular pathways have been demonstrated to play important roles during imatinib treatment, such as autophagy and apoptosis. Autophagy describes a dynamic and specifically regulated mechanism by which cells principally protect themselves from a variety of stresses, including starvation, hypoxia, and oxidative stress. Alternative survival pathways, normally present in eukaryotic cells, are then activated, leading to sequestration of the cytoplasmic material into lysosomes which are degraded. This degradation can promote cell survival by recycling the degraded nucleotides, amino acids, and fatty acids that maintain energy production or can promote cell death as a result of self-cannibalization. Fenotipically, autophagy is recognized by the presence of

vacuolated cells that are typically present in GIST tumors derived from surgical resection after imatinib treatment (El-Khattouti et al. 2013; Miselli et al. 2008). Genotipically, autophagy is regulated by a tumor-suppressor mechanism in which the major players are the activating beclin1/PI3KIII complex, the suppressing beclin1/bcl2 complex, and the presence of LC3-II strictly bound to autophagosomes. These autophagic markers have been demonstrated in imatinib-treated tumors (Miselli et al. 2008) and in GIST cell lines (Gupta et al. 2010). Thus, imatinib instead to induce cell death can also induce cell survival; and in this finding, an explanation for tumor cell reactivation in case of imatinib suspension can be found. Moreover, these opposite behaviors give also a justification for the incomplete tumoral remission often observed in GIST patients (Miselli et al. 2008; Gupta et al. 2010; Ravegnini et al. 2017). The "tumor dormancy" that fits well with the metabolic characteristics of autophagy, on the other hand, fits also with the activation of ANAPC-FZR1/CDH1-SKP2-CDKN1-p27^{Kip1} signaling axis mainly important for reinforcing a prolonged G1 phase of the cell division cycle and was demonstrated to be present in imatinib-induced quiescent GIST cells derived from PDX models (Boichuk et al. 2013).

By contrast, apoptosis demonstrated in GIST cell lines was not observed in GIST surgical specimens as sustained by the negative results of the biochemical and IHC experiments using caspase 3, caspase 7, and lamin A/C antibodies (Miselli et al. 2008). This is in keeping with the observation that in GIST, pro-apoptotic proteins, as BAX, are downregulated, while expression of anti-apoptotic factors, for example, the BCL2 family members, is generally observed. These data, all together, indicate that autophagy and apoptosis and their balance during imatinib treatment in GIST cells are governed by multi-factors, including the differential expression of miRNA and that we are only at the beginning of a road which has to be explored in detail.

3.4 Resistance Due to Other Mechanisms

By analyzing tumors derived from patients undergoing progression during imatinib treatment, or sunitinib treatment, only for a few percentage of them a molecular explanation has been determined. For this reason, alternative mechanisms of escape, not yet understood, should be present. It has also to be pointed out that surgical removal of the progressing disease in metastatic patients is not a common medical intervention; thus, the unavailability of the right material to investigate resistance represents a big limitation.

It has been proposed that the activation of other RTKs, such as AXL expression in gefitinib-treated lung adenocarcinomas, and the expression of an alternative receptor are generally accompanied by KIT loss of expression. The result is always a reactivation of the pathways leading to cell proliferation (Mahadevan et al. 2007).

Also, an upregulation of SRC and integrins possibly mediated by FAK has been observed. These interesting data point the attention on a possible role of microenvironment in which cells remain embedded as result of the pathological remission

of tumor cells due to imatinib. A deep analysis of this myxoid stroma can reveal the presence of growth factors able to keep the survival cells into a quiescent status which immediately disappears with imatinib suspension. Recently, in melanomas BRAF mutated and progressing to vemurafenib, it has been proposed that high levels of integrin beta 1 and FAK, which, in turn, reactivated ERK and MAPK, are responsible for drug resistance (Hirata et al. 2015). Intriguingly the reactivation of the ERK/MAPK pathway was observed in areas of high stromal density. It could happen also in GISTs. It is not speculation to imagine that through the block of tumoral cells inside (by imatinib or other inhibitors) and outside by inhibiting the stromal cells, an efficacious therapy can be achieved, and this perhaps represents a real challenge to definitively eradicate GIST tumor cells.

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Mechanisms of Resistance to Targeted Therapies in Chronic Lymphocytic Leukemia

Francesca Arruga and Silvia Deaglio

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Abstract

Even if treatment options for Chronic Lymphocytic Leukemia (CLL) patients have changed dramatically in the past few years, with the approval of targeted therapeutic agents, the disease remains incurable. Beside intrinsic genetic features characterizing the leukemic cell, signals coming from the microenvironment have a key role in promoting cell survival and in protecting CLL cells from the action of drugs. Consequently, the identification of previously unrecognized genetic lesions is important in risk-stratification of CLL patients and is progressively becoming a critical tool for choosing the best therapeutic strategy. Significant efforts have also been dedicated to define microenvironment-dependent mechanisms that sustain leukemic cells favoring survival, proliferation, and accumulation of additional genetic lesions. Furthermore, understanding

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the molecular and biological mechanisms, potentially driving disease progression and chemoresistance, is the first step to design therapies that could be effective in high-risk patients. Significant progress has been made in the identification of the different mechanisms through which patients relapse after "new" and "old" therapies. These studies have led to the development of targeted strategies to overcome, or even prevent, resistance through the design of novel agents or their combination.

In this chapter we will give an overview of the main therapeutic options for CLL patients and review the mechanisms of resistance responsible for treatment failure. Potential strategies to overcome or prevent resistance will be also discussed.

Keywords
Chronic lymphocytic leukemia • Resistance • Target therapy

1 Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western world, with an incidence rate of approximately 3.9/100,000/year, representing 30–40% of all adult leukemias (Hallek 2015). It is characterized by the progressive accumulation of mature $CD5^+/CD23^+$ B lymphocytes in the peripheral blood (PB), bone marrow (BM), and lymphoid tissues, with lymphocyte counts in the PB usually $\geq$5,000/mm^3 (Dighiero and Hamblin 2008). The clinical course is highly heterogeneous: some patients exhibit an indolent non-progressive disease, showing a life expectancy more or less identical to individuals of the same age. Other patients, on the contrary, experience an aggressive disease, require early treatment, and become quickly resistant to treatment. These patients are characterized by a significantly worse prognosis. This subset of patients is at risk to develop an aggressive high-grade non-Hodgkin lymphoma, usually a diffuse large B-cell lymphoma termed Richter syndrome (RS), which is the final stage of CLL evolution (Hallek 2015; Rossi and Gaidano 2016; Tsimberidou and Keating 2005).

Given the wide heterogeneity in the clinical presentation, staging and prognostic assessment at the time of diagnosis are critical to anticipate the disease course and to allow proper monitoring. CLL staging system is based on the RAI (stage 0 to IV) and Binet (stage A-B-C) classifications (Binet et al. 1981; Rai et al. 1975) and is important to stratify patients, predict prognosis, and interpret treatment results. Both systems rely on and combine together clinical parameters such as lymphadenopathy, hepatosplenomegaly, anemia, and thrombocytopenia. However, they do not take into account known biologic and molecular features of CLL cells that could also predict survival and response to treatment (Cramer and Hallek 2011; Kay et al. 2007). Beside clinical staging, in fact, several prognostic markers are used in clinical practice to improve patient stratification and to help assessing the need for therapy and selecting the best treatment option (Hallek et al. 2008; Pflug et al. 2014). These include cytogenetic profile, mutational status of immunoglobulin

heavy chain variable gene (*IGHV*), and expression of cell surface markers. In particular, unmutated *IGHV*, ZAP-70, CD49d, and CD38 overexpression, as well as specific chromosomal aberrations, such as trisomy 12, 11q, and 17p deletion or disruption of *TP53* gene, are considered unfavorable genomic and molecular prognostic markers (International CLL-IPI Working Group 2016; Damle et al. 1999; Dohner et al. 2000; Ghia et al. 2003; Rassenti et al. 2004). The advent of next generation sequencing (NGS) further implemented our knowledge of CLL mutational landscape by identifying previously unrecognized, recurrently mutated genes. Among others, mutations of *NOTCH1*, myeloid differentiation primary response 88 (*MYD88*), splicing factor 3b subunit 1 (*SF3B1*), and ataxia telangiectasia mutated (*ATM*) have offered new prognostic information and impacted CLL management, although the characterization of their functional contribution to CLL pathogenesis is still only partially understood and currently under investigation (Baliakas et al. 2015; Campregher and Hamerschlak 2014; Landau et al. 2015; Puente et al. 2011; Quesada et al. 2011).

Two essential features characterize CLL pathogenesis: first, an intrinsic genetic profile given by the different cytogenetic aberrations and genetic lesions impacting on cell homeostasis and genomic stability, and therefore likely promoting progressive accumulation of mutations (Landau et al. 2015). The most important in this respect are p53 lesions (mutations or 17p deletions) that disrupt the master regulator of DNA repair, cell proliferation, and apoptosis (Mohr et al. 2011). Among genetic defects, *SF3B1* mutations might be also linked to genomic stability and epigenetic modification, since evidence of altered pre-mRNA splicing has been detected in CLL cases with this genetic defect (Wan and Wu 2013).

Second, CLL cells display a characteristic dependence on extracellular stimuli coming from the microenvironment for survival and proliferation (Choi et al. 2016). Signals include those received through (auto)antigens via B-cell receptors (BCR), CD40/CD40L receptor-ligand pair, insulin-like growth factor, NOTCH1, chemokine- and toll-like receptors (Arruga et al. 2014; Burger 2010; Burger and Chiorazzi 2013; Lee et al. 2005; Muzio et al. 2009; Schattner 2000). In particular, NOTCH1 signaling is halfway between the genetic and the microenvironment-dependent pathogenic mechanisms. In fact, *NOTCH1* is one of the most recurrently mutated genes in CLL at diagnosis and is associated with aggressive/progressive chemorefractory disease (Villamor et al. 2013). Mutations impact on protein stability and prolong signaling, but the binding to the ligand, expressed by neighboring stromal cells, is indispensable to activate NOTCH1 pathway, even in the presence of mutations (Arruga et al. 2014). Activated NOTCH1 in turn contributes to remodeling of gene expression in leukemic cells through both a direct transcription-mediated regulation or by unbalancing nuclear protein interactions and fine-tuning of epigenetic mechanisms (Arruga et al. 2016). There is strong evidence that the microenvironment of the BM and secondary lymphoid organs exerts a protective effect on CLL cells, supported, for example, by the observation that CLL cells accumulate in vivo but invariably undergo spontaneous apoptosis in vitro, despite supporting culture conditions (Collins et al. 1989). Collectively, the pro-survival signals mentioned above attenuate the apoptotic network of CLL cells skewing towards survival. In line with this, apoptosis failure is considered a major

component in the dysregulation of B-cell homeostasis in all CLL subsets (Keating 1999). Of note, many pathways integrating extracellular survival stimuli ultimately converge on NF-κB activation that mediates CLL survival by driving the expression of anti-apoptotic genes, such as Bcl-XL and MCL1, which mediate resistance against p53-induced apoptosis (Leu et al. 2004; Pepper et al. 2009; Schott et al. 1995).

2 Treatment Options for CLL Patients

Treatment of CLL ranges from an observational approach with therapy indicated only in the presence of symptoms (the "watch and wait" approach) to a variety of therapeutic options, including alkylating agents, purine analogs, combination chemotherapy, monoclonal antibodies, tyrosine-kinase inhibitors, and transplant options (Gribben and O'Brien 2011). However, except for allogeneic BM transplants, which are infrequently considered an option for elderly patients, current treatments are not proven to be curative, making CLL a still incurable disease (Cassaday et al. 2015; Dreger et al. 2013). Since the probability of progression may vary from patient to patient, even in the presence of adverse prognostic markers at diagnosis, frequent and careful observation is required to monitor clinical course. Treatment of infectious, hemorrhagic, or immunologic complications is also part of the therapeutic management of CLL (Cooperative Group for the Study of Immunoglobulin in Chronic Lymphocytic Leukemia 1988; Kaufman et al. 2009; Mauro et al. 2000). Therapeutic decisions are essentially made on the basis of disease stage and the presence of clinical, cellular, and genetic markers. In general, treatment is delayed for as long as possible, until the patient becomes symptomatic or there are signs of bone marrow failure (Gribben and O'Brien 2011). The current treatment scenario for CLL includes different options, with significant variations between North America and Europe, due to a delay in the availability of targeted therapies in Europe, outside of clinical trials.

1. *Chemoimmunotherapy*. The current recommended frontline treatment of CLL includes a combination of cytotoxic chemotherapy together with a monoclonal antibody directed against CD20, an antigen selectively expressed on B-lymphocytes. Pivotal studies have shown better results coming from the combination of chemotherapy and immunotherapy compared to the same agents used alone. The combinations vary depending on the subset of patients: for younger and fit patients the most common choice is fludarabine, cyclophosphamide (FC), and rituximab (FCR), where the addition of the anti-CD20 monoclonal antibody resulted in a significant prolongation of the overall survival, compared to FC alone (Hallek et al. 2010; Robak et al. 2010). In elderly patients with major comorbidities, a combination of obinutuzumab (GA101, the novel humanized and glycoengineered anti-CD20) together with chlorambucil (G-Clb) was demonstrated to be effective and well tolerated (Goede et al. 2014, 2015). For the intermediate subset of elderly patients, with mild comorbidities, both the combination between bendamustine plus rituximab (BR) or dose reduced FCR

are therapeutic options considered feasible and effective (Eichhorst et al. 2016; Fischer et al. 2012; Foon et al. 2009, 2012). The presence of comorbidities raises the possibility of toxicities associated with FCR treatment, that are mainly represented by the high risk of infections due to severe myelosuppression, making FCR an unsuitable regimen for CLL patients >65 years. For its better tolerability, the combination of BR has been widely adopted as an alternative regimen in elderly patients (Eichhorst et al. 2016). However, FCR and BR are not indicated for patients with serious comorbidities. The majority of patients are diagnosed with CLL at an advanced age and, by the time the patients need therapy, they could have acquired additional medical comorbidities limiting their quality of life and performance status (Baumann et al. 2014). The goal for these patients is to choose a therapy that controls disease-related manifestations, at the same time preserving quality of life. In this respect, the G-Clb combination is a less intense regimen, proven to prolong survival (Goede et al. 2014).

2. *Ibrutinib*. Ibrutinib is an orally bioavailable selective and irreversible inhibitor of the Bruton tyrosine kinase (BTK), an essential player of the BCR signaling cascade (Byrd et al. 2013). It was recently approved for frontline use in CLL patients based on the randomized, multicenter, open-label phase 3 RESONATE-2 trial of ibrutinib vs. chlorambucil, in which ibrutinib achieved a 91% reduction in the risk for disease transformation and an 84% reduction in the death risk compared to chlorambucil (Burger et al. 2015). A significant number of clinical trials are still investigating the efficacy of ibrutinib both as a single agent and in combination in different subset of CLL patients (www.*clinicaltrials.gov*). Ibrutinib induces rapid and durable lymph node responses both in previously untreated CLL patients and in patients with relapsed or refractory CLL (Byrd et al. 2015; O'Brien et al. 2014). Similar to other kinase inhibitors developed for CLL therapy, ibrutinib interferes with the protective effect of stromal cells, prevents lymphocyte adhesion and homing, and interrupts the network of microenvironment-dependent stimuli by acting on leukemic cells (Wiestner 2012). For these reasons, during the first weeks of therapy, patients can undergo transient and massive lymphocytosis due to redistribution of CLL cells from the lymphoid niches to PB. This should not be confused with disease progression and should not lead to discontinuation of the drug (Woyach et al. 2014b). Although ibrutinib is currently approved for frontline therapy, particularly in CLL patients with 17p deletion, it must be taken into account that this therapeutic choice commits the patient to lifelong therapy and that continuous therapy started in a young patient can pose several issues, including lack of compliance with a daily oral medication and the possibility of long-term toxicities that have not yet been determined (Barrientos 2016).
   Novel btk inhibitors are currently in development and in clinical trials, showing promising effects as the non-specific effect on other Tec family kinases has been significantly reduced (Byrd et al. 2016; Herman et al. 2016; Walter et al. 2016; Wu et al. 2016).
3. *Idelalisib*. Idelalisib (CAL-101 or GS-1101) is an oral selective and reversible inhibitor of the p110δ isoform of the PI3K (Lannutti et al. 2011). In CLL, PI3K is a critical player in the BCR signaling cascade, but also integrates signals

coming from other cell-surface receptor, like CD40, CXCR4, and integrins (Herman et al. 2010; Hoellenriegel et al. 2011). As part of these pathways, PI3K also influences B cell trafficking by promoting chemotaxis towards a chemokine gradient, migration beneath stromal cells, and upregulation of chemokine secretion by CLL cells (Okkenhaug and Vanhaesebroeck 2003; Srinivasan et al. 2009). In the context of CLL pathogenesis, prosurvival and proliferative signals stimulated by BCR activation and stromal cell contact may be amplified by the convergence on common downstream players including PI3K (Brown 2016). Similar to ibrutinib, idelalisib is associated with early lymphocyte redistribution and mobilization from lymphoid tissues to blood circulation, with increased lymphocyte counts associated to nodal response (Cheson et al. 2012). Given that the lymphocytosis is often a persistent condition, a number of studies were designed based on the combination of agents, such as rituximab, in order to progressively abrogate lymphocytosis and to meet traditional IWCLL response criteria. For example, the idelalisib plus rituximab regimen is FDA approved since 2014 for relapsed CLL patients for whom rituximab would be an appropriate treatment option (Furman et al. 2014). In contrast, first-line treatment with idelalisib is not recommended given the high rate of toxicity, including an increased risk of opportunistic infection (Lampson et al. 2016).

4. *Venetoclax*. Venetoclax (ABT-199) is an orally administered small molecule that was recently FDA approved as monotherapy for second-line treatment of CLL patients who have relapsed or have been refractory to previous treatments and have *TP53* defects (Stilgenbauer et al. 2016). It is a selective inhibitor of the prosurvival protein BCL-2 and therefore acts by restoring the apoptotic machinery of malignant cells. BCL2 over-expression and activation are typical of the CLL founder clone and its selective inhibition by venetoclax acts on cancer cells in a TP53-independent way (Roberts et al. 2016). As for kinase inhibitors, venetoclax has been tested in trials as a continuous treatment, with all the potential implications on long-term safety, drug interactions, quality of life, compliance to treatment, and economic sustainability, which remain incompletely determined. In a phase 1 dose-escalation study, Roberts and colleagues observed a significant antitumor activity of daily oral venetoclax in patients with relapsed or refractory CLL. However, important side effects were reported to occur in a significant proportion of patients and including tumor lysis syndrome (prevented upon dose adjustment), mild diarrhea (in 52% of the patients), upper respiratory tract infections (in 48%), nausea (in 47%), and grade 3 or 4 neutropenia (in 41%) (Roberts et al. 2016). Nevertheless, in relapsed and refractory CLL with TP53 disruption, the response rates achieved by venetoclax treatment is so far the highest (excluding allogeneic stem-cell transplantation), and potentially translates into durable remissions (Rossi 2016).

Other novel treatment options, current trials, and future directions will be discussed later in the chapter.

3 Resistance to Chemotherapy and Chemoimmunotherapy: The Role of p53, NOTCH1, and Other Genetic Lesions

Despite the availability of different therapeutic options and novel targeted agents, *TP53* dysfunctions remain the most important adverse prognostic factor and have been shown to provide the most powerful predictive information on clinical outcome and on therapy responsiveness. Several studies indicated *TP53* aberrations as a negative prognostic factor, independent of other important negative features, and they represent the most important risk factor for treatment-free survival (TFS) and overall survival (OS). Thus far, *TP53* mutations or loss, as a result of 17p deletion, are the prognostic markers most influencing therapy decisions (Rosenquist et al. 2013; Te Raa and Kater 2016; Zenz et al. 2010). *TP53* alterations in CLL are most frequently represented by biallelic defects such as a deletion (17p-) in one allele together with a gene mutation on the other allele, although *TP53* mutations without a concurrent chromosome deletion are observed in ~30% of cases (Malcikova et al. 2009; Zenz et al. 2008b). Therefore, *TP53* status should be routinely analyzed both by FISH and by sequencing (Pospisilova et al. 2012). However, survival seems to be equally poor for patients carrying a biallelic defect or either the deletion or the mutation alone (Zenz et al. 2008a). More recently, given the increased prevalence of adverse outcome genetic defects in more advanced disease, NGS analysis has been used to improve the prognostic power of *TP53* alterations in the context of clonal evolution. The question here is whether more aggressive and chemoresistant subclones are already present at early stages of the disease, and are progressively selected, or whether they evolve and acquire driver defects during the treatment phase (Malcikova et al. 2014).

TP53 dysfunctions are a common feature in the majority of human cancers. The p53 protein is known as "the cellular gatekeeper" as it acts to transmit stress-inducing signals to antiproliferative cell responses. After activation as a consequence of DNA damage, oncogene signaling, or hypoxia, it subsequently orchestrates a wide spectrum of mechanisms including apoptosis, cell cycle arrest, senescence, or autophagy (Zilfou and Lowe 2009). In CLL, p53 is activated upon DNA damage through the activation of ATM and induces cell-cycle arrest through p21 and apoptosis through different targets including Puma. In addition, miR-34a is a direct transcriptional target of p53, and induces apoptosis and cell-cycle arrest through silencing of its potential targets as cyclin-dependent kinases 4 and 6, CCND1, MYCN, BCL2, and Sirtuin1 (Zenz et al. 2009) (Fig. 1). In keeping with its role as pivotal regulator of the DNA damage response, alterations of *TP53* are associated with resistance to conventional chemotherapy (i.e. fludarabine, cyclophosphamide) that target essentially every dividing cell by inducing double strand breaks (DSBs) (Te Raa and Kater 2016). Accordingly, none of the chemoimmunotherapy combinations that are available as frontline therapy for CLL has shown evidence of sustained clinical activity in patients with 17p deletion or in the presence of *TP53* mutations. As shown by the CLL8 trial, the FCR regimen, with the addition of rituximab to fludarabine and cyclophosphamide, significantly improved CLL management and was shown to induce long-term remission and prolong OS in the majority of CLL patient except for those with *TP53* defects (Hallek

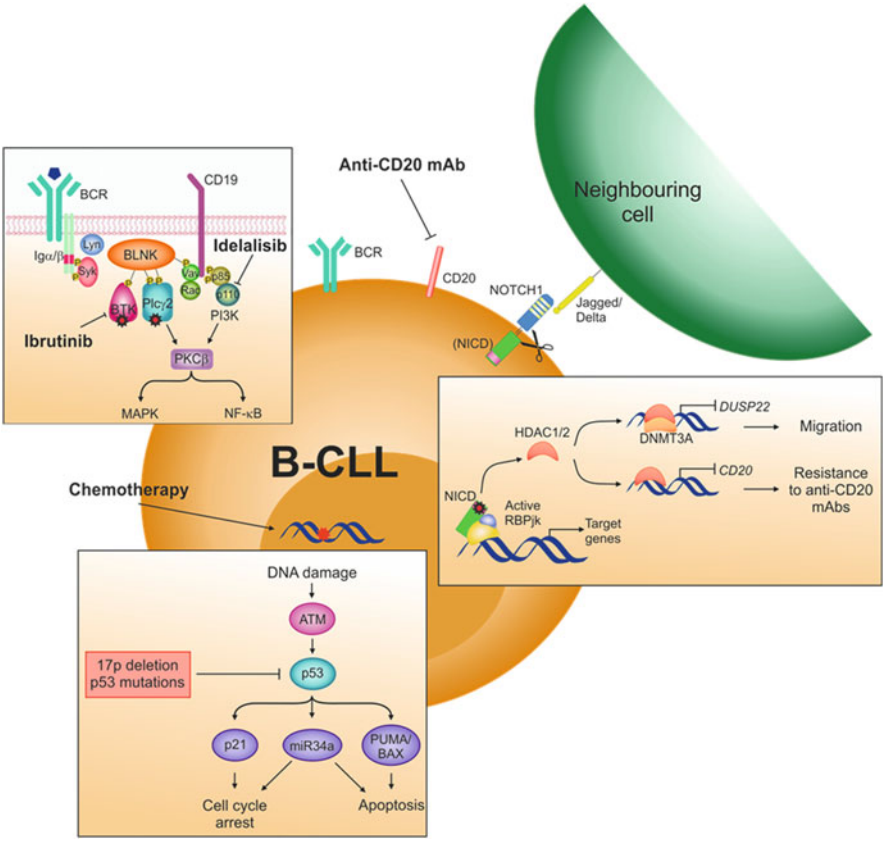

Fig. 1 Schematic view of the main molecular mechanisms that mediate therapy resistance in CLL. The upper box shows the BCR signaling pathway with drugs targeting key downstream players (e.g., ibrutinib and idelalisib). The red/black stars represent mutations of btk and Plcγ2, which represent the most frequent mechanisms of ibrutinib resistance. The box in the middle shows the effects of NOTCH1 pathway activation. Therapy resistance is the result of expression of NOTCH1 target genes and of gene expression remodeling mediated by HDAC1/2 displacement from RBP-jk complex. The red/black star indicates mutations in NOTCH1 PEST domain, which further exacerbate the effects. The bottom box shows the signaling cascade activated upon chemotherapy-mediated DNA damage. Activation of p53 is the master regulator of cell fate and genetic lesion disrupting the "cellular gatekeeper" are indicated

et al. 2010). Specifically, the FCR arm showed a median progression-free survival (PFS) of 51.8 months, compared to the PFS of 32.8 months in the FC group. FCR treatment didn't significantly modify the outcome in patients with 17p deletion and p53 mutations, as the median PFS was 11.3 and 15.4 months for FCR and FC, respectively (Stilgenbauer et al. 2014). A promising alternative strategy was represented by alemtuzumab, a recombinant, humanized, monoclonal antibody directed against CD52, and thus targeting both B and T lymphocytes. Alemtuzumab was found to be more effective than rituximab as a single agent in first-line therapy of

CLL and was believed to be effective also in the presence of TP53 defects (Lundin et al. 2002; Stilgenbauer and Dohner 2002). However, subsequent trials did not confirm that initial observation. Moreover, even if addition of alemtuzumab to fludarabine and cyclophosphamide resulted in a prolonged PFS in patients with high risk cytogenetics, this was not observed in 17p- patients showing a median PFS comparable to that observed in the CLL8 trial in patients with *TP53* defects (Geisler et al. 2014).

Different studies support ibrutinib as frontline treatment for patients with 17p- or *TP53* mutations (Byrd et al. 2013, 2015; O'Brien et al. 2016). The trial from Byrd et al., that included high-risk patients with relapsed or refractory CLL previously treated, showed a PFS at 30 months of 69%, with a median of 28 months for 17p- patients and 38.7 months for 11q– subjects. Despite the inferior PFS observed in patients with 17p-, compared to patients without this alteration, results are however significantly more promising than chemoimmunotherapy. For this reason ibrutinib is now approved for frontline use in this category of patients. Also, combination trials of ibrutinib with monoclonal antibodies are currently ongoing to determine whether addition of rituximab or other anti-CD20 antibodies could change long-term prognosis compared to ibrutinib monotherapy (Barrientos 2016).

It must be said that the response duration in CLL patients with *TP53* alterations is still very short even in the case of agents acting independently of p53 pathway, such as monoclonal antibodies. This is most likely because none of the therapeutic strategies currently used are able to achieve a complete eradication of the leukemic clone and small subclones of CLL cells, protected by microenvironmental niches, are prone to expand more rapidly when having a p53 defect (Malcikova et al. 2014).

Beside the well-defined role of TP53 defects in chemoresistance, other factors are emerging as markers of therapy resistance, even if their clinical significance is still a matter of debate and their biological mechanisms of action are only partially understood. Screening of recurrently mutated genes in fludarabine-refractory CLL (FR-CLL), combining approaches of whole-exome sequencing (WES) and copy number aberration (CNA) analysis, revealed that the majority of these patients carry mutations in genes that were recently recognized as recurrently mutated, including *NOTCH1* (~25%), *SF3B1* (~20%), and *BIRC3* (~15%) (Messina et al. 2014). These mutations are unfrequently simultaneous in the leukemic population, being more likely mutually exclusive within each other and with TP53 defects, and representing independent negative factors. Together with TP53 dysfunction they account for the majority of the genetic lesions underneath CLL chemorefractoriness. In particular, *BIRC3* abnormalities are specifically associated with a chemorefractory phenotype, as they were not observed in progressive, but fludarabine-sensitive, CLL. *BIRC3* disruption identifies patients with a poor outcome similar to patients with *TP53* defects and is associated with an abnormal microenvironment-independent activation of the noncanonical NF-κB pathway, which in turn sustains leukemic cell survival through the upregulation of anti-apoptotic genes (Rossi et al. 2012).

SF3B1 and *NOTCH1* mutations cluster in few spots within the gene and affect a relatively high number of patients (Fabbri et al. 2011; Wang et al. 2011). *SF3B1* is part of the splicing machinery and its mutations are frequently observed with

concurrent alternative splicing isoforms of protein-coding and non-coding genes, although SF3B1 functions and impact on RNA processing are still under investigation (Furney et al. 2013). In CLL, *SF3B1* mutations are enriched in the fludarabine-refractory cases, as their frequency was found significantly increased compared to CLL samples collected at diagnosis (Rossi et al. 2011). Mutations are somatically acquired and are generally represented by missense mutations clustering in three main hotspots (codons 662, 666 and 700). Given its role in pre-mRNA splicing, SF3B1 may contribute to CLL and chemoresistance through the generation of alternatively spliced transcripts. Recently, it was demonstrated that aberrant splicing, resulting from *SF3B1* mutations, may impact on multiple pathways that play a relevant role in CLL homeostasis, such as DNA damage response, telomerase activity, and NOTCH1 signaling (Wang et al. 2016). The final result is the accumulation of genetic lesions and a gene setting promoting leukemic cell survival. In particular, by perturbing the correct DNA damage response or by deregulating NOTCH1 activity through the disruption of the negative regulator *DVL2*, *SF3B1* mutations induce a phenotype largely similar to that of TP53 dysfunction or NOTCH1 mutations, respectively. The pleiotropic effects of *SF3B1* mutations, impacting on cellular processes that are frequently deregulated by genetic abnormalities in CLL, may also explain why *SF3B1* mutations, *TP53* defects, and *NOTCH1* mutations are mutually exclusive (Rossi et al. 2011).

Among the genetic lesions described in CLL, *NOTCH1* mutations represent the most frequent alteration, with prevalence increasing if we consider chemorefractory CLL. Mutations cluster at the exon 34 of the gene and hit the PEST domain, a region rich in proline, serine, and threonine residues. More recently, mutations in the the 3′ region (3′UTR) of NOTCH1 were also identified. These alterations cause aberrant splicing events, increase NOTCH1 activity, and result in a more aggressive disease (Puente et al. 2015). In a significant proportion of cases, *NOTCH1* mutations in CLL are represented by a 2 bp frameshift deletion at position 7,541–7,542 (c.7541-7542delCT), leading to the formation of a premature STOP codon, and resulting in a truncated protein. The final result is the loss of the PEST domain, that is determinant for the correct protein ubiquitination and degradation. For this reason, the mutations described in CLL are considered stabilizing mutations, rather than activating, as the resulting protein is predicted to remain transcriptionally active in the nucleus for a longer time (Arruga et al. 2014; Fabbri et al. 2011). In fact, NOTCH1 signaling is induced upon ligand binding, an event required also in the presence of PEST domain mutations, that triggers subsequent proteolytic cleavages resulting in the release of the intracellular portion of the protein (NICD). The NICD is the active form of NOTCH1 and acts as a transcription modulator through the interaction with the RBP-jk complex in the nucleus (Castel et al. 2013). On one side, this interaction activates a specific transcription pattern having *HES1* and *DTX1* as the most representative targets. On the other side, by displacing the repressor molecules bound to RBP-jk, NOTCH1 acts by remodeling nuclear protein interactions in a domino effect that in the end globally affects the genetic setting of the leukemic cell (Arruga et al. 2016; Pozzo et al. 2016). From a functional perspective, NOTCH1 mutations render CLL cells more

resistant to spontaneous or drug-induced apoptosis (Rosati et al. 2009; Secchiero et al. 2009). In particular, NOTCH1 activation by microenvironmental stimuli, mirrored in vitro by culturing CLL cells in the presence of nurse-like cells expressing NOTCH1 ligands, may favor chemoresistance and protect leukemic cells by fludarabine-induced apotosis, selectively in mutated cells, a condition that can be reverted by specific NOTCH1 inhibitors (Arruga et al. 2014). The association of *NOTCH1* mutations with poor response to therapy is also supported by the observations of the CLL8 trial that pointed the attention to the fact that, beside being more prone to undergo fludarabine resistance, *NOTCH1*-mutated patients did not benefit from the addition of rituximab, as the responses in the FC vs. the FCR arm of the trial were almost identical (Stilgenbauer et al. 2014). Clinical data are sustained by biological evidences that *NOTCH1*-mutated CLL cells express lower levels of CD20 on cell surface, and can therefore escape rituximab. Consistently, mutated cells are less efficiently lysed upon anti-CD20 exposure in vitro. Both phenotypes are rescued upon treatment of leukemic cells with γ-secretase inhibitors, small molecules acting on the key enzyme in NOTCH1 activation cascade. NOTCH1 is directly involved in the regulation of CD20 expression and it operates through the unbalance of nuclear protein interactions. In fact, it is known that in the absence of the NICD, RBP-jk is bound to negative regulators that repress its transcriptional activity. Among the negative interactors, the histone deacetylases (HDAC1 and HDAC2) play a fundamental role. The acetylation status of a chromatin region determines its accessibility for transcription and deacetylation results in a more condensed, less accessible, chromatin. When the NICD is released from the cell membrane and translocates to the nucleus, it binds RBP-jk and displaces the repressor complex. In particular, free HDACs can be recruited elsewhere in the genome and exert their epigenetic regulation on other targets. It was demonstrated that, in the presence of the NICD and particularly of a PEST mutated NICD, that persists in the nucleus for longer, HDACs were less complexed with RBP-jk and more bound to the CD20 promoter, resulting in epigenetic silencing of gene expression (Pozzo et al. 2016). This NICD-dependent epigenetic modulation of gene expression is likely to affect several targets and may contribute to CLL evolution by perturbing diverse cellular processes. Among them, the tumor suppressor gene *DUSP22* has been recently identified as a target, as its expression is downmodulated in the presence of an active NOTCH1 signaling through a methylation-dependent mechanism. In fact, the HDACs availability impacts on the activity of the DNA methyltransferase 3A (DNMT3A), being higher when the enzyme is complexed with HDACs. Consistently, in the presence of a PEST mutated NICD, *DUSP22* promoter is more methylated as the result of an increased amount of the HDAC-DNMT3A complex. *DUSP22* downregulation leads to constitutive MAPKs and STAT3 signaling and promotes leukemic cell growth and CCL19-driven chemotaxis (Arruga et al. 2016) (Fig. 1). As CCL19 regulates homing to lymphoid organs, it is conceivable that *NOTCH1* mutations might favor CLL recirculation to lymph nodes and spleen, where the local environment favors proliferation and protection from spontaneous and drug-induced

apoptosis, two conditions that are associated with more aggressive disease and unfavorable prognosis.

4 From Mechanisms of Resistance to Novel Therapeutic Approaches

Therapeutic opportunities for CLL patients have improved significantly with the advent of BCR signaling antagonists, such as ibrutinib and idelalisib. Before the approval of these agents, patients with relapsed disease or with evidence of a more aggressive disease had a generally poor outcome with standard chemoimmunotherapy. Stem cell transplantation can be an option for young and fit patients, but it can't be adopted as a strategy for the majority of CLL patients. Nevertheless, although both ibrutinib and idelalisib can induce rapid and durable remission, there are patients who fail to respond or who relapse even after long periods of remission (Woyach 2015).

Two main mechanisms underlying resistance to ibrutinib have been identified and observed in multiple CLL patients; in contrast, mechanisms of resistance to idelalisib are still to be fully elucidated. Whole exome sequencing, performed in six patients with late relapse after ibrutinib, revealed the presence of acquired mutations in BTK at the binding site of the inhibitor (C481) in which a cysteine was mutated to serine. In one patient, multiple mutations in phospholipase Cγ2 (PLCγ2; R665W, L845F and S707Y), the kinase immediately downstream of BTK, were identified (Woyach et al. 2014a). Functionally, the C481S mutation reduces the binding affinity of ibrutinib for BTK resulting in a reversible rather than irreversible inhibition. Given the relatively short half-life of the molecule, the result is only a transient inhibition of BTK phosphorylation. This mutation was confirmed in multiple patients, who relapsed under ibrutinib (Cheng et al. 2015) (Fig. 1). Recently, a novel BTK mutation potentially driving ibrutinb resistance has been described in a case report. Serial analysis of samples collected through the patient's clinical course pointed out a mutation at codon 316 post ibrutinib relapse. The mutation consists in the substitution of a threonine with an alanine (T316A) in the SH2 domain of BTK. However, this is not the kinase domain and it doesn't apparently directly interfere with ibrutinib binding to BTK, making the mechanism of resistance through this mutation still unclear (Sharma et al. 2016).

On the other hand, PLCγ2 mutations have been demonstrated to be potentially gain of function, with a consequent pathway activation overcoming the presence of inactive BTK (Liu et al. 2015; Zhou et al. 2012). More recently, additional CLL patients who relapsed after ibrutinib were genetically characterized using targeted deep sequencing, both at baseline and at relapse, and were all found to harbor mutations in BTK at C481S or in PLCγ2 (Maddocks et al. 2015) (Fig. 1). Importantly, while the association between BTK or PLCγ2 mutations and CLL progression was clear, they did not associate with increased risk of Richter transformation. Another study reported two patients who relapsed on ibrutinib but without BTK or PLCγ2 mutations, suggesting that there might be other mechanisms that can lead to

relapse (Woyach and Johnson 2015). Primary resistance to ibrutinib has been rarely observed and no mechanism has yet been identified. However, it is likely that activating mutations in central pathways such as MAPK or PI3K signaling might render a patient less sensitive to ibrutinib in the early phases of therapy.

CLL progression and relapse during ibrutinib therapy is, therefore, most likely the result of mutations in BTK or PLCγ2. What remains to be determined is whether these mutations are already present at the subclonal level when treatment is started, leading to the slow and progressive selection during ibrutibib, or whether they are acquired during therapy, as a result of drug-induced pressure. Computational evolutionary models suggest that resistance mutations should already be present before ibrutinib administration, but results in CLL samples remain controversial (Komarova et al. 2014). In fact, in some studies where mutations of BTK or PLCγ2 were identified as responsible for resistance, deep sequencing was performed in PB samples at baseline as well, and no mutations were found (Cheng et al. 2015; Liu et al. 2015; Maddocks et al. 2015; Woyach et al. 2014a; Zhou et al. 2012). In addition, another independent study reported deep sequencing in a large cohort of ibrutinib-naïve patients without finding mutations in BTK (Fama et al. 2014). However, these data suggest that BTK mutations are not commonly found in PB prior to ibrutinib exposure, but they do not exclude that mutations might be present in very small clones or in niches other than PB. Several groups studied and dissected evolution of ibrutinib resistance by using whole-exome and deep-targeted sequencing in small cohorts of serial CLL samples. Taking advantage of droplet-microfluidic technology, allowing single cell analysis, Burger et al. demonstrated the presence of ibrutinib resistant subclones before treatment initiation, supporting the idea that therapy may select and promote expansion of rare resistant subclones (Burger et al. 2016). Accordingly, in a recent study, Ahn et al. published results from a phase 2 clinical trial with ibrutinib as a single agent in a cohort of 86 patients and showed that mutations in BTK or in PLCγ2 could be detected up to 15 months before clinical manifestations of progression and resistance. Moreover, in a small group of patients, they described the presence of multiple subclones carrying different independent mutations, suggesting subclonal heterogeneity of resistant disease (Ahn et al. 2017).

Resistance to idelalisib has not yet been fully elucidated from the biological point of view and is currently under investigation, although some mechanisms of resistance and potential alternative targets have been proposed. Idelalisib selectively targets the p110δ isoform of the PI3K, that is encoded by the *PIK3CD* gene. A mechanism of resistance that is likely to occur is the upregulation of either *PI3KCD* or an alternative class 1A PI3K such as *PIK3CA* or *PIK3CB*. Research performed in cell line models of mantle cell lymphoma, for example, highlighted that a higher ratio of *PIK3CA*/*PIK3CD* predicts resistance to selective p110δ inhibition (Iyengar et al. 2013). Similar results were obtained in a breast cancer model harboring mutations of *PIK3CA*, where resistance to PI3Kα inhibitor was mediated by the upregulation of *PIK3CA*, so that signaling pathway and PI3K functions were maintained even in the presence of the inhibitor at saturating concentrations (Huw et al. 2013). Other studies in breast cancer models, performed

with PI3Kα inhibitors, observed and proposed other potentially relevant mechanisms of resistance outside the drug target, and having as a major player MYC amplification (Muellner et al. 2011). However, none of the mentioned mechanisms have yet been verified in CLL.

Mechanisms of resistance to venetoclax have not yet been described as well, also in consideration of the relatively recent entry in clinical practice. It must be considered, however, that the BCL2 family is composed of several members and therefore the most likely mechanism of resistance would be the upregulation of an alternative member of the family, such as BCL-XL, BCL-W, MCL1, or BCL2A1 (Youle and Strasser 2008). This hypothesis is also supported by evidence that ABT-737, an inhibitor of both BCL2 and BCL-XL, induced resistance in vitro by upregulating BCL-XL and BCL2A1 (Vogler et al. 2009). Similarly, other studies describe upregulation of BCL-XL or MCL1, as well as autophagy, as mechanisms underlying resistance to venetoclax (Woyach and Johnson 2015). Furthermore, acquired resistance in vitro has also been described after prolonged exposure to venetoclax and the appearance of mutations in the BCL2 BH3 domain and in BAX has been reported (Fresquet et al. 2014).

Strategies to overcome resistance to these agents are currently under establishment, but since they show distinct mechanisms of actions, the switch among the therapies is one of the first possibilities to be considered.

5 Microenvironment and Therapy Resistance

The dependence of cancer cells on a tumor microenvironment that promotes cell growth and allows escaping from apoptosis and immune surveillance is a central hallmark of cancer. This protective niche is characterized by the presence of multiple elements including tumor-associated macrophages, tumor-infiltrating lymphocytes, or other stromal cells which provide various cytokines and chemokines sustaining cancer cells (Hanahan and Weinberg 2011). Tumor microenvironment is particularly important in CLL given the high dependence of leukemic cells on external factors for proliferation and survival. Indeed, whilst the fraction of $CD5^+$ leukemic cells circulating in the PB are arrested in the G0 phase of the cell cycle, those in the BM or residing in the lymphoid tissues proliferate at a rate of 0,1–1% of the total leukemic clone per day (Herndon et al. 2017). The CLL microenvironment in the lymphoid organs is organized in pseudofollicular structures named proliferation centers, focal aggregates of pro-lymphocytes and para-immunoblasts that represent the major sites of neoplastic cell proliferation (Soma et al. 2006). Proliferation centers are marked by the presence of Ki-67^+ proliferating CLL cells that interact with multiple signals coming from the heterogeneous non-neoplastic compartment. Beside the central role played by the BCR signaling, other features of CLL microenvironment are of relevance as they are part of a network of signals potentially driving disease evolution. CLL microenvironment is constituted by non-transformed elements that promote homing, retention, and proliferation of CLL through both soluble and contact-dependent mechanisms. These elements include principally stromal cells, monocyte-

derived nurse-like cells (NLC), and T-lymphocytes. On the other hand, CLL cells exert a role in microenvironment remodeling through immunomodulatory signals that promote evasion from immune surveillance and shape the stromal compartment. This network of bidirectional interactions favors the establishment of a progressively abnormal environment that sustains proliferation and survival of leukemic cells and, by protecting them from chemotherapy, maintains a reservoir of cells potentially accumulating novel genetic lesions from which disease relapse may occur (Audrito et al. 2013).

NLCs are similar to tumor-associated macrophages and, despite being primarily reported as an in vitro derived population, they have been also found in the lymph nodes and BM of CLL patients (Filip et al. 2013). NLCs support leukemic cells through the adhesion molecules and secrete chemokines and cytokines including the B-cell activating factor (BAFF) and a proliferation inducing lingand (APRIL). The final result is the upregulation of anti-apoptotic genes such as *BCL2*, *SURVIVIN*, *BCL2A1,* and *XIAP* that promote leukemic cell survival and escape from spontaneous and drug-induced apoptosis. Interestingly, this population can be differentiated from circulating monocytes only in the presence of CLL cells, suggesting a bidirectional effect and supporting the idea of a CLL-dependent microenvironment remodeling (Filip et al. 2015). For example, it was shown that CLL cells can produce, and secrete in the extracellular space, cytokines and cytokine-like molecules to polarize monocytes towards tumor-supportive M2 macrophages by activating MAPKs, STAT3 and NF-κB signaling (Audrito et al. 2015). Moreover, CLL can directly recruit NLC precursors through the secretion of CCL3 and CCL4 in response to signals triggered by the BCR activation and by CD38 (Aydin et al. 2008; Burger et al. 2009; Zucchetto et al. 2009). These findings suggest that the reciprocal cross-talk between the leukemic population and the surrounding microenvironment may occur not only through cell–cell contact but also through secreted factors acting in a paracrine way.

Similarly to NLCs, mesenchymal stromal cells (MSC) constitutively secrete chemokines that recruit CLL cells. This cell population represents the "feeder" layer for hemopoietic progenitor cells in physiological conditions and is commonly found in secondary lymphoid tissues in CLL patients (Choi et al. 2016). It was shown that, upon direct contact with leukemic cells, MSCs not only induce up-regulation of factors promoting CLL cell survival and proliferation, but they also favor a metabolic switch of leukemic cells by increasing glutathione synthesis and glycolysis through the NOTCH1-c-Myc axis, in turn impacting on cell survival and drug resistance (Jitschin et al. 2015; Purroy et al. 2015). Metabolic reprogramming represents a hallmark of malignant cells and the finding that stromal cells impact on the glucose dependency of leukemic cells, and that this mechanisms is mediated by oncogenic signaling pathways known to be deregulated in CLL, such as NOTCH1 and its target c-Myc, could be exploited therapeutically to overcome drug resistance. Furthermore, an increasing number of studies are focusing on the pro-survival Wnt5a/ROR1 pathway. Results have shown that MSCs express high levels of Wnt5a and that high levels of ROR1 on CLL cells are frequently associated with an accelerated disease progression (Cui et al. 2016; Fukuda et al.

2008) (Fig. 2). As for NLCs, the cross-talk between MSCs and CLL cells is at the basis of a bidirectional network of interactions in which MSCs sustain leukemic cells survival and proliferation and the CLL counterpart acts by remodeling stromal cells to be the utmost supportive. MSCs are activated by direct contact with CLL, for example through the induction of protein kinase C β II (PKCβII) expression and the subsequent activation of the canonical NF-κB pathway. Stromal NF-κB in turn regulates the expression and secretion of proinflammatory cytokines that are required for CLL survival, such as IL-1α and -1β, IL-6, IL-10, IL-15 (Lutzny et al. 2013). Moreover, CLL-derived vesicles such as exosomes contain proteins and microRNAs that can induce a pro-inflammatory phenotype in target cells, such as MSCs, enhancing proliferation, migration, and secretion of cytokines, further supporting the idea that cell–cell contact is not necessarily required for tumor–host interactions (Paggetti et al. 2015).

CLL cells in the lymphoid niches are also in close contact with T lymphocytes that provide critical regulatory signals (Bagnara et al. 2011). Activated $CD4^+$ T lymphocytes are found in proliferation centers adjacent to leukemic cells, likely supporting the idea of adhesion-mediated bidirectional interactions. Furthermore, CLL cells themselves can drive T cells to the lymph nodes by secreting factors such as CCL22, CCL3, and CCL4, again suggesting a direct role of leukemic cells in

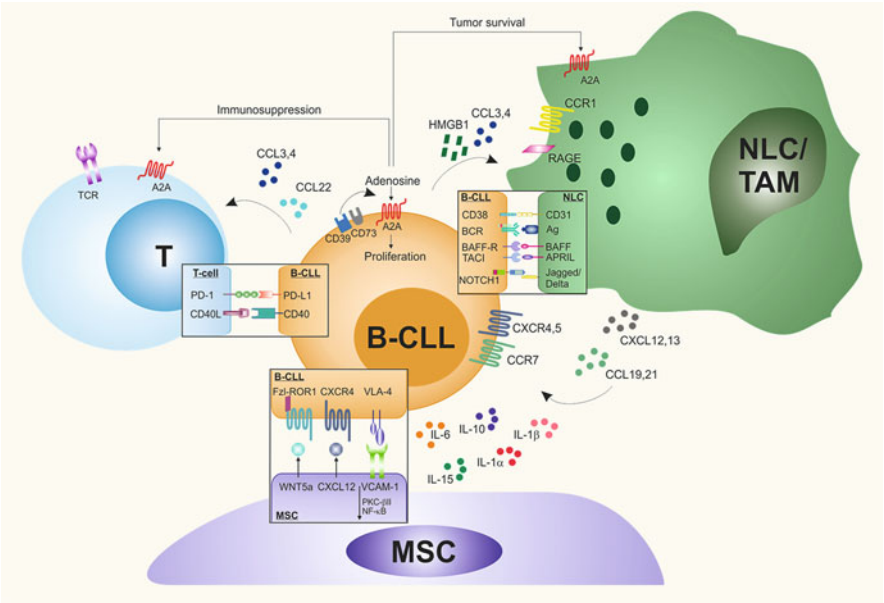

Fig. 2 Schematic view of the CLL microenvironment. In lymphoid niches, CLL cells interact with multiple partners, such as NLC, stromal cells (MSC), and T lymphocytes. All these elements provide signals promoting CLL survival and proliferation and release soluble mediators in the extracellular space, further sustaining leukemic cells. On the other side, CLL cells themselves reshape the microenvironment, by secreting chemokines and cytokines turning bystander cells into tumor-supportive elements

recruiting elements to shape the microenvironmental niche. T cells are thought to support CLL cells via the CD40L/CD40 axis that rescues leukemic cells from apoptosis by upregulating pro-survival and anti-apoptotic factors (Audrito et al. 2013; Gricks et al. 2004). In addition to a direct stimulation of leukemic cells, CLL T cells are notable for their functional defects as they are unable to form a fully functional immune synapse (Ramsay et al. 2008). Indeed, they show evidence of chronic activation and exhaustion with increased expression of markers such as CD244, CD160, and PD1. In particular, signaling through the PD-1/PD-L1 axis was shown to play an essential role in T cell dysfunction, with evidence of an active crosstalk between PD-1, expressed by $CD4^+$ and $CD8^+$ subsets, and PD-L1, expressed by the leukemic counterpart. This interaction in turn significantly decreases IL-4 and IFN-γ production and secretion by $CD4^+$ and $CD8^+$, respectively, with the final effect of a pronounced Th2 skewing of T-cell responses (Brusa et al. 2013). The consequence is impaired cytotoxicity that contributes to the generation of an immunosuppressive environment further promoting CLL survival and expansion. Targeting the PD-1/PD-L1 axis may be therefore of clinical interest to disrupt the immune tolerance of CLL microenvironment (Fig. 2).

T cell responses can be also restored by reprogramming autologous T lymphocytes to target specific tumor antigens following approaches of targeted adoptive cellular therapies. The most successful strategy was developed by fusing together an antibody-derived antigen-binding moiety with an internal signaling domain as CDRζ to form a chimeric antigen receptor (CAR). The advantages of CARs, compared to other T-cell-based therapies, reside mainly in the use of autologous T cells, avoiding the risk of graft versus host disease, and in the fact that the same CAR can be adopted to prime T cells of multiple patients. In a pilot study, autologous T cells, engineered to express CD19-targeted CAR and infused at low doses into CLL patients, were able to achieve a robust in vivo activation and to induce a persistent clinical response (Porter et al. 2011). Over the past years, an increasing number of reports by different groups have shown potent clinical activity using this treatment paradigm, including eradication of disease in late-stage patients with a variety of CD19-positive leukemias (Kalos 2016).

Immunomodulation as a therapeutic strategy is also at the basis of the use of lenalidomide in CLL treatment. Lenalidomide has a wide range of activities, including stimulation of T cells through CD28, up-regulation of cytokines such as IL-2 and IFN-γ, suppression of regulatory T cells and increase of NK- and antibody-mediated cytotoxicity (Riches and Gribben 2016). In addition, its effects appear mediated also by suppression of CLL proliferation and reduction of pro-survival factors in CLL microenvironment. A number of clinical trials are ongoing to determine its activity both as single agent or as part of combination strategies (Buhler et al. 2016; Liang et al. 2016; Wendtner et al. 2016).

Additional elements in CLL microenvironment include endothelial cells (ECs) and follicular dendritic cells (FDCs), which play an essential role for tissue homing and CLL retention within lymphoid tissues. Adhesion to endothelial cells promotes CLL cell survival, activation and drug resistance, primarily through integrin binding. For example, ligation of CD44 on CLL cells up-regulates Mcl1, a member of

the BCL2 family of anti-apoptotic proteins, and also interacts with other surface molecules such as CD38, CD49d, MMP-9, and ZAP-70 (Zhang et al. 2013). The CD100/PlexinB1 axis also appears to play a role in FDCs/CLL crosstalk (Granziero et al. 2003).

In addition to cell–cell contact, soluble factors may as well drive CLL survival and proliferation. For example, extracellular nucleotides and nucleosides, such as adenosine triphosphate (ATP) and adenosine (ADO), together with their receptors and the enzymes involved in their metabolism, may participate in creating favorable conditions that promote tumor growth and survival, while suppressing the host immune responses. It was demonstrated that the accumulation of ADO, as a consequence of the overexpression and activation of the ectoenzymes CD39 and CD73, creates local conditions that protect leukemic cells from spontaneous and drug-induced apoptosis. Furthermore, CD73-generated ADO inhibits migration of CLL cells towards CXCL12, in the sense that leukemic cells are attracted to lymph nodes by CXCL12, that provides a long-range signal, whereas local ADO induces a short-range stop signal that keeps cells in a growth-favorable environment (Serra et al. 2011, 2016) (Fig. 2). Targeting the adenosinergic axis might therefore have a considerable therapeutic impact in CLL, particularly to potentiate the effects of chemotherapy, as it was proposed in other disease models. For example, it was shown that the dual inhibition of CD73 and of the A2A ADO receptor might be of significant therapeutic benefit as it results in the restoration of the immune response, with increased immune infiltrated, and in a significant reduction of tumor growth and metastasis (Young et al. 2016).

6 Concluding Remarks and Future Directions

Over the past few years the treatment scenario for CLL patients was revolutionized with the development of targeted therapies that are progressively replacing conventional chemotherapy- and chemoimmunotherapy-based schedules. So far therapeutic responses obtained with these drugs are highly significant, at the price of a limited toxicity, even if none of these drugs appears to cure the disease.

A significant proportion of patients undergo therapy resistance, particularly as a consequence of long-time treatment or after several lines of therapies, that invariably results in disease progression and relapse. Another major limit is that conventional chemotherapy is not always a recommended choice, due to the presence of comorbidities, or because it's unlikely to work in the case of specific genetic lesions. The genetic background and the host microenvironment play a critical role in determining treatment outcome and disease progression and the identification of high risk patients represents a challenge in CLL management. As other tumor models, CLL is entering the era of the "precision medicine" where information about a patient's genetic background, protein functional characterization, and environment are used to improve the diagnostic classification and treatment options. Applied to CLL, NGS not only contributes to a better understanding of the genetic landscape of this complex disease, but is also acquiring a growing

prognostic power to discriminate subsets of patients that could or could not benefit from specific therapeutic interventions. Nevertheless, functional studies, new technologies, and experimental models are essential to validate findings, dissect the network of interactions within microenvironment, and identify potential therapeutic targets. The development and establishment of novel therapeutic strategies, tailored on specific biologic features of CLL, or based on synergies with available treatment options, could represent the way to overcome resistance and cure this disease.

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Mechanisms of Resistance to Targeted Therapies in Chronic Myeloid Leukemia

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Abstract

Patients with newly diagnosed chronic myeloid leukemia (CML) usually received as first-line treatment a first- or second-generation tyrosine kinase inhibitor (TKI). Although initial responses are high, therapy fails in up to 40% of patients and initial response is lost within 2 years in approximately 25% of patients. In the last few years, intensive efforts have been spent to explain

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treatment failure, and different mechanisms of resistance have been identified, ranging from BCR-ABL1 kinase domain mutations to lack of adherence to therapy. In this review, we briefly summarize the clinical efficacy of approved TKIs and describe the main mechanisms of TKI resistance.

Keywords
Chronic myeloid leukemia • Mechanisms of TKI resistance • Tyrosine kinase inhibitors

1 Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder due to an uncontrolled expansion of pluripotent hematopoietic cells. CML is characterized by a singular chromosomal abnormality, the t(9;22) translocation responsible for Philadelphia (Ph) chromosome generation (Rowley 1973). The resulting chimeric gene (BCR-ABL) (Shtivelman et al. 1985) codes for a constitutively activated tyrosine kinase (TK) (Lugo et al. 1990). In the last two decades, the prognosis of this disease has dramatically changed, thanks to the clinical approval in 2001 of imatinib (IM), the first BCR-ABL tyrosine kinase inhibitor (TKI). However, with the first imatinib-resistant cases, it became clear that Ph + cells could evolve to elude inhibition, leading investigators to intensive efforts in characterization of different mechanisms of resistance and subsequently in the development of novel inhibitors. In 2006 and 2007, second-generation TKIs were approved (nilotinib, NI, and dasatinib, DA) initially only for patients with CML intolerant or resistant to IM. More recently, due to their efficacy in obtaining faster cytogenetic and molecular response, NI and DA have been registered also for newly diagnosed CML (Kantarjian et al. 2010; Saglio et al. 2010). Bosutinib (BO) is the more recent second-generation TKI approved for CML patients resistant or intolerant to prior therapy (Cortes et al. 2012a), while ponatinib (PO), a third-generation TKI, is nowadays usable only in patients with T315I mutation or for those with unacceptable side effects emerging during treatment with DA or NI (Cortes et al. 2013).

These five drugs act occupying the nucleotide-binding pocket of the BCR-ABL protein and block the access to ATP (Fig. 1) with a consequent inactivation of the signal transduction pathways. The BCR-ABL kinase inactivity causes a transcriptional modulation of different genes involved in the control of the cell cycle determining the apoptotic death of Ph + cells (Druker and Lydon 2000; Hantschel et al. 2008; Liu and Gray 2006).

TKIs can be divided in two classes according to their molecular mechanism of action:

- Type 1 (DA and BO) target the ATP-binding site of the kinase in the catalytically active form.
- Type 2 (IM, NI, and PO) bind and stabilize the kinase domain in an inactive conformation.

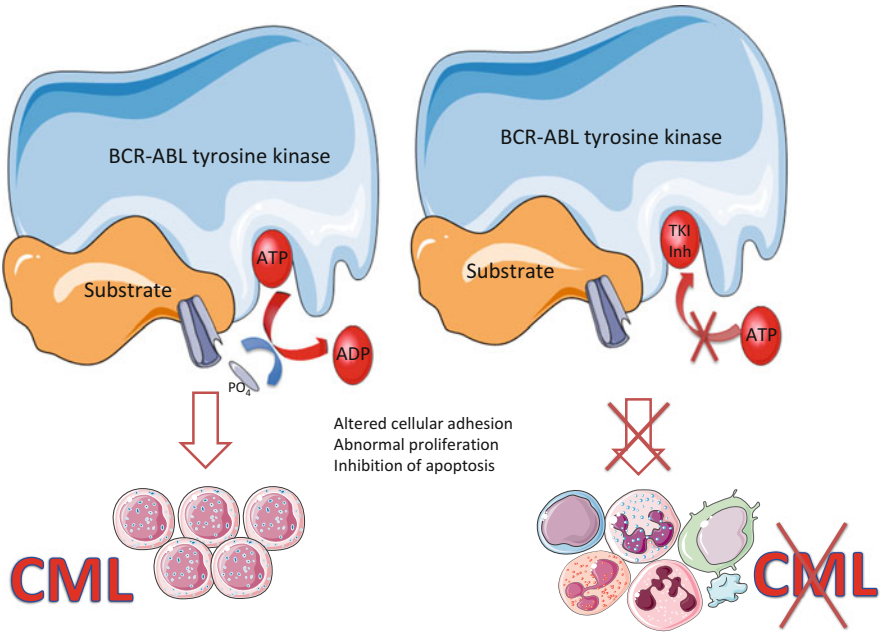

Fig. 1 Schematic representation of mechanism of action of TKI inhibitors

Despite the high rate of response using IM, NI, DA, and BO as frontline treatment of CML and TKIs, treatment failure occurs in some cases. According to European LeukemiaNet recommendations, the definition of TKI resistance has been defined as the inability to achieve an appropriate hematologic, cytogenetic, or molecular response at specific time points (primary resistance) or when the achieved response is lost (acquired or secondary resistance) (Baccarani et al. 2013). Different mechanisms potentially responsible for treatment failure have been described, such as mutations in the BCR-ABL domain, overexpression of BCR-ABL, elevated levels of the enzymes involved in the metabolism of TKIs, overexpression of drug influx and efflux pump, etc. In the daily clinical practice, a correct evaluation of the possible mechanisms of resistance is crucial to optimize the use of the different available TKIs with the key aim to prevent progression to the accelerated or the blastic phase of the disease.

2 Approved TKIs

2.1 First-Generation TKIs: Imatinib

The phase 3 International Randomized Study of Interferon and STI571 (IRIS) was the first clinical study showing a higher clinical activity and a lower toxicity of IM compared to interferon (IFN) plus cytarabine in CML (Hochhaus et al. 2009; O'Brien et al. 2003). In 2001, thanks to these results IM was approved for the

treatment of CML in any hematological phase. In the recent IRIS update, after a median follow-up of 10.9 years, about 65% of patients in the IFN arm switched to IM arm after a median duration therapy of 0.8 years. In the group of patients randomized to receive IM ($n = 553$), the rate of progression to the accelerated or blast phase was 6.9% with an estimated freedom from progression of 92%. The majority of events occurred during the first 4 years of treatment. The estimated event-free survival (EFS) and overall survival (OS) at 10 years were 80% and 83%, respectively. After 10 years of follow-up, 47% of patients are still alive receiving IM, 17.4% were alive not receiving the study drug, 15.6% died, and 20% had unknown survival status. About 25% of patients had cytogenetic assessment at 10 years; among these patients the complete cytogenetic response (CCyR) rate was 92%, while among 204 patients (40%) who had molecular evaluation, the major molecular response (MMR) was 93% and deep molecular response (MR) 63%. Imatinib was in general well tolerated; serious adverse events were registered in 9.3% of patients. Secondary benign or malignant neoplasms were reported in 11% of cases and cardiac events of any cause in 7.1% of patients (Hochhaus et al. 2017). Two independent studies aimed to assess the long-term outcome of patients treated with IM showed that CML-related deaths are very uncommon in patients achieving CCyR, and survival was not different from that of general population (Bower et al. 2016; Gambacorti-Passerini et al. 2011).

Consistent with these results is our experience of 203 consecutive CML patients treated in first line mostly with imatinib (92%). All patients received an accurate clinical, cytogenetic, and molecular follow-up according to ELN recommendations (Baccarani et al. 2013). The median age at diagnosis of the entire cohort was 58 years (range 15–85). After a median follow-up of 5.4 years (range 0.1–15.7), 175 patients (86%) are alive and 28 patients died. Most frequent causes of death were the development of other cancer (43%), CML progression (25%), and cardiac-related death (11%). Remarkably, the median age of patients dying for any reason was 74 (range 38–92). The 8-year OS is 85%, and the estimated risk of CML mortality at 8 years is only 5% (Fig. 2a, b). These findings confirm the reproducibility of the outstanding IRIS results in the daily clinical practice (Hochhaus et al. 2017).

2.2 Second-Generation TKIs

2.2.1 Dasatinib

Early in vitro studies conducted on IM-resistant cell lines showed the higher antileukemic activity of DA compared to IM (O'Hare et al. 2005). Among second-generation BCR-ABL inhibitors, DA was the first registered in 2006 initially for patients with CML resistant or intolerant to IM.

In the phase 3 randomized trial, DASISION patients were randomized to receive DA (100 mg once daily) or IM (400 mg daily) with the possibility to escalate the dose in case of suboptimal response (Kantarjian et al. 2010, 2012). The results of this study, now with a 5-year update, demonstrated the statistically significant higher rate of 5-year MMR and deep MR in patients randomized to DA (76% and 42%) compared

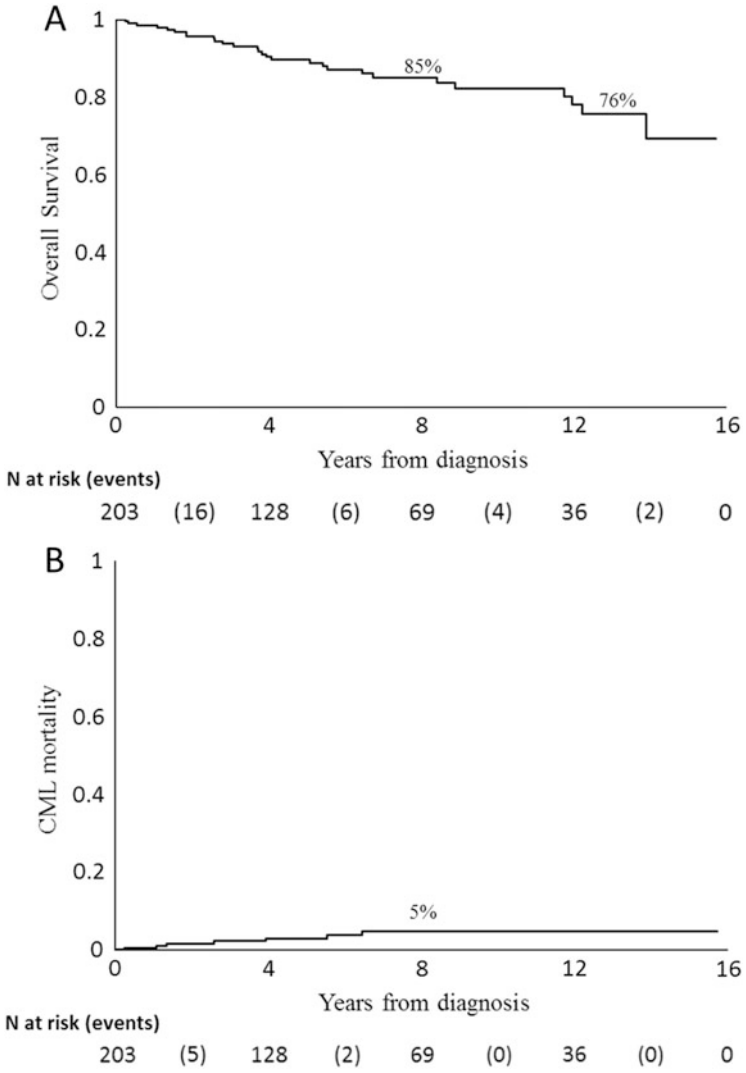

Fig. 2 Long-term results of CML treatment at the Hematology Unit, Ospedale Papa Giovanni XXIII, Bergamo. Panel (**a**), overall survival of the whole CML cohort diagnosed and treated front-line with a TKI. Panel (**b**), CML mortality of the same cohort of patients

to IM arm (64% and 33%). Despite that, the 5-year OS was similar in both arms 91% versus 90% as well as the 5-year PFS 85% versus 86% for DA and IM, respectively. In the intention to treat analysis, the risk of progression by 5 years was 4.6% in the DA group and 7.3% in IM group considering that two patients treated with IM had a CML progression between 3 and 5 years and none with DA. The probability to achieve a BCR/ABL reduction below 10% according to the international scale (IS) was

significantly higher in the DA arm (84% versus 64%). As it is known, achieving a BCR/ABL reduction $\leq$10% (IS) at 3 months is associated with a higher probability to reach a deep molecular response (Marin et al. 2012; Neelakantan et al. 2013).The risk of severe (grade 3 or 4) hematological toxicity was higher in patients treated with DA, as well as the risk of pleural effusion (28% in DA group with respect to 0.8% in IM); this side effect was more frequent in patients older than 65 years. The incidence of pulmonary hypertension (5% versus 0.4%) and arterial ischemic (5% versus 2%) was also higher and in patients treated with DA compared to IM, respectively.

Dasatinib was superior in achieving faster and deeper response with respect to IM, although 5-year PFS and OS were similar in the two arms with a survival rate comparable to that observed in the general population. In terms of safety, the most important side effect of DA is pleural effusion that occurred in about one third of patients, while pulmonary hypertension was reported only in some cases (5%).

2.2.2 Nilotinib

Nilotinib is a selected BCR-ABL second-generation inhibitor which is demonstrated to be more active than IM in vitro studies (Weisberg et al. 2005). The good results in terms of cytogenetic and molecular response in the first phase 3 randomized trial evaluating the efficacy of NI compared to IM for newly diagnosed CML patients named ENESTnd allowed to register NI in 2007 for the treatment of CML in first line (Saglio et al. 2010). The study was designed as a three-arm trial to evaluate two different doses of NI 300 and 400 mg twice a day compared to IM 400 mg single dose. Based on these results, NI was registered at the dose of 300 mg twice daily for first-line therapy (Saglio et al. 2010). The 2-year update of ENESTnd study showed that about 95% of patients in each arm were still in follow-up and the rate of drug discontinuation was 29% and 38% in NI 300 twice daily and IM 400 mg daily, respectively. Treatment discontinuation occurred in 20% of patients allocated to IM due to disease progression, treatment failure, or suboptimal response. In terms of efficacy by 3 years, the MMR rate was significantly higher in NI arm (73%) with respect to IM (53%) (Kantarjian et al. 2011). Considering the probability to achieve deep molecular response, the MR (Kantarjian et al. 2010) rate at 3 years was 50% in NI and 26% in IM ($P < 0.0001$), while the $MR^{4.5}$ rate was 32% versus 15%, respectively. The frequency of CML progression on treatment or during follow-up after drug discontinuation was 3.2% in the NI arm and 6.7% in the IM arm. The OS at 3 years was 95.1% and 94% in NI and IM group, respectively, while considering only CML-related deaths, the estimated rates of OS at 3 years were 98.1% and 95.2%. The principal concern of using NI was related to cardiovascular side effects. The ENESTnd update showed that the risk of peripheral arterial occlusive disease was 1.4% in the NI arm, while none of the case was registered in the IM arm; the risk of ischemic heart disease was higher for patients treated with NI (3.2%) with respect to those receiving IM (1.1%) (Larson et al. 2012). The more recent 5-year update of this study presented at ASCO Meeting in 2014 confirmed the significant superior

efficacy to achieve deep molecular response with NI despite the risk of cardiovascular toxicity which increased to 8% versus 2% in the IM group (Larson et al. 2014).

2.2.3 Bosutinib

In 2012 BO was approved by the FDA only for adult patients with CML resistant or intolerant to other TKIs (Cortes et al. 2011). The BELA (Bosutinib Efficacy and Safety in Newly Diagnosed Chronic Myeloid Leukemia) trial designed to evaluate the efficacy of BO (500 mg once a day) with respect to IM (400 mg) as frontline therapy failed to demonstrate a great superiority, and due to a major gastrointestinal toxicity, the drug did not receive the approval for first-line use (Cortes et al. 2012a). At the time of the recent 24-month analysis, 63% and 71% of patients were on treatment with BO and IM, respectively; the major reasons for discontinuation were severe toxicity (24% and 7%), disease progression/treatment failure (4% and 13%), and death (1% and <1%). As regard to the efficacy, at 24 months the rates of CCyR (58% versus 65%) and MMR (47% versus 41%) with 16% versus 12% of patients who achieved a deep molecular response (MR 4) for BO and IM, respectively, were not significantly different. More patients receiving BO had a BCR/ABL reduction below 10% according to the IS at 3 months, 86% versus 66%. The cumulative incidence of disease progression was 2% and 5% in the BO and IM arm, while the EFS at 24 months was 92% and 88%, respectively. The OS at 24 months was 97% for BO and 95% for IM; deaths due to CML occurred in 6 of 7 patients treated with BO and in 10 of 13 on IM. The major toxicity registered in the BO arm was due to gastrointestinal disorder: the rate of grade 3/4 diarrhea was 12% with respect to 1% in the IM arm. This side effect typically occurred during the first month of treatment. Another frequent side effect was the increase of the transaminase (grade 3/4) that occurred between 12 and 23% of patients in the BO arm with respect to less than 5% in the IM group. No cases of permanent hepatic damage were registered. Cardiovascular adverse effects were uncommon in both arms (<3% for grade >3); no cases of myocardial infarction or PAOD were registered (Brummendorf et al. 2015).

These results demonstrated the efficacy of BO to induce MMR faster than IM reducing the risk of disease progression with an acceptable safe profile characterized by a low to moderate gastrointestinal toxicity.

2.3 Third-Generation TKIs

2.3.1 Ponatinib

Ponatinib is a potent oral kinase inhibitor structurally designed to include a carbon-carbon triple bond that extends from the purine scaffold, permitting the molecule to occupy a position without steric hindrance from the bulky isoleucine residue at position 315 of the BCR-ABL mutant T315I (Cortes et al. 2012b; Goldman 2012). At concentration that is "clinically achievable," PO has shown preclinical activity

against all BCR-ABL mutants tested and has uniformly suppressed the emergency of single-mutant clones in a mutagenesis assay (O'Hare et al. 2009). In a phase 1 study, PO showed substantial antileukemic activity in patients with Ph + leukemias who had a resistance to or unacceptable side effects from previous treatment with tyrosine kinase inhibitors (Cortes et al. 2012b). The efficacy of PO was confirmed in an open-label multinational phase 2 trial enrolling 449 heavily pretreated patients who had CML or Ph + acute lymphoblastic leukemia with resistance to or unacceptable side effects from DA or NI or who had the BCR-ABL T315I mutation (Cortes et al. 2013). Among 267 patients with chronic-phase CML, 56% had a major cytogenetic response by 12 months, 46% had a CCyR, and 34% had a MMR. Responses were observed regardless of the baseline BCR-ABL kinase domain mutation status, and the estimated rate of sustained response of at least 12 months was 91%. Among 83 patients with accelerated-phase CML, the rate of major hematologic response and major cytogenetic response was 55% and 39%, respectively, while among 62 patients with blast-phase CML was 31% and 23%, respectively (Cortes et al. 2013). It is worth noting that serious arterial thrombotic events were observed in 9% of patients, prompting the FDA to issue boxed warnings. Thus, the European Medicines Agency recommends that patient's cardiovascular status be investigated before starting and during therapy with PO, with any risk factors actively managed and their treatment optimized (Hoy 2014).

3 Definition of TKI Resistance

The term "resistance" to a drug should be used when a drug is unable to hit its pharmacological target, due to inability to reach it (as a consequence of reduced bioavailability, in vivo inactivation, negative interaction with other substances) or to alterations of the target. In the last few years, the problem of "TKI resistance" has been largely emphasized in the medical literature, although the term has been used to refer more broadly to treatment failures based on clinical outcomes. The accepted definition of TKI resistance is treatment failure (primary resistance) or when the achieved response is lost (acquired or secondary resistance). Primary resistance can be subdivided in primary hematologic resistance that occurs in 2–4% of patients and cytogenetic resistance that occurs in 15–25% of patients (Shah 2007). Resistance to TKIs based on clinical outcomes can be explained by genomic mechanisms but also by BCR-ABL1-independent mechanisms that should never be laid aside. In this regard, a recent study showed that a lack of compliance accounted for the majority of poor TKI response and was the only significant mediator of poor clinical outcome (Noens et al. 2014). Therefore, a wide evaluation of potential different causes of clinical resistance ranging from patient-related to drug-related factors is necessary. In Table 1 are reported the main potential mechanisms of TKI resistance.

Table 1 Mechanisms of TKI resistance

Mechanisms of TKI resistance
BCR-ABL dependent
ABL1 kinase domain mutations
Increased BCR-ABL1 expression
BCR-ABL independent
Poor compliance
Drug influx and efflux
Activation of alternative signaling pathways
Plasma TKI concentration
Insensitivity of quiescent stem cells

4 BCR-ABL-Dependent Mechanisms of Resistance

4.1 Mutations in the BCR-ABL Domain

The most frequent mechanism of resistance is ABL kinase domain point mutation that directly or indirectly impairs appropriate TKIs binding to the kinase pocket either by altering BCR-ABL conformation with reduced affinity to the specific inhibitor or by interfering with a TKI-binding site. Mutations are located in different structural subunits of kinase domain and have been classified into four categories: (1) the direct binding site, (2) the phosphate-binding loop (P-loop), (3) the activation loop (A-loop), and (4) the catalytic loop (C-loop). The acquisition of point mutations in the ABL tyrosine kinase domain of BCR-ABL is observed in more than 50% of CML patients with clinical resistance and is more frequently found in patients with acquired resistance rather than primary resistance (Ernst et al. 2011; Hochhaus et al. 2002; Jabbour et al. 2006; Lahaye et al. 2005; Soverini et al. 2006). The first report of kinase domain mutation for IM-resistant was in 2001 when Sawyers and colleagues described that BCR-ABL1 could escape from inhibition by changing the shape of IM binding pocket (Gorre et al. 2001). The described mutation results in an amino acid substitution at position 315 in BCR-ABL, from a threonine (T) to an isoleucine group (I) (Soverini et al. 2006). This mutation is one of the most frequent mutations detected in 4–15% of IM-resistant patients (Jabbour et al. 2006, 2008; Soverini et al. 2006) and confers the highest resistance to IM and to the second-generation TKIs DA, NI, and BO (Mauro 2013). This mutation and others similarly affecting phosphate-binding loop (P-loop) of BCR-ABL are associated with a greater level of resistance (Khorashad et al. 2008; Nicolini et al. 2006), whereas other mutations have different clinical properties, and some of them are functionally irrelevant (Branford et al. 2003; Hochhaus 2003). To date, about 100 different BCR-ABL1 kinase domain mutations have been related to IM and to a lesser extent to second-generation TKI resistance (Milojkovic and Apperley 2009; O'Hare et al. 2012). The most frequent mutations detected in IM-resistant patients

were M244V, G250E, Y253F/H, E255K/V, T315I, F317L, M351T, E355T, F359V, and H396R/P (Soverini et al. 2011). Among patients relapsing on NI, the most frequent mutations observed were Y253H, E255K/V, F359V/C/I, or T315I, while among those relapsed on DA were V299L, F317L/V/I/C, and T315A/I mutations (Baccarani et al. 2013; Soverini et al. 2011; Cortes et al. 2016). For BO, L299V and T315I mutations induce a high degree of resistance. In Table 1, we summarize the sensitivity of more frequent BCR-ABL kinase mutations to second- and third-generation TKIs.

Patient carriers of TKI-resistant clones seem to be at higher risk to accumulate additional genomic aberrations leading to progression to blast crisis (Soverini et al. 2005). In particular, IM-resistant patients had higher likelihood of relapse associated with development of further mutations compared to patients who did not have mutations (Soverini et al. 2009).

Interestingly, resistance due to kinase domain mutations may pre-exist before any TKI exposure and selection pressure (Roche-Lestienne et al. 2002; Willis et al. 2005). Limited data are available from IM-naive patients regarding the incidence of BCR-ABL kinase domain mutations and their correlation with the therapeutic response (Carella et al. 2010). A study involving 100 patients with newly diagnosed CML showed that a pre-existing ABL kinase domain mutations in CD34+ cells were detected in about one third of patients, and their presence (including F311L, M351T, and T315I) was associated with IM resistance. These results suggest a potential importance to detect pre-existing BCR-ABL mutations as basis for selection of appropriate first-line drug therapy (Iqbal et al. 2013). However, at present, there is no definite demonstration of an association with clinical events conditioning cost-effective changes in patient management. In addition, highly resistant clones may pre-exist and emerge rapidly also in patients receiving second-generation TKIs at CML diagnosis (Kantarjian et al. 2012; Larson et al. 2012; Hochhaus et al. 2013; Rosti et al. 2009). Lastly, the probability to develop mutations increases with advancing phases of disease (Branford et al. 2009).

4.2 Overexpression of BCR-ABL

The mechanisms involved in resistance due to amplification of BCR-ABL remain to be elucidated. The first evidence of overexpression was observed in animal models (le Coutre et al. 2000) and subsequently in CML patients (Gorre et al. 2001). Recently it has been hypothesized a relationship between BCR-ABL overexpression and the generation of mutations (Tang et al. 2011), suggesting that this mechanism may represent a first step to the development of resistance. In addition, BCR-ABL overexpression seems to enhance self-renewal of leukemic stem cells (Abrahamsson et al. 2009).

5 BCR-ABL-Independent Mechanisms of Resistance

5.1 TKI Influx and Efflux

Drug efficacy depends also from its ability to reach the pharmacological target as a consequence of correct intracellular drug availability. In this regard, the relation between TKIs and their transporter proteins is very relevant, since these proteins regulate the intracellular drug concentrations. The mechanisms of resistance may be due to defective influx or conversely excessive efflux. Both mechanisms can be responsible for a decreased intracellular concentration of IM (Eechoute et al. 2011). The consequences of a reduced intracellular availability of IM might be an inferior ability to cause cell apoptosis, favoring the persistence of subclones that can acquire mutations of BCR-ABL kinase domain conferring insensitivity to the drug.

The drug-intake protein human organic cation transporter 1 (*h*OCT1) regulates the import of IM into the cell. Low activity of *h*OCT1 has been associated with suboptimal response to IM and OS (Crossman et al. 2005; White et al. 2007, 2010a). However, studies using single nucleotide polymorphisms (SNPs) to evaluate the impact of *h*OCT1 variants in response to IM showed controversial results that may be partly due to the small sample size of studies or the use of different criteria for response evaluation and different ethnical population in the studies (Angelini et al. 2013; Giannoudis et al. 2013; Koren-Michowitz et al. 2014; Vine et al. 2014; White et al. 2010b).

Several members of the adenosine triphosphate (ATP)-binding cassette (ABC) transporter protein family have also been associated with resistance to TKIs. Overexpression of P-glycoprotein (P-gp) encoded by subfamily B, member 1 ABCB1 gene, known as multidrug resistance 1 (MDR1) gene, causes an excessive efflux that influences negatively the therapeutic efficacy of TKIs (Deenik et al. 2010; Galimberti et al. 2005; Hegedus et al. 2002; Mahon et al. 2003; Ni et al. 2011; Peng et al. 2012; Widmer et al. 2003). Furthermore, ABCA3, ABCC2, and ABCG2 also seem to confer resistance to IM, NI, and DA (Choudhuri and Klaassen 2006; Shukla et al. 2011).

5.2 Activation of Alternative Signaling Pathways

A mechanism of TKI resistance is the activation of alternative signaling pathways, such as PI3K/AKT, JAK/STAT, Ras/MAPK, and SRC, that compensate the suppression of BCR-ABL kinase activity (Agarwal et al. 2008; Burchert et al. 2005; Donato et al. 2003; Gioia et al. 2011; Wang et al. 2007). As a consequence, cells continue to proliferate despite an effective inhibition of BCR-ABL1. The clinical relevance for this ABL1-independent mechanism of resistance is that some CML patients may be refractory to all available TKIs. In this regard, Cortes and colleagues in patients with refractory Philadelphia-positive leukemia treated with PO observed

a lower molecular response in patients without evidence of BCR-ABL1 mutations compared with carriers of a mutation (Cortes et al. 2012b), suggesting that the involvement of a BCR-ABL-independent mechanism may be responsible for the poor response to PO.

5.3 Liver Metabolism

TKIs are metabolized by the liver, and a part of interindividual variability in response has been demonstrated to correlate with plasma concentration of the drug. The plasma concentration may be influenced by the metabolic activity of the hepatic cytochrome P450, 3A4, and 3A5 that can be inhibited or induced by several different drugs (Peng et al. 2005). Thus, the interference with TKI metabolism by other drugs that are frequently given to patients is very important and should be taken into account. All concomitant medications should be investigated for potential interactions, particularly in patients with several comorbidities and in those with unexpected unsatisfactory response to the treatment (Iurlo et al. 2014). However, at present, there is not enough evidence to recommend in the clinical setting the measurement of TKI concentration.

5.4 CML Stem Cells

There is increasing evidence that most patients relapse after discontinuing TKI therapy even if they had obtained a sustained deep molecular response before stopping treatment (Mahon et al. 2010; Ross et al. 2013). The main hypothesis to explain these results is related to the peculiar biology of CML stem cells, which quite often can be primarily resistant to TKIs (Graham et al. 2002). Leukemic stem cells are CD34+/CD38- and BCR-ABL1 positive and account for less than 1% of CD34+ cells at diagnosis (Copland et al. 2006). These cells are not dependent on BCR-ABL activity for their survival (Zhang and Li 2013) and, accordingly, are generally primarily resistant to TKIs. Thus, they persist dormant during treatment and represent a reservoir to the possible relapse (Chomel and Turhan 2011), as well as an obstacle to successful implement treatment discontinuation strategies. Understanding what controls the persistence of CML stem cells, what is necessary to fully eradicate them, and how that might be approached therapeutically are crucial questions in order to increase the possibility to obtain a definite cure of CML. The available TKIs targeting only mature proliferating cells may not be sufficient to cure CML, and, therefore, it is necessary to identify drugs with new targets to eradicate the residual stem cells of CML. In this regard, omacetaxine is a promising drug approved by FDA (Cortes et al. 2012c) that has been shown effectively targeting CML leukemia stem cells in vivo (Chen et al. 2009).

5.5 Patient Poor Compliance

A suboptimal adherence to the therapy is an important possible cause for an inadequate response (Noens et al. 2014). The main causes of poor compliance go from the perception of stable remission equating to have been cured to simply forgetting. A study analyzed the relation between adherence to IM measured with microelectronic monitoring systems and the probabilities of treatment failure in 87 patients on long-term therapy. An adherence rate less than or equal to 85% was associated with a higher probability of treatment failure (Ibrahim et al. 2011). In this regard, before defining a patient as having TKI resistance and modifying therapy treatment, the lack of adherence to the medication needs to be always considered and ruled out.

6 Conclusions

In the last few years, the problem of CML resistance has been greatly emphasized in the medical literature, and different mechanisms of resistance have been extensively described ranging from BCR-ABL kinase domain mutations to lack of adherence to therapy. In addition, most patients relapse after discontinuing TKI therapy, due to the existence of CML stem cells, which have been demonstrated to be primarily resistant to TKIs. As a consequence, although most patients with CML would have a life expectancy close to normal (Bower et al. 2016; Gambacorti-Passerini et al. 2011), they require to continue TKIs for life and to be carefully monitored for signs of resistance in order to change therapy promptly. The future challenge is to identify all mechanisms of resistance, to optimize the use of different TKIs, and to combine them with new drugs that specifically target CML leukemic stem cells with the aim to prevent transformation and to eradicate the disease. The clinical implications of obtaining the cure of CML are potentially important both for patients and health-care systems, considering the issues of compliance to indefinite treatment, side effects, and costs (Table 2).

In Fig. 3, we summarize our present suggestions in the management of CML patients, according to the mutation type and patients' risk profile.

Table 2 Sensitivity of more frequent BCR-ABL1 kinase mutations to second- and third-generation TKIs

	Nilotinib	Dasatinib	Bosutinib	Ponatinib
Less sensitive	E255K/V	E255K/V	E2555K/V	E255K/V
	Y253H	Q252H		
	F359C/V	V299L		
		F317L		
Resistant	T315I	T315A/I	T315I	T315M
			V299L	

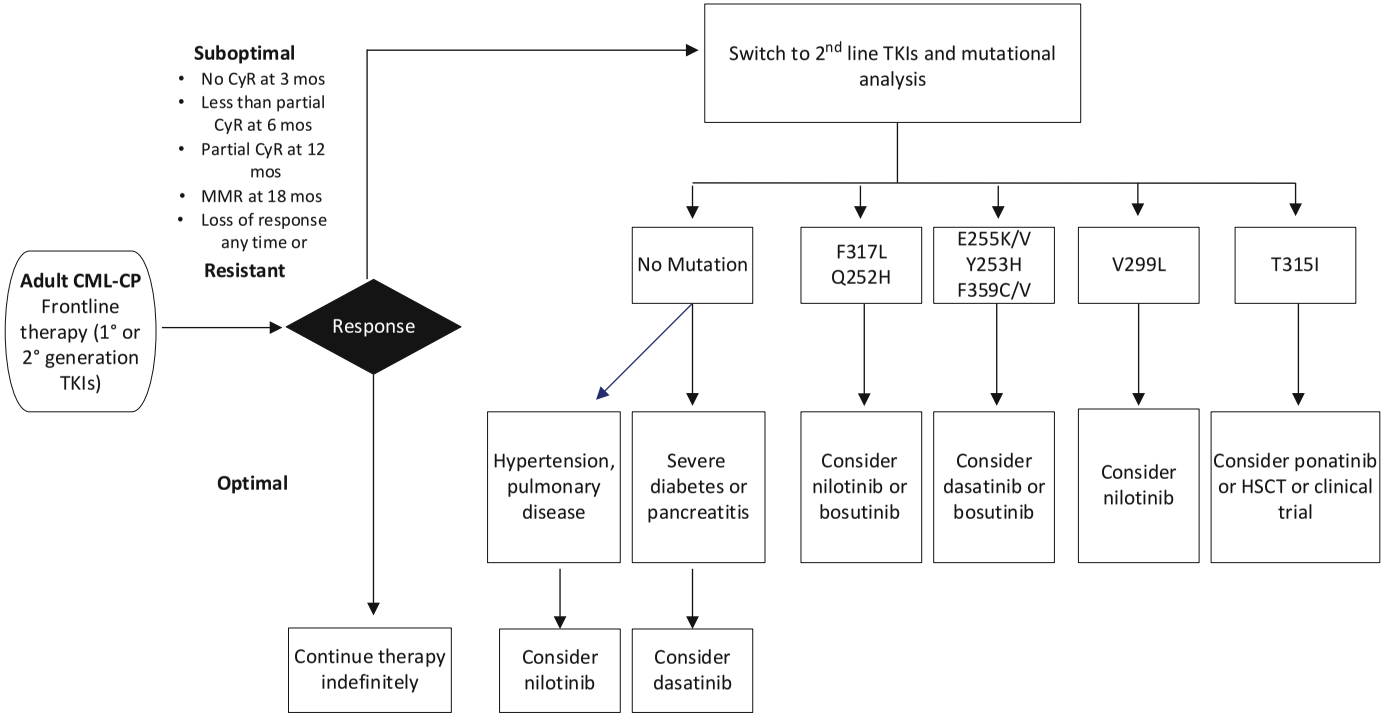

Fig. 3 Algorithm for management of CML patients, according to the mutation type and patients' risk profile. *CML* chronic myeloid leukemia, *CP* chronic phase, *CyR* cytogenetic response, *MMR* major molecular response, *HCST* hematopoietic stem cell transplantation, *mos* months

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Mechanisms of Resistance in Multiple Myeloma

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Abstract

Multiple myeloma (MM) is an incurable hematopoietic cancer that is characterized by malignant plasma cell infiltration of the bone marrow and/or extramedullary sites. Multi-modality approaches including "novel agents," traditional chemotherapy, and/or stem cell transplantation are used in MM therapy. Drug resistance, however, ultimately develops and the disease remains incurable for the vast majority of patients. In this chapter, we review both tumor cell-autonomous and non-autonomous (microenvironment-dependent) mechanisms of drug resistance. MM provides an attractive paradigm highlighting a number of current concepts and challenges in oncology. Firstly, identification of MM cancer stem cells and their unique drug resistance attributes may provide rational avenues towards MM eradication and cure. Secondly, the oligoclonal evolution of MM and alternation of "clonal tides" upon therapy challenge our current understanding of treatment responses. Thirdly, the success of MM "novel agents" provides exemplary evidence for the impact of therapies that target the immune and non-immune microenvironment. Fourthly, the rapid pace of drug approvals for MM creates an impetus for development of precision medicine strategies and biomarkers that promote efficacy and mitigate toxicity and cost. While routine cure of the disease remains the ultimate and yet unattainable prize, MM advances in the last 10–15 years have provided an astounding paradigm for the treatment of blood cancers in the modern era and have radically transformed patient outcomes.

Keywords

Drug resistance • Immunotherapy • Microenvironment • Multiple myeloma • Signaling pathways

1 Multiple Myeloma (MM): A Tumor of Plasma Cells, the "Snipers" of the Immune System

MM is an (oligo)clonal malignancy characterized by the proliferation of plasma cells that produce clonal immunoglobulins (Ig). Plasma cells represent a terminal differentiation stage of B-lineage lymphocyte development. Plasma cells are endowed with expanded endoplasmic reticulum (ER) content, mechanisms for protein quality control, as well as active secretory pathways. Malignant plasma cells most commonly synthesize and secrete monoclonal Ig of the IgG and IgA isotypes (Maclennan and Chan 1991). MM plasma cells may secrete clonal intact immunoglobulin and/or Ig components (e.g., free light chains), known as M-protein. Excess M-protein accumulates in the bloodstream and/or the urine of MM patients (Bataille and Harousseau 1997). MM plasma cells thrive in a state of symbiosis with their microenvironment: they receive pro-survival signals from cellular components of their niche and in turn, remodel their immune and non-immune microenvironment to promote local expansion, immune evasion, and metastasis. One of the best-known and most prevalent consequences of this

bidirectional cross-talk is MM bone disease: malignant plasma cells promote aberrant osteoclast activation and osteoblast dysfunction that translates into significant clinical morbidity from pathological fractures and hypercalcemia. End-organ damage in addition to bone lytic disease includes renal damage and increased susceptibility to infections and cytopenias (Kyle and Rajkumar 2008).

MM is the second most common blood cancer (after non-Hodgkin's lymphoma) and represents approximately 1% of all cancers in white individuals and 2% of all cancers in black individuals (Rifkin et al. 2016). MM is almost always preceded by a premalignant disease known as monoclonal gammopathy of undetermined significance (MGUS) (Landgren et al. 2009). Annual risk of progression of MGUS to MM is approximately 1% (Edwards et al. 2005) in low-risk cases.

Unusually for a hematological malignancy, MM is characterized by multiple and complex numerical and/or structural abnormalities, even at its pre-malignant stage, MGUS (Anderson and Carrasco 2011). Thus, MM has been called the hematological malignancy with "carcinoma-like" cytogenetics. The fact that overt MM is similar at the (cyto)genetic level to its pre-malignant counterpart (MGUS) has led to the hypothesis that progression is due to non-tumor cell autonomous mechanisms operating at the level of the microenvironment. These can be immune or non-immune in nature.

2 Molecular Features and Pathophysiology

Development of MM proceeds via a multistep transformation process that includes chromosomal abnormalities, oncogene activation, and microenvironmental remodeling (Zingone and Kuehl 2011).

2.1 Cytogenetic Abnormalities

Primary translocations occur as early and perhaps initiating events during the pathogenesis of MM, whereas secondary translocations occur as progression events (Bergsagel and Kuehl 2001). Primary translocations juxtapose a partner chromosome (at a breakpoint near an oncogene) to an Ig enhancer or other regulatory element at the IgH locus on chromosome 14. They are thought to occur in germinal center B cells. The prevalence of IgH translocations depends on the disease stage: nearly 50% in MGUS or SMM (smoldering MM) and 55–73% in symptomatic MM (Avet-Loiseau et al. 2002; Fonseca et al. 2003). There are five recurrent chromosomal partners (oncogenes) that are involved in IgH translocations in MGUS and MM: 4p16 (*MMSET* and usually *FGFR3*), 6p21 (*CCND3*), 11q13 (*CCND1*), 16q23 (*c-MAF*), and 20q11 (*MAFB*) (Chesi et al. 1996, 1997, 1998a, b; Kuehl and Bergsagel 2002; Shaughnessy et al. 2001). Breakpoints in primary translocations are characteristic of isotype switch recombination, an event dependent on the actions of activation-induced deaminase (AID) (Bergsagel et al. 1996). On the other hand, secondary translocations are typically not characteristic of antibody gene rearrangement processes. Secondary translocations may include unbalanced

and complex translocations and insertions that can involve three chromosomes, sometimes with associated amplification, duplication, inversion, or deletion (Bergsagel and Kuehl 2005).

Cytogenetic abnormalities constitute molecular drivers of disease and powerful prognostic factors. A combination of traditional cytogenetics and interphase fluorescence in situ hybridization (FISH) is currently being employed to stratify tumors into high-and standard cytogenetic risk disease at diagnosis (Table 1). Kapoor et al. (2010) examined retrospectively 290 patients with newly diagnosed symptomatic MM treated with novel agents and demonstrated that high-risk cytogenetics (i.e., deletion 13, monosomy of chromosome 13 and/or hypoploidy) conferred significantly shorter median overall survival (29 versus 69 months).

Aneuploidy is one of the most common findings in MM. Two categories are recognized: Hyperdiploid MM ($\geq$47 and $<$75 chromosomes; H-MM) and non-hyperdiploid MM (NH-MM). NH-MM is further divided into three subgroups: hypodiploid ($\leq$44 chromosomes), pseudodiploid (45–46 chromosomes), and near tetraploid ($>$75 chromosomes). Hypodiploidy purports poorer survival, whereas hyperploidy indicates good prognosis (Smadja et al. 1995). Hyperdiploid MM is characterized by trisomies in odd numbered chromosomes (3, 5, 7, 9, 11, 15, 19, and 21). Non-hyperdiploid MM is characterized by high frequency of primary translocations.

Because MM cells are mostly non-cycling, interphase FISH has been used to detect chromosomal abnormalities with prognostic significance in quiescent MM cells. FISH is a cytogenetic technique that uses fluorescent probes that bind to only those parts of the chromosome with a high degree of sequence complementarity. Based on FISH analysis, prognostic groups can be delineated as as shown in Table 1 (Fonseca et al. 2003).

"Primary" translocations in MM involving 14q32, the site of the immunoglobulin heavy chain (IgH) locus, carry widely variable impacts on prognosis. t(11;14), which involves *BCL-1* oncogene [the locus encoding cyclin D1 (*CCND1*)] and results in the up-regulation of cyclin D1, is found in about 15% of patients, and appears to be associated with a favorable outcome (Garand et al. 2003; Robillard et al. 2003). t(4;14) translocation carries a relatively poor prognosis regardless of whether patients are undergoing conventional or high-dose therapies (Stewart and Fonseca 2005). However, San Miguel et al. (2008) indicated that bortezomib may overcome the adverse prognostic effect of t(4;14) and thus this abnormality is now considered of intermediate risk. On the other hand, deletion of 17p, including the *TP53* locus, is found in 10% of MM patients and is correlated with an adverse

Table 1 Cytogenetic risk stratification of MM

High risk	Intermediate risk	Standard risk
17p13 deletion	t(4;14)	Trisomies (hyperdiploidy)
t(14;16)	Deletion 13 or hypodiploidy by conventional karyotyping	t(11;14)
t(14;20)	Gain 1q	t(6;14)

prognosis, which is complicated by rapid disease progression (Reece et al. 2009; Xiong et al. 2008).

2.2 Mutational Landscape

Identification of driver mutations in MM provides insight into the pathogenesis of the disease and impetus towards the development of targeted therapies. The first study that elucidated MM genome content (Chapman et al. 2011) analyzed samples from 38 patients. Whole genome sequencing was performed for 23 patients and whole exome sequencing for 16 patients, with one analyzed by both approaches. The authors identified *KRAS* and *NRAS* mutations as the most common point mutations (10 and 9 cases, respectively), followed by *TP53* (3 cases). Two point mutations of *CCND1* (Cyclin D1) were identified. Notably, *CCND1* constitutes the target of the t(11;14) chromosomal translocation (Takimoto et al. 2008).

Mutations in *DIS3* locus were detected in 4 out of 38 patients. DIS3 encodes a highly conserved RNA exonuclease which regulates the processing and abundance of all RNA species (Dziembowski et al. 2007). The four observed mutations occurred at highly conserved regions facing the enzyme's catalytic pocket and likely resulted in loss of function (three of the tumors exhibited loss of heterozygosity via deletion of the remaining *DIS3* allele). Thus, *DIS3* mutations may dysregulate RNA processing as an oncogenic mechanism in MM (Chapman et al. 2011).

FAM46C gene mutations were observed in 5 out of 38 patients. To shed light on FAM46C function, Chapman et al. (2011) examined *FAM46C* expression across 414 MM samples and findings showed a strong correlation with eukaryotic initiation and elongation factors involved in protein translation.

LRRK2 gene mutations were detected in 3 out of 38 patients (Chapman et al. 2011). *LRRK2* encodes a kinase that phosphorylates translation initiation factor 4E-binding protein (4EBP). Although *LRRK2's* role is established in the context of Parkinson's disease (Zimprich et al. 2004), its role in MM remains still unclear. However, Parkinson's disease is characterized by dysfunctional unfolded protein responses (Forman et al. 2003), suggesting an analogous role for the product of this gene in MM. Consistent with this hypothesis, mutations in the unfolded protein response (UPR) gene *XBP1* were detected in 5% of cases (Chapman et al. 2011). Spliced *XBP1* induces an MM-like syndrome when expressed in transgenic mice (Carrasco et al. 2007). Together, these findings indicate that mutations affecting protein translation and its quality control are common in MM (42% of patients). Mutations affecting various components of the NF-κB pathway were also detected (*BTRC*, *CARD11*, *CYLD*, *IKBIP*, *IKBKB*, *MAP3K1*, *MAP3K14*, *RIPK4*, *TLR4*, *TNFRSF1A*, and *TRAF3*). These data were corroborated by Keats et al. (2007) who identified mutations in upstream components of the NF-κB pathway in 20% of patients.

Subsequent to the Chapman analysis Lohr et al. (2014) analyzed 203 cases. In line with Chapman et al. (2011), Lohr et al. (2014) identified frequently mutated

pathways that have been causally implicated in MM, such as NF-κB, chromatin-modifying enzymes (*MLL, MLL2, MLL3, UTX, WHSC1, WHSC1L1*) and RNA processing loci. MAPK pathway (*KRAS*, *NRA*S, and *BRAF*) mutations were again detected at high rates. Moreover, 39 coding and noncoding mutations were identified in the *CCND1* locus, target of the t(11;14) translocation.

2.3 Cell of Origin

The identity of the target cell undergoing mutations leading to myelomagenesis can be speculated upon but little definitive evidence exists. It is likely that primary translocations occur as by-products of isotype switch recombination in a cell participating in the germinal center reaction, as evidenced by the identification of characteristic breakpoints. However, it is unclear if secondary mutations occur in a cell that is committed to the plasma cell fate [i.e., a cycling plasmablast expressing the transcription factor PRDM1 (Blimp1)] or another, non-plasmacytic progenitor. Moreover, it is not established whether secondary mutations, such as *RAS* mutations, may occur before or after the migration to the bone marrow or other metastatic tissues. Rasmussen et al. (2010) detected products of primary translocations, but not *RAS* mutations, in flow-sorted B cells from a MM patient. *RAS* mutations were clearly present in malignant plasma cells from the same patient. These results indicate that *RAS* mutations occur in a plasma cell lineage-restricted fashion.

2.4 MM Cancer Stem Cells (MMSC)

The term "cancer stem cell" captures the idea that a stable, minor, quiescent, and phenotypically definable subpopulation with enhanced self-renewal and regenerative capacity sustains tumor propagation. Cancer stem cells may differ in their ability to resist challenges such as genotoxic stress compared to more mature tumor cells. It has been a matter of some dispute, whether MMSC represent clonotypic $CD138^-$ cells or $CD138^+$ cells. Matsui et al. (2004) suggested that MMSC may be $CD138^-$ clonotypic cells. In this work, $CD138^-$ cells from both human MM cell lines and primary patient samples had greater clonogenic potential both in vitro and in immunodeficient mice, compared to corresponding $CD138^+$ cells. In subsequent work from the same group (Matsui et al. 2008), $CD138^-$ clonogenic progenitors were proven to be resistant to dexamethasone, lenalidomide, bortezomib, and 4-hydroxycyclophosphamide. These findings were attributed to the high drug efflux capacity and intracellular drug detoxification activity characterizing this compartment. Recently, the clinical success of chimeric antigen receptor cells (CAR-T) targeting CD19 (Garfall et al. 2016), a molecule present on the surface of most B cells, but not normal or malignant plasma cells, has been interpreted to suggest that clinical activity of this cellular therapy is due to its targeting of a CD19-expressing, clonogenic MMSC, distinct from the tumor bulk that is CD19-negative.

However, the assertion that MM clonotypic cells may not be committed plasmacytic precursors has been controversial. Kim et al. (2012) compared the engraftment potential of $CD38^{high}/CD138^{+}$ and $CD19^{+}/CD38^{low}/CD138^{-}$ human B and plasma cells in immunodeficient mice. They concluded that only xenografts derived from $CD38^{high}/CD138^{+}$ cells were clonally identical to patient MM cells. Another approach taken to identify MM clonogenic cells has focused on the "side" population (SP cells). SP cells are known as enriched source of cancer initiating cells with stem cell properties and they show a distinct low-staining pattern with the Hoechst 33342 dye (Kim et al. 2014). Interestingly, Challen and Little (2006) described that SP cells show a strong ABC (ATP-binding cassette) transporter activity resulting in high ability to efflux compounds across the membrane, while Jakubikova et al. (2011) demonstrated that SP phenotype in MM contains both $CD138^{+}$ and $CD138^{low}$ cells.

2.5 MM Microenvironment: "Canonical" and "Non-canonical" MM Niches

The local microenvironment is of crucial importance for MM pathogenesis. MM typically propagates within the bone marrow ("canonical" MM niche) but malignant plasma cells can also thrive in extramedullary, "non-canonical" niches. The BM microenvironment (stroma) is a complex network of extracellular matrix, soluble mediators (e.g., cytokines), and MM accessory cells (mesenchymal stem/stromal cells (MSC), osteoclasts, osteoblasts, immune cells as well as vascular components). Our laboratory has had a longstanding interest in infiltrating myeloid cells ("the myeloid-in-myeloma" compartment) consisting of myeloma-associated macrophages and neutrophils, dysfunctional dendritic cells, and myeloid-derived suppressor cells (MDSC) (Asimakopoulos et al. 2013, 2017). In MM, the interaction between tumor cells and microenvironment influences the survival, migration, and proliferation of malignant plasma cells as well as their response to therapy (Noonan and Borrello 2011). Thus the environment is crucial in supporting tumor progression (Reagan and Ghobrial 2012) (Fig. 1). This cross-talk is bidirectional: whereas proliferative and/or anti-apoptotic signaling pathways are activated in tumor cells (Borrello 2012), the latter remodel the microenvironment in crucial aspects, for example to promote osteoclastogenesis and angiogenesis (Di Raimondo et al. 2000).

2.6 Growth and Survival Factors and Pathways

Numerous studies have identified MM cell growth factors and signaling pathways supporting survival and proliferation of MM cells. These factors can be further classified into three groups. The first group of factors activate the NF-κB pathway (BAFF, APRIL, TNF) (Klein et al. 2003), the second one triggers JAK/STAT and

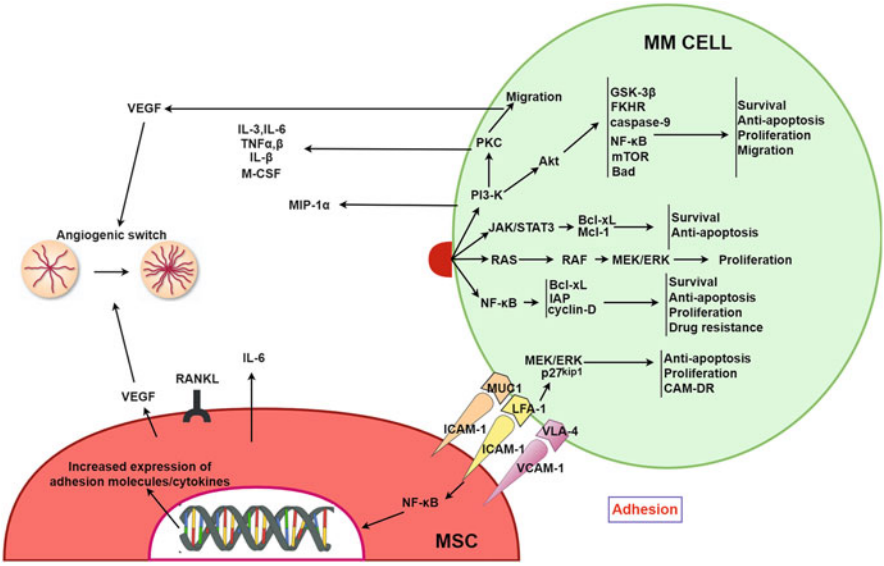

Fig. 1 Tumor-stromal cell interactions in the MM niche and their functional consequences. Cross-talk between MM stromal tumor and stromal cells results in activation of key growth and apoptosis pathways. This interaction is also a key regulator of neo-angiogenesis in the nascent MM lesion

MAP-kinase pathways (IL-6, IFNα, IL-10, IL-21), while the third group triggers PI3-Kinase/AKT and MAP-kinase pathways (IGF-1, insulin, EGF family, HGF).

2.6.1 NF-κB

Consititutive activation of the NF-κB pathway, whether intrinsic or extrinsic, is essential for the homeostasis of MM cells. Extrinsic ligands include BAFF and APRIL, which belong to TNF family. They can activate at least three receptors of the TNF receptor family: BAFF-R, BCMA, and TACI (Mackay and Kalled 2002). The receptors for BAFF and APRIL are mainly expressed by B cells (Darce et al. 2007; Ng et al. 2004). Activation of the former receptors triggers the NF-κB pathway in MM cells (Mackay and Schneider 2009). BAFF and APRIL are potent survival and proliferation factors for MM cells and can protect malignant MM cells from dexamethasone-induced apoptosis (Moreaux et al. 2004).

NF-κB may contribute to MM pathogenesis through various mechanisms. Firstly, by triggering antiapoptotic mediators (Li et al. 2008); secondly, through promotion of cell-cycle progression (Duyao et al. 1990; Guttridge et al. 1999), and thirdly, through facilitating MM cell invasion and metastasis (Hecht et al. 2008).

2.6.2 Interleukin-6 (IL-6)

IL-6 is a key growth and survival factor in MM predominantly produced by bone marrow stromal cells (BMSCs) and osteoblasts (OBs) (Hope et al. 2014; Klein et al.

1995). It mediates paracrine MM cell growth and can also be secreted by MM tumor cells in an autocrine manner, particularly in advanced and drug-resistant stages (Hideshima et al. 2001). In addition, IL-6 secretion is upregulated by other molecules/cytokines including CD40, TNF-α, VEGF, IL-1β, TGF-β. For instance, the paracrine production of IL-6 by BMSCs is mainly driven by the secretion of IL-1β by the MM cells and/or immune accessory cells (Dinarello 2011; Hope et al. 2014). IL-6 in its turn functions as a survival and proliferation factor for neoplastic plasma cells, thus creating an amplification loop. After binding to its receptor, IL-6 triggers activation of JAK/STAT3, PI3K/Akt, and MAPK signaling pathways (Hideshima et al. 2007; Podar et al. 2005). Specifically, IL-6 activation of the JAK/STAT3 pathway induces tumor cell survival by upregulation of Mcl-1, Bcl-xL, and c-Myc (Podar et al. 2005), whereas blockade of IL-6 induces upregulation of the pro-apoptotic BH3-only protein Bim and activation of Bax, thus inducing MM cell apoptosis (Le et al. 2004). Moreover, IL-6 may inhibit the antiproliferative effects of cyclin-dependent kinase (CDK) inhibitors p21 and p27 in MM cells through the PI3K/Akt pathway (Hideshima et al. 2001b), while IL-6 induced tyrosine phosphorylation of scaffolding proteins Gab1 and Gab2, which are expressed by MM cells, can also promote tumor cell survival through MAPK pathway (Podar et al. 2004). Interestingly, Carrasco et al. (2007) demonstrated that IL-6 may upregulate *XBP1* gene expression and therefore trigger plasma cell differentiation: serum IL-6 levels were increased in *Eμ-Xbp-1s* transgenic mice relative to control littermates.

2.6.3 Other Growth Factors and Pathways

Besides IL-6, other important cytokines such as IL-10 and growth factors (IGF-1, TGF-β, HB-EGF, TNF-α) are considered regulators of the proliferation and homeostasis of MM cells. IL-10 is a potent immunosuppressive factor produced by various normal and malignant cell types of hematopoietic origin (Benjamin et al. 1994). IL-10 induces LIFR (leukemia inhibitory factor receptor) and IL-11R expression on MM cells, thus promoting MM proliferation (Gu et al. 1996; Lu et al. 1995). Otsuki et al. (2000) described a paracrine network for IL-10-mediated support of MM cells. Alexandrakis et al. (2015) reported a strong association between the expression of angiogenic cytokines (VEGF and Ang-2) and IL-10.

IGF-1 is a survival and proliferation factor for most MM cell lines independently of IL-6 (Ferlin et al. 2000). IGF-1 induces the PI-3Kinase pathway, triggering an anti-apoptotic response, through activation of anti-apoptotic Bcl-2 and Bcl-xL (Jourdan et al. 2000) and downregulation of pro-apoptotic Bim (De Bruyne et al. 2010). Sprynski et al. (2009) also demonstrated a major effect of IGF-1 in MM cell growth: autocrine IGF-1/IGF-1R stimulation was active in 4 out of 5 CD45+ human MM cell lines. This autocrine loop (IGF-1/IGF-1R) synergized with growth-promoting IL-6, HB-EGF, or HGF when MM cells expressed IGF-1R.

TGF-β triggers IL-6 and vascular endothelial growth factor (VEGF) secretion by BMSCs, therefore enhancing paracrine IL-6 and VEGF-related tumor cell growth in the BM microenvironment (Yasui et al. 2008). HB-EGF (heparin-binding epidermal growth factor-like growth factor) cooperates with IL-6 to induce an optimal

survival of MM cells mainly through PI-3K/AKT pathway (Wang et al. 2002), while TNF-α also works synergistically with IL-6. Moreover, TNF-α accounts for NF-κB activation and induction of ICAM-1 or VCAM-1, thus promoting adhesion of MM cells to BMSCs (Hideshima et al. 2001).

2.7 Angiogenesis

Neo-angiogenesis is crucial to the success of the nascent MM tumor. Angiogenesis is partly driven by release of pro-angiogenetic factors from the tumor plasma cells, BMSC, and osteoclasts: these factors include VEGF, bFGF, and matrix metalloproteinases (MMPs). Adhesion between MM cells and BMSCs upregulates angiogenic cytokines especially VEGF and bFGF (Podar and Anderson 2005). Pro-angiogenic factors may also be produced cell-autonomously as a result of oncogene activation (Rajkumar and Witzig 2000). However, inflammatory cells such as macrophages and mast cells may also participate in angiogenesis through "vasculogenic mimicry" (Nico et al. 2008; Scavelli et al. 2008). Blood vessels are required for tumor growth and progression for provision of vital oxygen and nutrients. Scavelli et al. (2008) demonstrated that MM macrophages exposed to VEGF and bFGF acquired endothelial cell markers and formed capillary-like structures mimicking bone marrow endothelial cells. Bone marrow biopsies of active MM harbored "mosaic" pattern vasculature, being formed by MMECs and macrophages. Conversely, macrophages from nonactive MM, MGUS, or controls displayed weaker potential for vasculogenic mimicry than macrophages derived from overt MM.

2.8 Osteoclastogenesis

The balance between bone resorption and new bone formation is radically redressed in many cases of MM, resulting in clinically significant bone destruction and osteolytic lesions (Bataille and Harousseau 1997). Malignant plasma cells stimulate osteoclastogenesis mainly by increasing RANKL and reducing the levels of osteoprotegerin (OPG) (Terpos et al. 2003). RANKL is a member of TNF family that plays a pivotal role in the increased osteoclastogenesis implicated in MM bone disease. RANK is a transmembrane signaling receptor expressed by osteoclast cells. OPG is a decoy receptor for RANKL and by binding RANKL, OPG blocks the RANKL-RANK interaction between osteoblast/stromal cells and osteoclasts, thus preventing excessive bone resorption. MM cell binding to BMSC results in increased RANKL expression. Moreover, MM cells may promote antiapoptotic effects on osteoclasts through M-CSF secretion, which in turn potentiates bone resorption (Dib et al. 2008). Restoring the balance between RANKL and OPG not only reverses MM-induced osteolysis, but also prevents development of resistance to chemotherapeutics (Liu et al. 2012).

Tian et al. (2003) demonstrated that osteoblast dysfunction in MM is due to inhibition of WNT signaling through MM-secreted WNT inhibitor, DKK1. Qiang

et al. (2008) showed that DKK1 directly increased RANKL to OPG ratio in the MM microenvironment. Blocking DKK1 resulted in a bone anabolic effect and reduced tumor burden in MM mice (Yaccoby et al. 2007).

3 Drug Resistance in MM

The last 10–15 years have witnessed a revolution in MM treatment with the introduction of "novel agents" and advances in stem cell transplantation. The first generation of "novel agents" included proteasome inhibitors (PIs) and the immunomodulatory drugs (IMiDs) (thalidomide, lenalidomide, and pomalidomide). The incorporation of "novel agents" into induction, consolidation, maintenance, and salvage therapies has markedly improved progression-free and overall survival. However in spite of multiple efficient therapeutic regimens for MM patients, drug resistance is still the major culprit for relapse and the chief harbinger of adverse clinical outcomes.

3.1 Risk-Adapted Approaches to MM Therapy

The initial therapy of patients with symptomatic MM varies depending on risk stratification, eligibility for autologous hematopoietic cell transplantation (HCT), and the resources available (Fig. 2). All patients receive induction therapy, although there is no general consensus as to the preferred induction regimen. Hence, patients eligible for HCT receive induction therapy for 2–4 months prior to stem cell collection, in order to reduce the number of tumor cells in the bone marrow and peripheral blood, whereas standard-risk patients ineligible for HCT receive induction lenalidomide plus dexamethasone (Palumbo et al. 2014a). Post induction therapy for HCT-eligible patients consists of high dose chemotherapy (usually melphalan 200 mg/m^2) followed by single or tandem autologous HCT (early transplant strategy). Alternatively, "late transplant" approach may be taken, consisting of continued therapy usually with the same induction regimen, reserving autologous HCT until first relapse.

For a variety of practical and reimbursement-related issues, many patients in the USA opt for few induction cycles followed by upfront ("early") transplant. In early trials evaluating the efficacy of autologous HCT vs. conventional chemotherapy alone, autologous HCT-treated arms demonstrated superior event-free survival and overall survival (Attal and Harousseau 2001; Attal et al. 1996; Barlogie et al. 1997; Child et al. 2003; Palumbo et al. 2004, 2014b). Taking into account that virtually all patients who receive autologous HCT for MM eventually develop relapsed disease, various trials have investigated the use of chemotherapeutic and biologic agents in an attempt to control residual cancer MM cells after HCT (Attal et al. 2012, 2013; Holstein et al. 2015; McCarthy et al. 2012, 2016; Palumbo et al. 2014b). Maintenance therapy after HCT is now routinely recommended. In HCT-ineligible patients, maintenance therapy after limited-duration induction has not proven to

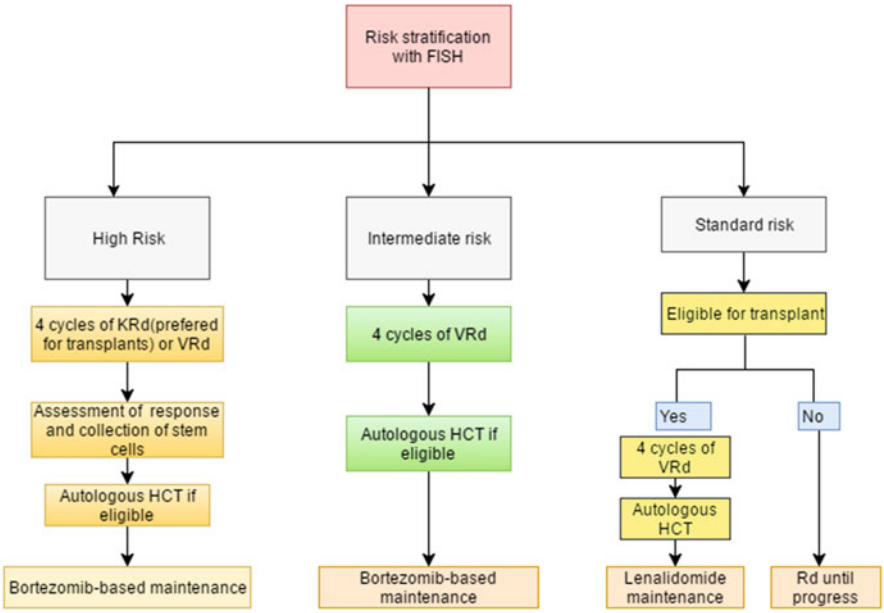

Fig. 2 MM state-of-the-art treatment algorithm. VRd: bortezomib, lenalidomide, low-dose dexamethasone, KRd: carfilzomib, lenalidomide, low-dose dexamethasone; Rd: lenalidomide plus low-dose dexamethasone; HCT: hematopoietic cell transplantation

increase overall survival so far (Benboubker et al. 2014; Dimopoulos et al. 2013; Palumbo et al. 2012; Stewart et al. 2015; Zweegman et al. 2016). Until more data are available, many authorities recommend observation with retreatment at the time of progression rather than maintenance therapy for transplant-ineligible standard-risk patients who have been treated with a triplet regimen. For intermediate or high-risk patients maintenance therapy is suggested, since the risk for relapse is high (Palumbo et al. 2014a, 2015). Transplant-ineligible patients receiving the combination of lenalidomide and dexamethasone continue until progression.

Patients who relapse after regimens containing bortezomib may respond to a newer proteasome inhibitor such as carfilzomib or ixazomib. Similarly, patients who relapse on lenalidomide-containing regimens may respond to a regimen containing pomalidomide. Patients whose disease progresses despite IMiDs and PIs can be treated with regimens containing the recently FDA-approved monoclonal antibody, daratumumab. Promising evidence from latest clinical trial data (Dimopoulos et al. 2016) points out that the addition of daratumumab to lenalidomide and dexamethasone significantly lengthened progression-free survival among patients with relapsed or refractory MM. Moreover panobinostat, a histone deacetylase inhibitor, has demonstrated activity in MM when administered with bortezomib and dexamethasone. Its approval was based on PANORAMA 1 trial, which evaluated the addition of panobinostat to bortezomib plus dexamethasone in 768 patients with relapsed/refractory MM. The panobinostat-treated arm showed

longer median progression-free survival (PFS) (San-Miguel et al. 2014). Finally, elotuzumab [an antibody targeting signaling lymphocytic activation molecule F7 (SLAMF7)] has also been approved from the FDA for use in combination with lenalidomide (Rev) and dexamethasone (Dex) for MM patients who have received one to three prior therapies (Lonial et al. 2015). Likewise, a randomized phase 2 trial pointed out improved PFS for patients assigned to etoluzumab plus bortezomib/dexamethasone compared to those assigned to bortezomib/dexamethasone for relapsed/refractory MM (Jakubowiak et al. 2016).

3.2 Response Assessment and Minimal Residual Disease

Patients should be evaluated before each treatment cycle to determine how their disease is responding to therapy. The International Myeloma Working Group (IMWG) uniform response criteria are the preferred criteria to determine the patient's best response to treatment and to define when relapse has occurred (Palumbo et al. 2014a; Rajkumar et al. 2011) (Table 2). The preferred method is the measurement of monoclonal (M) protein in serum and urine. Additional methods include the evaluation of bone marrow plasma cell percentage and serum free light chain (FLC) measurements. The latter is particularly valuable for patients with unmeasurable M protein in the serum and urine or oligosecretory disease (serum M protein levels $\leq$1 g/dL and/or urine M protein levels $\leq$200 mg/24 h) (Durie et al. 2006). At present, it is generally accepted that there is a positive correlation between the depth of response, particularly complete response, and prolonged progression-free survival (PFS) as well as overall survival (OS), particularly in high-risk patients. This association has been demonstrated in many different individual studies (Cavo et al. 2010; Dimopoulos et al. 2007; Rosiñol et al. 2012; San Miguel et al. 2008) and proved in meta-analyses among transplant-eligible and transplant-ineligible patients (Gay et al. 2011; van de Velde et al. 2007). However, almost all patients with MM who survive initial treatment will eventually relapse and require further therapy. Therapy for relapsed disease is indicated if there is a clinical deterioration or a rapid rise in paraproteins (Palumbo et al. 2014a; Rajkumar et al. 2011).

In the last few years, principles of measuring minimal residual disease (MRD), extrapolated from other hematological malignancy paradigms, have been explored in MM. MRD monitoring is one of the most relevant prognostic factors in MM patients regardless of age and cytogenetic risk in both transplant-eligible and -ineligible settings (Paiva et al. 2008, 2016). A meta-analysis by Munshi et al. (2016) provided sufficient evidence to support the integration of MRD-assessment as an end point in MM clinical trials.

There are several techniques for MRD assessment. Multiparametric flow cytometry (MFC) has been used to differentiate between normal and abnormal plasma cells in patients with MM by enabling detection of aberrant cell-surface marker expression (Mailankody et al. 2010). Its prognostic value in MRD monitoring was first introduced in 2002 by the Spanish (San Miguel et al. 2002) and British groups (Rawstron et al. 2002); both studies demonstrated improved

Table 2 Treatment response criteria

IMWG 2011 treatment response criteria for multiple MM
Immunophenotypic complete response
Stringent complete response, plus an absence of phenotypically aberrant plasma cells in bone marrow with a minimum of 1 m cells analyzed by multicolor flow cytometry (4 or more colors)
Molecular complete response
Stringent complete response, plus a negative ASO-PCR test
Stringent complete response
Meets the criteria for complete response, plus normal FLC ratio and an absence of clonal plasma cells by immunohistochemistry or 2–4-color flow cytometry
Complete response
• Negative immunofixation results in serum and urine
• Disappearance of any soft-tissue plasmacytomas
Very good partial response
Serum and urine M-component detectable by immunofixation but not by electrophoresis, or $\geq$90% reduction in serum M-component plus urine M-component $<$100 mg per 24 h
Partial response
$\geq$50% reduction of serum M-protein levels and reduction in 24-h urinary M-protein levels by $\geq$90% or to $<$200 mg per 24 h
In addition, a 50% reduction in the size of soft tissue plasmacytomas (if present at baseline)
Stable disease
• Criteria not met for complete response, very good partial response, partial response or progressive disease
Progressive disease
• Increase of 25% from lowest response value in any of the following: serum M-component (absolute increase must be $\geq$0.5 g/dL); and/or urine M-component (absolute increase must be $\geq$200 mg per 24 h); and/or definite development of new bone lesions or soft-tissue plasmacytomas, or definite increase in the size of existing bone lesions or soft-tissue plasmacytomas

FLC: free light chain, IMWG: International Myeloma Working Group. Modified with permission from the American Society of Hematology (Mailankody et al. 2015; Rajkumar et al. 2011)

progression-free survival and overall survival in MRD-negative patients regardless of their cytogenetic risk. The major limitation of MFC was the variability between different studies in what is considered MRD-positive or MRD-negative disease: recently a consensus statement has proposed a threshold limit of detection of 0.001%, and ideally a limit of quantification of 0.001% (Arroz et al. 2016). Besides MFC, ASO-PCR (allele-specific oligonucleotide PCR) of variable diverse joining (VDJ) heavy rearrangements is used for the assessment of MRD status in patients with MM (Puig et al. 2014). Similarly to MFC, the clinical utility of ASO-PCR has been established in a range of studies (Korthals et al. 2012; Martinez-Lopez et al. 2014). Finally, next generation sequencing (sensitive type of VDJ sequencing) may detect one clonal cell in 10^6 background cells. Martinez-Lopez et al. (2014) demonstrated that MRD-negative status detected by next generation sequencing was correlated with prolonged time to disease progression and overall survival.

3.3 Clonal Alternation and Clonal Tides

The contribution of clonal heterogeneity to disease progression and resistance to therapy is increasingly being recognized in MM. Deep sequencing of plasma cell genomes at different time points over the course of the disease has uncovered a previously unsuspected model of genomic evolution in MM. Keats et al. (2007) described three distinct evolutionary patterns between initial treatment and subsequent relapses. One third of patients had stable genomes that were associated with hyperploidy and favorable outcomes. The second third included either differential clonal response or "clonal tides," where distinct subclones dominated at different time points. The final third of patients displayed a pattern consistent with linear evolution, where a new subclone emerged later in the natural history. The presence of variants detectable only at alternating time points suggests that all clonal precursors were present at diagnosis, but selection pressures from treatment and clonal evolution caused dominance of these clones to rise and fall over time. In line with this study, Lohr et al. (2014) demonstrated that MM tumors are highly heterogenous with point mutations in the most significantly mutated genes (*BRAF*, *NRAS*, *KRAS*) being found to be clonal in some patients but subclonal in others.

Egan et al. (2012) analyzed a single case, harboring a t(4;14) mutation, that progressed to secondary plasma cell leukemia (sPCL) after 53 months of treatment. They perfomed whole-genome sequencing (WGS) to investigate the genetic events associated with the natural history of progressive MM in this patient. WGS was conducted at 4 time points during tumor evolution (diagnosis, first relapse, second relapse, and sPCL). They identified variants that were only detectable at alternating points, suggesting the "waxing and waning"of different clones with time and treatment. The concept of clonal tides and inta-clonal heterogeneity becomes very important in terms of treatment strategies for relapsed disease. It may be appropriate to retreat a patient with the same regimen that proved to be effective at an earlier point, since the current clone may not have been selected for resistance to that treatment (Egan et al. 2012; Keats et al. 2012). It will be of crucial importance to evaluate the extent of clonal heterogeneity in patients being evaluated for response to targeted therapies and probably employ drug combination regimens targeting multiple subclones simultaneously.

3.4 Resistance to Proteasome Inhibitors (PI)

26S proteasomes are composed of a 20S core which binds one or two 19S regulatory subunits (Voges et al. 1999). PI agents that target specific catalytic subunits have been introduced in MM clinical practice over the last 15 years: bortezomib and carfilzomib obtained FDA approval for treatment of relapsed/refractory MM in 2003 and 2012, respectively. Many hypotheses have been put forward to explain why MM cells may be sensitive to PIs: their copious immunoglobulin production likely contributes to synthetic lethality with PI therapy. This increased protein load lowers the threshold for proteotoxic stress, rendering the cells susceptible to

unfolded/misfolded proteins and apoptotic signals triggered by endoplasmic reticulum stress response (Obeng et al. 2006). PIs are thought to exert their cytotoxic effects through additional mechanisms including activation of c-Jun NH2 terminal kinase (JNK), inhibition of NF-κB pathway, and induction of reactive oxygen species.

One of the first reported mechanisms of PI resistance was the presence of mutations and/or aberrant expression of proteasome subunits, which impedes the ability of the drug to bind and inhibit proteasomes. Oerlemans et al. (2008) showed that PSMB5 (a β5-proteasome subunit) overexpression is associated with bortezomib resistance. Moreover, point mutation of the *PSMB5* gene (G322A mutant) conferred resistance to bortezomib. Ri et al. (2010) showed that mutated G322A allele contributed to resistance against bortezomib-induced apoptosis in MM cells. Expression of G322A-mutant PSMB5 was associated with the prevention of accumulation of unfolded proteins and thus suppression of apoptotic cell signaling. However, although *PSMB5* mutations have been identified in various in vitro studies, their clinical relevance is still unclear. On the other hand, Kortum et al. (2016) revealed that drug-refractory MM patients harbored mutations in the MAPK pathway genes *KRAS*, *NRAS*, and/or *BRAF* (72%). Resistance to bortezomib has also been associated with activation of the PI3K/AKT pathway (Ikeda et al. 2010; Meng et al. 2008).

Bortezomib may inhibit NF-κB activation by blocking proteasome degradation of IκBα (nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α) and decreasing NF-κB nuclear translocation (Mitsiades et al. 2002). However, whether aberrant NF-κB activation constitutes a mechanism of underlying resistance to proteasome inhibitors, or a compensatory strategy that only minimally affects cytotoxicity, remains a matter of controversy. Interestingly, Markovina et al. (2008) demonstrated that primary tumor cells from MM have relatively high constitutive NF-κB activation due to genetic abnormalities or microenvironment alterations. Subsequent analysis revealed that lack of inhibition of constitutive NF-κB activity by bortezomib was not due to ineffective proteasome inhibition: rather, NF-κB activity was triggered by an NF-κB canonical inducer, TNF-α. Taking into account that components of the microenvironment, such as bone marrow stromal cells (BMSCs), are well known to support MM disease, Markovina et al. (2010) further demonstrated that MM BMSCs may activate bortezomib-resistant NF-κB activity through secretion of soluble proteinaceous factors in conjunction with IL-8. Intriguingly, Hideshima et al. (2009) showed that bortezomib induces canonical NF-κB activation in MM cells through proteasome-independent downregulation of IκBα linked with IKKβ activation, which potentiates bortezomib-induced cytotoxicity. The authors suggested the potentially synergistic combination of bortezomib with IKKβ inhibitor. Finally, Fabre et al. (2012) showed that PBS-1086, a promising dual inhibitor of canonical and noncanonical NF-κB pathway, overcame the proliferative and antiapoptotic effects of the bone marrow milieu and strongly enhanced the cytotoxicity of bortezomib in resistant MM cell lines.

Efflux transporters have been implicated in resistance to proteasome inhibitors. Up-regulation of P-glycoprotein (also referred to as MDR1), a member of the ABC

(ATP-binding cassette) superfamily of transporters encoded by *ABCB1*, has frequently been observed in MM and strongly associated with relapse and drug resistance (Epstein et al. 1989; Grogan et al. 1993). O'Connor et al. (2013) examined the interaction of bortezomib with multidrug transporters including P-gp. The P-gp-overexpressing cell line, DLKP-A, was less sensitive to bortezomib than its parental non-P-gp-overexpressing line, DLKP, and the combination of a potent P-gp inhibitor, elacridar, with bortezomib produced strongly synergistic toxicity in the DLKP-A cell model. These observations suggest that P-gp can play an important role in bortezomib resistance. Moreover, Hawley et al. (2013) also showed that overexpression of P-gp is related to carfilzomib resistance, a newly FDA-approved, second-generation PI. Finally, Zhou et al. (2013) showed that a chromosomal instability (CIN) gene, *NEK2*, was highly correlated with bortezomib resistance, rapid relapse, and poor outcome in MM. High expression of *NEK2* induced drug resistance mainly through activation of the efflux pumps and it was demonstrated that down-regulation of *NEK2* reversed bortezomib resistance and induced bortezomib-mediated tumor growth inhibition.

Alonso et al. (2016) suggested that crosstalk between Hedgehog and retinoid signaling may confer bortezomib resistance. Expression of CYP26 [P450-like retinoic acid (RA) 4-hydroxylase] in BM stromal cells promotes a retinoic acid-low (RA-low) microenvironment that prevents the differentiation of normal and malignant hematopoietic cells (Ghiaur et al. 2013; Su et al. 2015). Since retinoid signaling promotes plasma cell differentiation and Ig production (Ertesvag et al. 2007) and low-secretory B cell phenotype is correlated with bortezomib resistance (Obeng et al. 2006), Alonso et al. (2016) found that the BM niche triggers bortezomib resistance by preventing plasma cell differentiation through stromal CYP26 activity. Moreover, paracrine Hedgehog secretion by MM cells upregulated stromal CYP26 and further reinforced a bortezomib-resistant microenvironment. Notch signaling has also been reported to contribute to bortezomib resistance. Notch receptors are expressed by MM cells and Notch ligand Dll1 is present on bone marrow (BM) stromal cells (Xu et al. 2012). In this study, Xu et al. (2012) demonstrated that Dll1 can activate Notch signaling mostly through Notch2 receptor and can promote drug resistance to bortezomib by upregulating CYP1A1, a cytochrome P450 enzyme involved in drug metabolism, both in murine and human MM cells. Finally, heat shock proteins (HSPs) have been implicated in bortezomib resistance. HSPs are molecular chaperones that are rapidly upregulated when cells are exposed to stress conditions, such as genotoxic stress or ER stress. Chauhan et al. (2003) showed that silencing Hsp27 in bortezomib-resistant SUDHL4 cells restores sensitivity to bortezomib, while overexpression of Hsp27 induces bortezomib resistance in bortezomib-sensitive SUDHL6 cells. The mechanism behind this interesting observation remains to be elucidated.

3.5 Resistance to Traditional Chemotherapy and Corticosteroids

3.5.1 Efflux Pump-Mediated Resistance to Chemotherapy

The development of drug resistance to chemotherapeutic agents remains one of the primary obstacles in cancer treatment. Membrane drug-efflux pumps such as P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and ABCG2 have been demonstrated to produce resistance to several commonly used chemotherapeutic agents. Turner et al. (2006) found that ABCG2 expression in MM cell lines increased after exposure to topotecan and doxorubicin, and was higher in log-phase cells when compared to growth-inactive cells. Notably, MM cells obtained from patients treated with high-dose melphalan and topotecan demonstrated increased ABCG2 expression after treatment and at relapse. Methylation-specific PCR indicated that expression of ABCG2 was regulated by promoter methylation both in cell lines and in patient plasma cells. Hence, drug-induced demethylation of the promoter increased ABCG2 mRNA and protein expression in response to topotecan. In addition, Grogan et al. (1993) and Cornelissen et al. (1994) demonstrated that expression of P-gp was increased upon exposure to "traditional" chemotherapy agents such as vincristine, doxorubicin, and dexamethasone.

3.5.2 Resistance to Alkylating Agents

Alkylating agents, such as melphalan and cyclophosphamide, act by forming cross-links between the two strands of DNA, therefore impairing DNA synthesis and cell replication. Spanswick et al. (2002) suggested that the principal mechanism of resistance to melphalan was the increased repair rate of DNA inner strand cross-links, mediated by the Fanconi anemia (FA)/BRCA pathway (Chen et al. 2005). Chen et al. (2005) demonstrated that knocking down Fanconi anemia genes using siRNA in melphalan-resistant cells could reverse drug resistance, while their overexpression leads to increased cell survival following melphalan treatment. A novel mechanism of resistance to alkylating agents was proposed by Cho et al. (2016): the authors showed that *MAGE-A* gene expression (Type I Melanoma Antigen Genes of the *MAGE-A* family) promoted drug resistance through differential regulation of anti-apoptotic protein Bcl-2. MAGE-A expression conferred resistance not only to melphalan but also to vorinostat (pan-histone deacetylase inhibitor) and bortezomib.

3.5.3 Resistance to Corticosteroids

Prednisone and dexamethasone are among the most commonly used drugs in the treatment of MM. Resistance to dexamethasone has been found to be due to steroid-receptor mutations. Moalli et al. (1992) reported that a truncated glucocorticoid receptor lacking the C-terminal hormone binding domain rendered MM cells resistant to dexamethasone. Sánchez-Vega and Gandhi (2009) suggested that glucocorticoid resistance can be induced by transcription elongation block in the glucocorticoid receptor gene *NR3C1*. In addition, Nojima et al. (2009) showed that epigenetic inactivation of *RASD1* (a small GTPase, member of Ras superfamily) plays a key role in the development of dexamethasone resistance in MM.

3.6 Resistance to Thalidomide Analogues

Although several mechanisms have been put forward to explain the activity of thalidomide, lenalidomide, and pomalidomide in MM, including demonstrable antiangiogenic, anti-proliferative, and immunomodulatory effects, the precise targets and the underlying molecular mechanisms have only recently become clearer. A landmark study (Ito et al. 2010) revealed that CRBN (cereblon) is the primary mediator of thalidomide-induced teratogenicity. Later, it was demonstrated that CRBN is also required for the anti-MM activity of thalidomide analogues, collectively called immunomodulatory drugs (IMiDs). Cereblon (CRBN) forms an E3 ligase complex with damaged DNA binding protein 1 (DDB1) and Cul4A, known as the CRL4 (CRBN). Krönke et al. (2014) and Lu et al. (2014) reported that treatment with lenalidomide, and other agents in this class, selectively promotes the ubiquitination and degradation of two lymphoid transcription factors, Ikaros (IKZF1) and Aiolos (IKZF3) by the CRL4 (CRBN) ubiquitin ligase. Lower CRBN expression was associated with lenalidomide resistance, indicating that CRBN is essential for lenalidomide and related IMiDs activity (Zhu et al. 2011).

Kortum et al. (2016) confirmed the crucial role of CRBN mutations in MM progression and responsiveness to treatment. The authors found 3 CRBN mutations in MM patients with acquired resistance to lenalidomide (these mutations were undetectable before MM became refractory). These mutations conferred lenalidomide-resistance in vitro. Xu et al. (2016a, b) identified AGO2 as a CRBN-binding protein. Silencing AGO2 halted MM cell growth and was associated with concomitant high CRBN expression (Fig. 3). Thus, AGO2 could be considered as a novel drug target to overcome IMiD resistance in MM cells.

3.7 Resistance to Immunotherapies

Antibody-based immunotherapy is a promising area of continued progress that has expanded our therapeutic armamentarium against MM. Daratumumab, which was granted FDA approval in 2015 for treatment of relapsed/refractory MM, is directed against CD38, a transmembrane protein that is highly expressed on malignant plasma cells. Daratumumab acts through induction of CDC (complement-dependent cytotoxicity), modulation of enzymatic activation, and ADCC (Tai et al. 2009). Despite the well-established clinical efficacy of daratumumab, not all heavily pretreated patients respond well to single-agent daratumumab, and the majority of patients who initially respond eventually progress. This implies the need for new insights into mechanisms of resistance. Upregulation of CD38 expression correlates with improved ADCC- and CDC-mediated activity in vitro and hence better daratumumab efficacy (Nijhof et al. 2015). Pretreatment CD38 levels on MM cells were significantly higher in patients who achieved at least partial response compared with patients who achieved less than partial responses (Nijhof et al. 2016). Complement-inhibitory proteins (CD55 and CD59) may

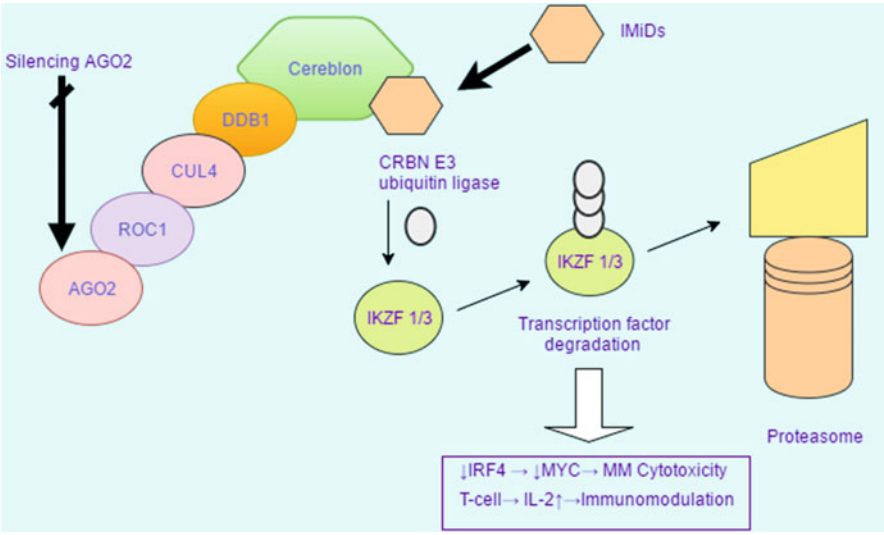

Fig. 3 Key downstream targets of cereblon (CRBN). Resistance against IMiDs, whose anti-MM activity depends heavily on CRBN downstream signaling, is observed in CRBN-depleted MM cells. Silencing AGO2 (cereblon binding protein argonaute 2) may restore responsiveness to IMiDs

represent a broad resistance mechanism for monoclonal antibodies that function through CDC. In particular, although expression levels of CD55 and CD59 were not associated with response in MM patients treated with daratumumab monotherapy, analysis of serial blood and BM samples revealed that CD55 and CD59 levels were increased on MM cells at the time of progression compared with baseline values. ATRA (all trans retinoic acid) may overcome daratumumab resistance by increasing CD38 levels.

PD-L1/PD-1 axis has recently emerged as a master immune checkpoint that controls antitumor immune responses against MM (Fig. 4). PD-L1-expressing tumor cells in the tumor microenvironment engage PD-1 on tumor-infiltrating T lymphocytes to repress antigen-driven activation (Pardoll 2012). Currently, two ongoing phase III clinical trials (*KEYNOTE 183* and *KEYNOTE 185*) are examining the efficacy of checkpoint inhibitors in combination with pomalidomide and low-dose dexamethasone in refractory and in newly diagnosed/naïve MM, respectively. Although it is premature to draw broad conclusions, findings from *KEYNOTE-023* clinical trial (San Miguel et al. 2015) demonstrated progression-free survival benefit when checkpoint inhibitors were combined with lenalidomide and dexamethasone.

Zaretsky et al. (2016) sought to identify mutations associated with resistance to anti-PD1 therapy in melanoma. In this study, it was demonstrated that loss of function of interferon-gamma signaling through acquired *JAK1* and *JAK2* truncating mutations rendered cancer cells resistant to interferon-induced growth arrest. Another patient demonstrated a truncating mutation in β_2 microglobulin (B2M), as

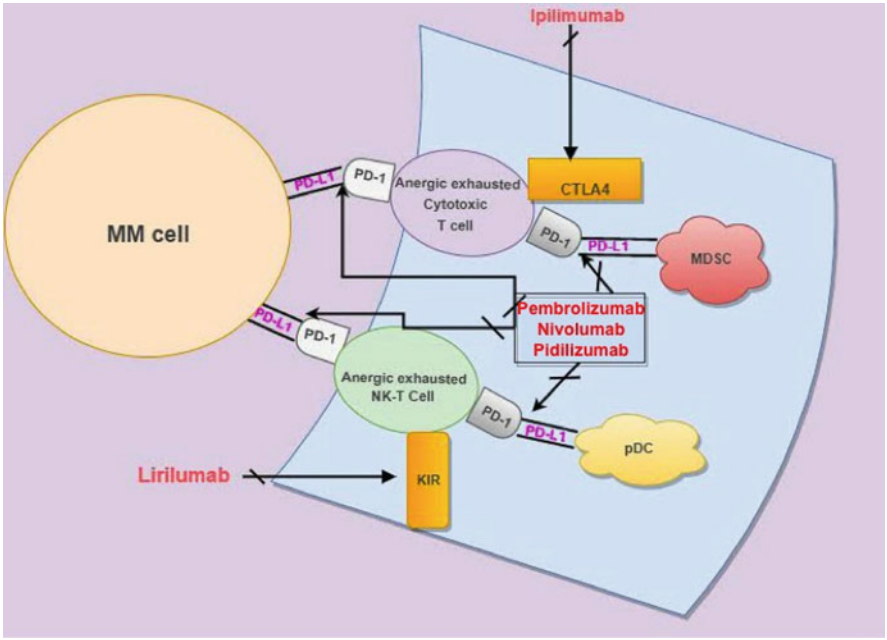

Fig. 4 Immune checkpoint inhibitors in MM therapy. PD-1 and its ligand PD-L1 are depicted as complementary transmembrane structures on effector and target cells (MMCs [multiple myeloma cells], MDSCs [myeloid-derived suppressor cells], pDCs [plasmacytoid dendritic cells]), respectively. CTLA4 (cytotoxic T-lymphocyte-associated protein 4) and killer cell immunoglobulin-like (KIR) are also presented on effector cells (Bianchi et al. 2015)

previously alluded to by Restifo et al. (1996). B2M is a structural component shared by all major histocompatibility complex (MHC) class I molecules. The loss of B2M from tumor cells prevents recognition by tumor-specific CD8+ T cells.

4 Microenvironment-Dependent Mechanisms of Resistance

MM microenvironment-mediated drug resistance can be subdivided into two categories: (1) soluble factor-mediated resistance (SFM-DR), which relies on cytokines, chemokines, and growth factors, and (2) cell adhesion-mediated resistance (CAM-DR) resulting from adhesion of tumor cells to BM stromal cells or to ECM components.

Key components of the SFM-DR include the interleukins IL-6 and IL-8, the growth factor IGF and the chemokine SDF-1. The important role of IL-6 in MM cell growth and survival has been previously addressed (Sect. 2.6.2). Additionally, IL-6 plays a role in therapy resistance. Frassanito et al. (2001) reported that autocrine IL-6 production was associated with high tumor burden and conferred resistance to dexamethasone-mediated apoptosis. Grigorieva et al. (1998) described a protective

effect of BMSCs toward glucocorticoid-induced apoptosis of MM cells through IL-6 production. In addition, IL-6 was found to induce antioxidant pathways by upregulation of NF-κB dependent MnSOD (manganese superoxide dismutase) expression, therefore rendering MM cells resistant to radiation and dexamethasone. Finally, it has been demonstrated that neutralizing IL-6 with the monoclonal antibody siltuximab increases sensitivity of MM cells to bortezomib (Voorhees et al. 2007). IL-8 also contributes to BM stromal cell-induced NF-κB activity in MM cells and the consequent resistance to bortezomib (Markovina et al. 2010). IGF-1 and the stromal cell-derived factor (SDF-1)/CXCR4 axis play also important role in MM survival, growth, and angiogenesis. IGF-1, which is produced by both MM cells and BM stromal cells, has been proven in vitro and in vivo to confer resistance to bortezomib (Kuhn et al. 2012). Several preclinical studies targeting the IGF-1/IGF-1R pathway are underway (Bieghs et al. 2016). Activation of the SDF-1/CXCR4 axis promotes formation of pro-metastatic BM niches and confers resistance to treatment (Alsayed et al. 2007). In vivo CXCR4 blockade (Roccaro et al. 2015) as well as SDF-1 inhibition (Roccaro et al. 2014) in murine xenografted mouse models inhibited MM bone-to-bone cell dissemination and disease progression. Likewise, Azab et al. (2009) found that AMD3100, an inhibitor of CXCR4, disrupted the interaction of MM cells with the bone microenvironment and sensitized them to therapy.

CAM-DR denotes mechanisms whereby MM cells overcome the cytotoxic effects of anti-cancer therapy via adhesive interactions with BM stromal cells and/or ECM components. Hazlehurst et al. (2000) demonstrated that integrin β1-mediated adhesion of MM cells to fibronectin resulted in G_1 arrest, whereas disruption of binding led to a rapid recruitment of cells into S phase and sensitized them to etoposide. Neri et al. (2011) showed that integrin β_7 silencing reduced CAM-DR and sensitized MM cells to melphalan and bortezomib. Signaling by another member of the integrin family, VLA-4 (Pulido et al. 1991) conferred resistance to vincristine and dexamethasone, while bortezomib was able to overcome VLA-4-mediated resistance (Noborio-Hatano et al. 2009). Bjorklund et al. (2014) demonstrated that CD44-expressing MM cells were resistant to lenalidomide by binding to the glycosaminoglycan hyaluronan (HA), while blocking the CD44/HA interaction sensitized MM cells to lenalidomide. Lin et al. (2016) showed that the ECM protein, reelin, produced by MM cells, enhances the adhesion of tumor cells to fibronectin via activation of α5β1 integrin, therefore potentiating drug resistance.

Oncogene activation can directly promote CAM-DR. Bustany et al. (2016) showed that ectopic cyclin D1 overexpression increased MM cell adhesion to stromal cells and fibronectin, upregulated expression of ICAM-1, as well as the synthesis of pro-inflammatory chemokines IL-8, IP10 and RANTES, collectively re-shaping the interplay between tumor microenvironment and MM cell. The 1q– amplicon contains *CHD1L* (chromodomain helicase/ATPase DNA binding protein) whose overexpression induced the expression of adhesion molecule CD49d (α4 integrin, a subunit of VLA-4) as well as CXCR4 (Xu et al. 2016a, b). Finally, epigenetic regulators can modulate CAM-DR. Direct contact of MM cells with

BMSCs triggered phosphorylation-mediated inactivation of the H3K27 histone methyltransferase, EZH2, that led to induction of antiapoptotic gene expression in MM cells (Kikuchi et al. 2015).

5 Deciding the Next Line of Therapy: Precision Medicine and Biomarkers

Precision medicine embodies the principle of matching the right drug to the right patient. Ex vivo cultivation and manipulation of tumor cells not only provides deep biological insight into tumor components and tumor–stroma interactions, but also permits the rapid evaluation of tumor responses to specific drugs (Friedman et al. 2015). Our group has previously described the development of such an ex vivo chemosensitivity and resistance assay (CSRA) based on a microfluidic system, MicroC^3 (microfluid cis-coculture) (Young et al. 2012). The reprise of key aspects of tumor microenvironment constitutes a major advantage of MicroC^3, enabling patient-specific microscale prediction of individual drug responses. Considering the promising responses of MM cells to bortezomib in MicroC^3 (Pak et al. 2015), further preclinical studies are required to establish ex vivo CSRA's role in everyday clinical practice.

Another critical question that should be addressed in the near future is whether time is ripe for predictive biomarker-driven MM therapy. A biomarker is typically defined as any characteristic (e.g., gene, protein, clinicopathologic variable, imaging feature) that can be objectively and reproducibly measured to serve as indicator of disease biology or response to a therapeutic intervention (Biomarkers Definitions Working Group 2001). When it comes to MM, the new generation of prognostic biomarkers, including cytogenetics (Avet-Loiseau et al. 2007; Fonseca et al. 2009), novel imaging (Usmani et al. 2013), bone turnover (Patel et al. 2014), gene expression profile (GEP) signature (Decaux et al. 2008; Dickens et al. 2010; Kuiper et al. 2012; Shaughnessy et al. 2007) may provide promising tools for improved classification of MM patients into specific therapies and trials. As discussed in Sect. 3.2, flow cytometric biomarkers have gained popularity in MRD monitoring (Rawstron et al. 2008). While traditional cytogenetics and FISH biomarkers along with whole gene expression profiling play a major role in risk stratification and overall prognosis, little progress has been made in applying predictive biomarkers routinely in clinical decision-making. Prognostic value does not always imply clinical utility; thus, emphasis should be placed on the optimization of predictive biomarker-guided treatments in the foreseeable future.

6 Overcoming Drug Resistance and Emerging Therapies

Manipulating p53 turnover in MM has been considered as a promising strategy to re-sensitize drug-resistant cells to therapy. Stuhmer et al. (2005) and Saha et al. (2010) showed that direct inhibition of the p53-negative regulator MDM2, through

treatment with an MDM2 antagonist (nutlin), or by blocking proteasomal degradation of p53 by bortezomib, can stabilize p53 and activate the p53 apoptotic signaling pathway. Moreover, Chauhan et al. (2012) showed that inhibition of ubiquitin-specific-protease 7 (USP7), which normally stabilizes MDM2, induces apoptosis in MM cells resistant to conventional and bortezomib-based therapies.

Another promising approach is to target drug resistance pathways through epigenetic modulation. Recently, small molecule inhibitors of oncogenic, chromatin-dependent signal transduction (e.g., bromodomain inhibitor JQ1) have been shown to possess activity against myeloma cells, acting through MYC transcriptional regulation, both in vitro and in vivo (Delmore et al. 2011).

Overcoming drug resistance through BH-3 mimetics that target anti-apoptotic proteins has recently been shown to possess significant activity against relapsed/refractory myeloma. In a phase 1 open-label study, venetoclax, a potent, selective small molecule Bcl-2 inhibitor demonstrated clear anti-myeloma activity in a cohort of 66 patients. Best responses correlated with presence of t(11;14) and high Bcl-2 as well as low Bcl-xL and/or MCL-1 expression levels (Kumar et al. 2016).

Inhibitors of nuclear export (e.g., selinexor) have been shown to produce meaningful clinical responses in patients who have progressed following therapy with IMiDS, PIs as well as the novel anti-CD38 antibody, daratumumab (penta-refractory MM population). This population of MM patients has exhausted all currently approved treatment options. Selinexor, a selective exportin XPO1 inhibitor, showed potent induction of apoptosis of MM cells independent of p53 signaling (Rosebeck et al. 2016). These principles were tested in a phase II clinical study (STORM): Vogl et al. (2016) reported significant anti-tumor activity of selinexor in combination with low-dose dexamethasone in penta-refractory MM as well as MM with adverse cytogenetic risk factors.

Immunotherapy is revolutionizing oncology and drug-resistant MM should be among the most fertile grounds to employ this strategy. Tran et al. (2016) elicited a polyclonal CD8+ T-cell response against mutant G12D *KRAS* expressed by metastatic colorectal cancer. The authors observed an objective regression of all lung metastases after the infusion of KRAS-directed tumor-infiltrating lymphocyte (TIL) clonotypes. This approach may constitute an effective strategy against *RAS*-mutant MM (Sect. 2.2). Moreover, promising results from ongoing clinical trials show that the prospects for checkpoint inhibition immunotherapy for MM are enticing, particularly when used in combination with "novel agents" (Badros et al. 2015; San Miguel et al. 2015) (Fig. 4). Future combinatorial immunotherapy approaches will test the efficacy and toxicity of combining checkpoint inhibitors with cellular therapies, e.g., CAR-T cells (see next paragraph).

CAR-T cell therapy has been investigated in MM (Maus et al. 2014). Ali et al. (2016) conducted a first-in-human clinical trial of CAR-T cells targeting B-cell maturation antigen (BCMA), expressed on MM plasma cells. Two patients were treated on the fourth dose level of $9 \times 10(6)$ CAR(+) T cells/kg body weight – both responded with long remissions, albeit at the expense of cytokine release syndrome and prolonged cytopenias.

Tumor vaccines seem to be a promising emerging therapy in MM. Cancer vaccines are designed to increase the frequency of antigen-specific T cells or antibodies through enhanced in vivo priming. To date, several vaccination approaches have been used for MM, such as idiotype vaccines, dendritic cell (DC)-based vaccines, and GM-CSF-based cellular vaccines (Hoyos and Borrello 2016). Based on this concept, Rosenblatt et al. (2011, 2013) developed an MM vaccine in which patient-derived tumor cells are fused with autologous dendritic cells, so that a variety of tumor antigens, including neo-antigens generated from mutational aberrations, are cross-presented to effector T-cells (Rosenblatt and Avigan 2016).

7 Concluding Remarks and Future Prospects

Treatment options for MM continue to develop at a rapid pace. It is becoming increasingly challenging to determine the optimal therapeutic approaches, since many years of follow-up are required to demonstrate a clear survival benefit. In the last 10–15 years, identification of a variety of molecules/signaling pathways with distinct modes of contribution to MM progression has opened new avenues for the development of novel targeted therapy strategies. The role of microenvironmental mechanisms in modulating treatment responses has recently begun to be systematically explored but several "black boxes" remain. In the light of the constantly evolving targeted therapy landscape, there is a call for a detailed, integrated, and multi-scaled approach for the analysis of the genomic, epigenomic, metabolomic, and immunophenotypic profile of MM cells, as well as a deeper insight into the role of cross-talk between MM cells and their microenvironment.

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Cancer Evolution as the New Frontier of Precision Medicine

Giuseppe Curigliano

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Abstract

New experimental breast cancer therapies directed against novel targets are currently in clinical These experimental agents are likely to be effective for a niche of breast cancers with specific "driver mutations". The ability to perform comprehensive molecular profiling of individual tumors has rapidly expanded over the last few years, and new DNA sequencing technologies require relatively limited quantities of fresh or archived paraffin-embedded or snap-frozen tumor tissue and provide rapid turnaround of sequencing results within a few weeks or less. All these technologies provide an unprecedented opportunity to identify patients with rare "driver" molecular alternations that are candidates for proof-of-concept clinical trials with matched targeted therapy, in the context of basket trials. The aim of this chapter on molecular profiling is to summarize the known recurrent molecular alterations in breast cancer that are potentially amenable to investigational targeted therapy, to provide an overview of the existing technological platforms for molecular profiling and ongoing or planned institutional/national screening initiatives and to outline a vision for molecular screening that may be integrated into the future activities of breast cancer research.

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1 Introduction

There is an exciting array of experimental breast cancer therapies directed against novel targets that are currently in clinical development. These investigational agents are likely to be effective for small subsets of breast cancers with specific "driver mutations". The ability to perform comprehensive molecular profiling of individual tumours has rapidly expanded over the last few years, as the cost of DNA sequencing technologies that allow for targeted multiplex "hotspot" mutation testing or deeper targeted exome and whole genome DNA sequencing has become cheaper than traditional Sanger-based DNA sequencing methods. New DNA sequencing technologies require relatively limited quantities of fresh or archived paraffin-embedded or snap-frozen tumour tissue and provide rapid turnaround of sequencing results within a few weeks or less. These technological advances allow for the prospect of point-of-care molecular profiling that can be used to guide the development of personalized breast cancer medicine therapy. For an international collective of academic breast cancer researchers, this provides an unprecedented opportunity to identify patients with rare "driver" molecular alternations that are candidates for proof-of-concept clinical trials with matched targeted therapy. The aim of this report on molecular profiling is to review the known recurrent molecular alterations in breast cancer that are potentially amenable to investigational targeted therapy, to provide an overview of the existing technological platforms for molecular profiling and ongoing or planned institutional/national screening initiatives and to outline a vision for molecular screening that may be integrated into the future activities of breast cancer research.

2 Background and Rationale

Personalized Medicine and New Drug Development The "oncogene revolution" has led to an explosion of molecularly targeted therapeutics in preclinical and clinical development over the last decade (Hanahan and Weinberg 2000). It is estimated that there are more than 800 targeted anticancer therapies currently in various stages of clinical development. Disappointingly, historical data indicate that only 5% of these investigational therapies will ultimately progress to registration for widespread use. These high attrition rates have multiple causes, including lack of efficacy and excessive toxicity (Kola and Landis 2004). In particular, when patients are selected for phase III trials based on histopathology alone, a targeted drug with a 5–10% single-agent response rate runs a high risk of failure (Stewart and Kurzrock 2009). Recent efforts to systematically sequence cancer genomes have revealed

that individual tumours frequently harbour multiple "driver" somatic mutations that confer growth advantage and positive selection (Stratton et al. 2009).

The increasing identification of specific somatic mutations and other genetic aberrations that drive cancers leaves us on the threshold of a new era of "personalized cancer medicine", in which specific biomarkers will be used to direct targeted agents only to those patients deemed most likely to respond. The potential medical, scientific and economic benefits of such a personalized approach to cancer therapy are immense and self-evident. Yet despite some important advances, only a limited number of approved targeted agents have had their approvals predicated on specific biomarkers of sensitivity or resistance. The premises behind personalized cancer medicine include (1) genetic aberrations exist in human malignancies; (2) a subset of these aberrations, often present across multiple cancer types, have functional relevance as "drivers" for oncogenesis and tumour progression; (3) such genetic aberrations are potentially "druggable" targets; and (4) there are tolerable medicinal compounds that can effectively modulate such targets (Greenman et al. 2007). A key requirement of this new, personalized approach to anticancer therapy is that specific patients must be matched to a particular drug or combination of drugs. Molecular profiling of tumours to identify somatic mutations and/or other genetic aberrations are examples of enrichment strategies to assist in matching patients to drugs or treatments that have gained increasing interest in the oncology community (Callaway 2010). The true merits of such personalized medicine strategies remain to be established. However, proof-of-concept clinical trials that establish the value of matching targeted treatments to rare molecular alterations in breast cancer and other malignancies are beyond the scope of any single pharmaceutical sponsor, cancer treatment facility or national cancer agency and will ultimately require international collaboration. Recent examples demonstrate that sequential testing of infrequent genomic alterations to identify candidates for clinical trials with matched targeted is inefficient, expensive and wasteful of scarce archived tumour tissue resources. Comprehensive molecular screening programs, which provide simultaneous testing of multiple biomarkers early in the course of a patient's natural history of disease, are most likely to advance personalized cancer medicine.

Genomic Alterations in Breast Cancer Somatic mutations are responsible for approximately 90% of breast cancers. Although data from comprehensive, large-scale breast cancer DNA sequencing projects are still awaited (Ellis et al. 2007), key features of the genomic breast cancer landscape have begun to emerge. First, although multiple regions of copy gain are observed, none occurs as frequently as 17q12 which harbours *ERBB2/HER2*; second, there are high-frequency somatic point mutations in three "gene mountains" (Greenman et al. 2007) – *TP53* (44%), *PIK3CA* (26%) and *CDH1* (19%) – but low-frequency recurrent point mutations (<5%) are also seen in genes that are validated drug targets in other types of cancer (i.e. *KRAS*, *BRAF* and *EGFR*); third, genes with somatic point mutations are also frequently regions of copy number gain in independent tumour samples (i.e. *PIK3CA*, *ERBB2*), highlighting their importance as oncogenes; and fourth,

point mutations are observed in multiple components of a signalling pathway at a higher rate than expected by chance alone (i.e. *PIK3CA*, *PTEN*, *AKT1*) indicating the relevance of the signalling pathway as a therapeutic target in mutated tumours. Additional data from large-scale sequencing projects, such as the Cancer Genome Atlas (TGCA) and the International Cancer Genome Consortium (ICGC), should provide additional insight with regard to the characteristic genome alterations that define the intrinsic molecular subtypes of breast cancer.

3 Molecular Screening Programs

Clinical Application of Targeted Genomic Sequencing Recent advances in DNA sequencing technology allow for rapid testing of multiple hotspot mutations using limited quantities of tumour DNA isolated from archival paraffin-embedded tumour material at an affordable cost (MacConaill et al. 2009; Dias-Santagata et al. 2010; Thomas et al. 2007). Studies by Thomas et al., MacConaill et al. and Dias-Santagata et al. examined between 250 and 1,000 individual tumour specimens for 120–400 mutations in 13–33 known oncogenes and tumour suppressor genes. These studies found at least one mutation in 30–37% of tumour samples (Thomas et al. 2007; Dias-Santagata et al. 2010; MacConaill 2013). Recently, Sequist et al. published their experience at Massachusetts General Hospital (MGH) with molecular screening of 552 non-small cell lung cancer patients using the multiplex PCR-based SNaPshot assays, which detects ~50 mutations and 14 genes, and FISH for ALK translocations (Sequist et al. 2011). They identified $\geq$1 mutation in 51% of patients who underwent successful profiling and directed 70 (22%) of 353 patients with advanced disease to a genotype-directed therapy. There are two reported studies that have investigated if therapy matched to molecular profile (MP) improves outcome. Von Hoff et al. conducted a study of matching treatments to MP in 86 patients across 9 different centres in the United States (Von Hoff et al. 2010). Only 66 patients proceeded to MP, wherein 64 targets were examined using a combination of immunohistochemistry (IHC), FISH and gene expression microarrays. Each aberration was matched to a predefined treatment. In 18 of 66 patients, they demonstrated progression free survival (PFS) for matched treatment to be 1.3 times greater than PFS for the treatment patients received immediately prior. Tsimberidou et al. performed molecular analysis on 1,283 patients, with success in 1,144 (89%) (Tsimberidou et al. 2011). They used polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) in examining for 11 separate molecular aberrations. In their cohort, 40% of patients had at least one aberration. They matched each aberration to a targeted treatment when available and demonstrated that patients who received matched targeted therapy had better response rates and improved time to treatment failure.

Molecular Screening Platforms The advantage of multiplex PCR-based platforms such as Sequenom's Oncocarta or OncoMap and Applied Biosystem's SNaPshot assay is that they provide excellent coverage of frequently mutated "druggable"

oncogenes when mutations cluster in a limited number of DNA sequence regions, such as *KRAS* (9 bases account for >99% of all mutations), *BRAF* (15–18 bases account for >90% of all mutations) and *PIK3CA* (12–15 bases account for >80% of all mutations). However, for clinically relevant tumour suppressor genes, such as *TP53*, *PTEN*, *BRCA1* or *BRCA2*, where mutations are more widely distributed across a much larger DNA coding region, the ability to detect mutations is limited to a few selected hotspots. In addition, the published molecular screening panels using these platforms are only able to detect known base-pair substitutions and limited deletions or insertions (indels) and gene amplification. They do not include translocations, larger indels or novel base-pair substitutions. The Sequenom MassARRAY Analyzer has developed methods to evaluate copy number variation (CNV); however, this has not been validated for point-of-care molecular profiling using human tumour samples.

Next-Generation Sequencing Sequenom, SNaPshot and other PCR-based multiplex assays are constrained by bandwidth and throughput. Next-generation sequencing (NGS) refers to technological platforms that allow for massive parallel sequencing of millions of DNA templates. "Second"-generation deep sequencing refers to clonal amplification of DNA templates on a solid support matrix followed by cyclical sequencing with short reads. These instruments are currently used to sequence entire genomes, exomes, transcriptomes and methylomes that often require weeks for sample template preparation, sequence generation and data analyses. As a result, their use is largely confined to large genome centres. Since "second-generation" DNA sequencing instruments are not employed in diagnostic settings, additional validation of potential candidate mutations is required using clinical-grade sequencing assays in certified diagnostic laboratories. The advent of "third"-generation sequencers such as Pacific Biosciences PacBio RS and Life Technologies' Ion Torrent Personal Genome Machine (PGM) provides increased speed of sequencing due to their use of sensors that detect nucleotides as they are added to DNA molecules in synthesis, although parallelization and machine throughput currently is much lower than with second-generation technologies. In addition to the Ion Torrent PGM, other so-called "bench sequencing" machines have recently been released by Illumina (MiSeq) and Roche/454 (GS Junior), which are moderate-throughput platforms with fast run times, long DNA reads and automated library preparation that are well-suited to clinical applications. The appeal of these low-cost ($\leq$125,000€ per instrument) "bench sequencing" platforms is that they offer the opportunity to comprehensively test a large targeted panel of relevant cancer genes (1,000 or more) with 30–50$\times$ or greater coverage to identify rare (<5% prevalence) mutations and copy number alterations that are potentially relevant to clinical care with a rapid turnaround time to results of 1 week or less. One of the major obstacles to NGS for cancer diagnostics is the ability to assess DNA extracted from limited formalin-fixed paraffin-embedded (FFPE) material, such as archival tumour blocks or small core tumour biopsies. Preliminary experience suggests that NGS is feasible from FFPE core tumour biopsies, although the quality DNA isolated

from archival tumour material that is routinely stored for >5 years and the robustness of methods of sequence enrichments remain questionable.

Ongoing Molecular Screening Programs Recognizing that cancer genome sequencing is likely to be integrated in routine clinical decision-making in the near future, many leading cancer research institutions and national cancer agencies have recently launched or are soon to launch broadscale molecular screening programs for solid tumours, including breast cancer (Tuma 2011). Massachusetts General Hospital (MGH) has implemented a phased roll out of the SNaPshot testing (which now includes ~120 mutations in 16 oncogenes) using archival tumour tissue in four tumour types: lung, colon, breast and glioblastoma multiforme (GBM). The Vanderbilt-Ingram Cancer Center (VICC) also initiated a similar program of SNaPshot screening of archival tumour tissue in non-small cell lung cancer and melanoma in 2010 including ~40 mutations in 6–8 genes. They integrated the molecular screening results into the patient's electronic medical record. Their "My Cancer Genome" (www.mycancergenome.org) website includes information about common activating mutations in "druggable" oncogenes and includes links to clinical trials with molecular selection based upon molecular profiling. In July 2011, they expanded their program to include PI3-kinase pathway-specific mutation panel for breast cancer. The Dana-Farber Cancer Institute (DFCI) in partnership with the Brigham and Women's Hospital has recently announced an ambitious US$43-million program (PROFILE) to perform mutation profiling using OncoMap (which includes ~470 mutations in 41 genes) in selected tumours, including colon, lung, breast and some sarcomas and leukaemias. Their project will include patients with early stage and advanced disease, linking genomic information with clinical outcomes and response to matched targeted therapies. It has been estimated that the program will include up to 10,000 patients annually (Tuma 2011). In Canada, the Ontario Institute for Cancer Research (OICR) and Princess Margaret Hospital (PMH) opened a pilot feasibility with biopsy of metastatic lesions involving patients with advanced solid tumours for profiling using the Sequenom Oncocarta (v1.0) and the third-generation NGS platform PacBio RS analyser for the same 19 genes as are included on the Oncocarta v1.0 panel. The initial results for the first 30 patients accrued were presented at the 2011 AACR-NCI-EORTC Molecular Target and Cancer Therapeutics Meetings (Tran et al. 2011). PMH will soon launch its own internal program entitled the Integrated Molecular Profiling in Advanced Cancers Trial (IMPACT) to perform mutation profiling using a customized Sequenom panel that includes ~277 mutations in 25 genes for patients with advanced non-small cell lung cancer, colorectal cancer, ovarian cancer, breast cancer and patients considered for phase I clinical trials. The IMPACT study will initially include 500 patients annually and will be expanded to include additional disease sites and NGS technology.

Investigators at the University of Michigan also recently published their pilot experience with real-time high-throughput whole exome sequencing for two patients enrolled in the MI-ONCOSEQ protocol (Roychowdhury et al. 2011).

They successfully performed whole exome sequencing of fresh tumour biopsies from two patients – with colorectal cancer and melanoma – on the Illumina HiSeq platform and reviewed the results at a Sequencing Tumour Board within 4 weeks from the time of tumour biopsy. There are plan to perform deep whole exome sequencing of approximately 100 patients with advanced solid tumours per year, with the aim of matching patients to investigational clinical trials with targeted therapies. In Europe, there are also molecular screening programs that are underway. At the Institut Gustavy Roussy (IGR) in Paris, the ongoing MOSCATO (Molecular Screening for Cancer Clinical Trial Optimization) clinical trial protocol will perform molecular profiling using array comparative genome hybridization (aCGH) and Sanger sequencing for selected mutation hotspots in 600 patients over 3 years who are candidates for phase I clinical trials. Similarly, the ZAFIR01 clinical trial protocol at IGR will perform aCGH and targeted Sanger sequencing (PIK3CA and AKT1) in 400 patients with advanced breast cancer who undergo tumour biopsies for molecular screening. Cancer Research UK has recently launched the "Stratified Medicine Program" across seven cancer research hospitals in the United Kingdom which will perform molecular profiling for ~20 alterations in 8 genes using archival tumour material from 9,000 patients with advanced melanoma, breast, prostate, ovarian, colorectal and non-small cell lung cancer over 2 years. The details of the platform that will be used for molecular profiling have not been publicly disclosed. In the Netherlands, hospitals from Amsterdam, Rotterdam and Utrecht have launched a molecular screening to perform next-generation sequencing of fresh tumour biopsies from patients who are candidates for phase 1 clinical trials. Approximately 1,200 patients will be enrolled over the next 3 years, with plans to profile approximately 2,000 genes per patient using targeted sequencing on the Illumina HiSeq platform. The Breast International Group is also running a molecular screening program in metastatic breast cancer named AURORA project. AURORA has two broad purposes:

1. To analyse breast cancer samples using techniques including but not limited to targeted DNA sequencing and RNA sequencing, in order to better understand the genetic aberrations related to breast cancer. This part of AURORA could help us understand breast cancer disease evolution (this will be done in all patients) and determine why some patients respond well to a certain treatment while others don't (this will only be done in a minority of patients). This may not provide you with any benefit directly, but your participation is likely to help us find answers to questions which could help to improve the treatment and/or quality of life of future breast cancer patients.
2. To identify patients potentially eligible to participate in approved studies testing new therapeutic strategies based on known breast cancer-related molecular aberrations found in the breast cancer samples. Such identification is done when the aberrations of your primary and/or metastatic tumour DNA found by targeted sequencing match an ongoing therapeutic clinical trial testing a drug against the aberration. These trials might not be available at the time being, but your treating physician shall inform you in case they become available. If you are found to be eligible for an ongoing trial, your treating physician will give you more

information and an additional informed consent form to sign, specific to that particular trial. Note that enrolment in one of these candidate trials is completely up to you. Please note that aberrations for which therapeutic clinical trials are available may be found only in a minority of patients. To start with, this research project will involve 1,300 patients from hospitals mainly located in Europe.

4 Future Perspectives

It is likely that future clinical trials in breast cancer with targeted therapies will be conducted in molecularly defined subpopulations of disease. Advances in high-throughput DNA sequencing technology allow for screening a large number of genes simultaneously at a relatively low cost to molecularly characterize individual tumours for triage of clinical trials with targeted therapies. These molecular screening programs are rapidly being developed by large cancer research hospitals and national cancer societies in North America and Europe. It is very unlikely that a single pharmaceutical sponsor will be able to support the large-scale molecular screening programs to identify relatively rare subpopulations ($\leq$5%) of breast cancer that are amenable to clinical trials with matched targeted therapies. The existing model of sequential prescreening for individual clinical trials – with separate informed consent forms, processes of tumour material retrieval and shipping and methods of laboratory testing and reporting – is expensive, inefficient and not well-suited to the current era of molecularly targeted drug development. We need to find new paths to access innovations to clinical research and daily practice. To ensure that continued innovation meets the needs of patients, the therapeutic alliance between patients and academic-led research should to be extended to include relevant pharmaceutical companies and drug regulators with a unique effort to bring innovation into clinical practice. We need to bring together major players from the world of breast cancer research to map out a coordinated strategy on an international scale, to address the disease fragmentation, to share financial resources and to integrate scientific data. The final goal will be to improve access to an affordable, best standard of care for all patients in each country.

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