Resistance to Targeted Anti-Cancer Therapeutics 16
Series Editor: Benjamin Bonavida

Jenifer R. Prosperi *Editor*

Resistance to Targeted Therapies in Breast Cancer

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"Resistance to Targeted Anti-Cancer Therapeutics": Aims and Scope

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For several decades, treatment of cancer consisted of chemotherapeutic drugs, radiation, and hormonal therapies. Those were not tumor specific and exhibited several toxicities. During the last several years, targeted cancer therapies (molecularly targeted drugs) have been developed and consisting of immunotherapies (cell mediated and antibody) drugs or biologicals that can block the growth and spread of cancer by interfering with surface receptors and with specific dysregulated gene products that control tumor cell growth and progression. These include several FDA-approved drugs/antibodies/inhibitors that interfere with cell growth signaling or tumor blood vessel development, promote the cell death of cancer cells, stimulate the immune system to destroy specific cancer cells, and deliver toxic drugs to cancer cells. Targeted cancer therapies are being used alone or in combination with conventional drugs and other targeted therapies.

One of the major problems that arise following treatment with both conventional therapies and targeted cancer therapies is the development of resistance, preexisting in a subset of cancer cells or cancer stem cells and/or induced by the treatments. Tumor cell resistance to targeted therapies remains a major hurdle, and, therefore, several strategies are being considered in delineating the underlining molecular mechanisms of resistance and the development of novel drugs to reverse both the innate and acquired resistance to various targeted therapeutic regimens.

The new Series *Resistance to Targeted Anti-Cancer Therapeutics* was inaugurated and focuses on the clinical application of targeted cancer therapies (either approved by the FDA or in clinical trials) and the resistance observed by these therapies. Each book will consist of updated reviews on a specific target therapeutic and strategies to overcome resistance at the biochemical, molecular, and both genetic and epigenetic levels. This new Series is timely and should be of significant interest to clinicians, scientists, trainees, students, and pharmaceutical companies.

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Series Editor Biography

Dr. Benjamin Bonavida, Ph.D. (Series Editor), is currently Distinguished Research Professor at the University of California, Los Angeles (UCLA). His research career, thus far, has focused on basic immunochemistry and cancer immunobiology. His research investigations have ranged from the mechanisms of cell-mediated killing, sensitization of resistant tumor cells to chemo-/immunotherapy, characterization of resistant factors in cancer cells, cell-signaling pathways mediated by therapeutic anticancer antibodies, and characterization of a dysregulated NF-κB/Snail/YY1/RKIP/PTEN loop in many cancers that regulates cell survival, proliferation, invasion, metastasis, and resistance. He has also investigated the role of nitric oxide in cancer and its potential antitumor activity. Many of the above studies are centered on the clinical challenging features of cancer patients' failure to respond to both conventional and targeted therapies. The development and activity of various targeting agents, their modes of action, and resistance are highlighted in many refereed publications.

Acknowledgments

The Series Editor acknowledges the various assistants that have diligently worked in both the editing and formatting of the various manuscripts in each volume. They are Leah Moyal, Kevin Li, and Anne Arah Cho.

Editor's Biography

Dr. Prosperi received her BA in Microbiology from Miami University (OH), and went on to get a Ph.D. in Integrated Biomedical Science (focus: Cancer Biology) at The Ohio State University. She joined a laboratory focused on breast cancer research, and started volunteering at The James Cancer Hospital and with The Komen Foundation. She then completed postdoctoral studies at the University of Chicago, where she started to focus on the APC tumor suppressor and developing targeted therapies for breast cancer. In 2012, she was recruited to Indiana University School of Medicine, South Bend, with an adjunct faculty position at the University of Notre Dame. Through these affiliations, she has been a member of both the Simon Cancer Center and the Harper Cancer Research Institute since 2012. Her laboratory is focused on the understanding of resistance to chemotherapy in breast cancer patients, specifically how the APC tumor suppressor impacts this process.

Objective

Resistance to Targeted Therapies Against Human Breast Cancer

Breast cancer is a collection of multiple different subtypes based on differential gene expression. The different subtypes, including luminal and basal, vary in their response to standard chemotherapy. Given the differing chemotherapeutic responses and the molecular subtypes, researchers and physicians have invested in the development of targeted therapeutics. Tamoxifen and trastuzumab were among the first targeted therapies for estrogen receptor (ER+) or Her2+ breast cancer, respectively. Triple negative breast cancers (TNBC), which lack expression of ER, Her2, and progesterone receptor (PR), are generally treated with broad-spectrum chemotherapeutic agents. While it is evident that breast cancers often develop resistance to chemotherapy, recent studies have also shown resistance to targeted therapies. Patients exhibiting resistance to targeted therapy will often have tumor recurrence and decreased survival. This book will explicate the necessity for targeted therapy in breast cancer, but also discusses how breast tumors develop resistance even to these specific therapies. Finally, it considers ways to overcome resistance and the landscape of the future of targeted therapies in breast cancer.

Preface

Breast cancer, previously studied as one disease, is really a compilation of multiple subtypes that have differential gene expression and vary in response to standard chemotherapy. Based on the response to chemotherapy, the specific molecular subtypes, and the ability of tumors to become resistant to therapy, recent investigations have focused on developing targeted therapeutics. The earliest of these included the introduction of estrogen receptor (ER) inhibitors and targeted therapy for Her2 positive breast cancer. Triple negative breast cancers (TNBCs), which lack expression of ER, Her2, and progesterone receptor (PR), are generally treated with broad-spectrum chemotherapeutic agents. While it is evident that breast cancers often develop resistance to chemotherapy, recent studies have also shown resistance to targeted therapies. Despite much advancement that has occurred in this field, breast cancer is still likely to become resistant to therapeutic regimens resulting in tumor recurrence and decreased overall patient survival. This book is designed to focus on some of the most commonly used targeted therapies, to understand how breast cancer develops resistance, and to discuss the potential ways to overcome resistance.

This book first provides a brief overview of standard chemotherapy for breast cancer and the development of chemoresistance. Dr. David Morrison and colleagues delve into how well we are hitting the target in precision medicine. We will continue to focus on the primary targeted therapies for breast cancer and a discussion of the ability of breast cancer to evade even these specific targeted therapies. The first of these targeted therapy chapters was contributed by Dr. Susan Kane and colleagues. They are focused on the development of resistance to HER2-targeted therapy and detail the available antibody inhibitors, antibody-drug conjugates, small molecule inhibitors, and combination therapies. Finally, they provide valuable information on the multiple mechanisms of resistance to HER2-targeted therapy. Dr. Irida Kastrati contributed a chapter on endocrine resistance and the relationship to breast cancer stem cells (CSCs). She details ER-mediated signaling pathways, the multiple types of endocrine-targeted drugs, and the development of resistance to these compounds. She further discusses the possibility of targeting CSC-mediated pathways to overcome endocrine resistance. Drs. Laura Bourdeanu and Landon have provided insight into the EGFR pathway. They discuss both primary and acquired resistance

in addition to the crosstalk with other signaling pathways in breast cancer. Dr. Michael Wendt and his colleagues contributed a chapter focused on targeting the FGFR in breast cancer. He has described some of the classical mechanisms of resistance to targeted therapy and has focused on the available compounds for FGFR inhibition. Finally, I have provided an overview of some of the upcoming and less investigated targeted therapies in breast cancer. We have included iNOS, the PI3K pathway, PARP, PTK6, CDK4/6, and the Wnt signaling pathway. Some of these targets have been mentioned in other components of the text; however, in our chapter, we provide basic science information in addition to the ongoing clinical work and information about resistance to these therapies. This volume concludes with an analysis of the future of targeted therapy in breast cancer from Dr. Ravi Velaga. He has focused on cancer genomics and the robust increase in information on circulating tumor DNA (ctDNA). In this, there is a discussion about the future of precision medicine, specifically focused on breast cancer.

I thank the series editor, Dr. Ben Bonavida, for the opportunity to assemble this book, and the contributing authors, co-authors, and reviewers for sharing their valuable time and expertise to compile this book. The result is an excellent assembly of the currently used targeted therapies in breast cancer, the development of resistance to these drugs, and insights into ongoing and future efforts to circumvent resistance in breast cancer.

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Contents

Invasive Breast Cancer Therapy 2017: How Well Are We Hitting the Target?

Georges E. Tanios, Matthew E. Burow, Bridgette Collins-Burow, and David G. Morrison

Abstract Invasive breast cancer is a major cause of death due to cancer for American women. Progress against this disease has only been achieved through a better understanding of the cellular and molecular aspects of breast cancer. To begin with cancer of the breast is not just a loco-regional control issue. In fact, metastatic disease from invasive breast cancer is not a one-way street in that metastases metastasize and send clusters of tumor cells that migrate to other sites of the disease including back to the primary tumor. Since invasive breast cancer at diagnosis is a systemic disease, adjuvant therapy has been initially started with relatively nonselective cytotoxic agents and latter with the use of endocrine therapy. The response rate to endocrine therapy improved once we knew which cancers expressed the actionable target. Endocrine therapy was the first targeted therapy but resistance to it is common in the adjuvant setting and essentially universal in the metastatic setting. Use of everolimus and palbociclib to interdict additional signaling pathways are proof positive that new-targeted agents can overcome resistance to endocrine therapy. In the adjuvant setting the risk of a patient's having metastatic disease can be approximated by knowing the histological type of breast cancer, size of invasive component of the primary, presence or absence of lymph node or lymphovascular space involvement, the degree of histological atypia, the presence or absence or estrogen, progesterone or Her-2 neu and the patient's menopausal status. Her-2 neu positive breast cancers have been converted from the most aggressive tumors to very curable cancers in the adjuvant setting by the use of Her-2 neu specific antibodies, such as trastuzumab, to the cell surface portion of the molecule. The use of adjuvant chemotherapy has been further refined on 2 fronts. First, smaller estrogen positive node negative breast cancers benefit from tamoxifen and some benefit more or less from additional therapy with chemotherapy based on the use of predictive models. For larger node positive breast cancers, adjusting the schedule of mostly S-phase cytotoxic chemotherapy to account for tumor cell growth kinetics has improved overall survival. Unfortunately, triple negative breast cancers lack a defined actionable target. Thus, the poorer survival in

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this group is not understood despite the initial good response to cytotoxic chemotherapy. Refinements in the pharmacokinetics of paclitaxel by using an albumin carrier have also proved beneficial to patients with metatstatic breast cancer. Additional refinements in drug carriers or other delivery systems should help in the care of those with metastatic disease. Only by accounting for the tumor's cellular biology, growth kinetics, and the targeted drug's pharmacokinetics as well as pharmacodynamics will blocking or overcoming resistance to targeted therapy be accomplished.

Abbreviations

AC	Doxorubicin and cyclophosphamide
ACD	Doxorubicin, cyclophosphamide and docetaxel; chemotherapy given with AC first then docetaxel
ACP	Doxorubicin, cyclophosphamide and paclitaxel with the doxorubicin and cyclophosphamide given together for 4 cycles followed by the paclitaxel
AI	Aromatase Inhibitor such as letrozole, anastrozole, or exemestane
AKT/PI3K	Protein kinase b/phosphoinositide 3 kinase
ASCO	American Society of Clinical Oncology
BCIRG	Breast Cancer International Research Group
BRCA	Breast Cancer as in breast cancer susceptibility genes 1 and 2
CALGB	Cancer and Leukemia Group B
CALOR	Chemotherapy as Adjuvant for Locally Recurrent breast cancer
CD 4/6	Cyclin dependent kinases 4/6
Chx	Chemotherapy
CMF	Cyclophosphamide, methotrexate and 5-fluorouracil
DAF	Decay Accelerating Factor
DFS	Disease Free Survival
DNA	Deoxyribonucleic Acid
EBCTGCG	Early Breast Cancer Trialists Cooperative Group
EC	Epirubicin and Cyclophosphamide
EGFR	Epidermal Growth Factor Receptor; erb-1
Endo	Endocrine therapy such as tamoxifen
ER	Estrogen Receptor
Erb	Epidermal growth factor receptor b
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor; a diverse family of ligands and receptors
FISH	Fluorescent In Situ Hybridization
Her-2 neu	Human epidermal growth factor receptor b 2/ Neu oncogene; a transmembrane receptor protein with no known ligand it may heterodimerize with erb-1, erb-3 or erb-4; dimerization activates the intracellular signaling from these proteins so as to need no cell surface ligand binding. Neratinib blocks the tyrosine kinase part of these molecules.

IHC	Immunohistochemistry
IHC4	An immunohistochemical panel of 4 stains used to subclassify patients who need or probably do not need adjuvant chemotherapy
Ki-67	An immunohistochemical stain used to identify dividing cells
Level I	Refers to lymph node groups as define anatomically for dissection in breast cancer; level I nodes are closer to the primary cancer than level II
LHRH	Luteinizing Hormone Releasing Hormone; drugs altering this hormone are either agonists or antagonists but in either case they work to shut off estrogen production in the ovaries
microRNA	Microribonucleic acid; a family of short RNA species that modify cell growth
MINDACT	Microarray In Node negative and 1–3 positive lymph nodes Disease May Avoid chemotherapy; a research study using Mammaprint to indicate what patients do or probably do not need chemotherapy
mTOR	Mechanistic target of rapamycin; everolimus blocks this pathway
N2	In the Tumor, Node and Metastasis classification of the stage of breast cancer it refers to patients who have 4–9 lymph nodes involved with breast cancer
NCCT	Northern California Cancer Trialists
NeoSphere	Neoadjuvant pertuzumab and trastuzumab in locally advanced, inflammatory and early Her-2 neu breast cancer
NSABP	National Surgical Adjuvant Bowel and Breast Program
OS	Overall Survival
p53	The protein product of a major tumor suppressor gene; loss of function of this gene contributes to the virulence of breast and other cancers
PARP	Poly-adenosinediphosphate polymerase
pCR	Pathological Complete Response
PD l-1	Programmed Death 1 Ligand
PD-1	Programmed Death 1 Receptor
PR	Progesterone Receptor
q	Every as in every 2 weeks-q14 days; every 3 weeks-q21 days
RFS	Relapse Free Survival
RNA	Ribonucleic acid
RR	Response Rate
SEER	Surveillance, Epidemiology and Endpoint Results; a large data base that has information on breast cancer patients
SLN	Sentinel Lymph Node; the first lymph node that breast cancer cells will metastasize to which can be identified by both a dye and radioactive tracer during dissection of the axilla
S-phase	The part of the cell cycle when DNA is synthesized
T	Paclitaxel; an antimicrotubule based chemotherapy drug
T DM1	Aldo-traztuzumab emantsine; a chemically modified version of traztuzumab linked to an antimicrotubule agent
T1b	In the Tumor, Node and Metastasis classification of breast cancer stage it is a cancer less than 1 cm but greater than 0.5 cm

T2 or T3	In the Tumor, Node and Metastasis classification system for the staging of breast cancer it refers to breast cancers that are >2.0 cm to <5 cm and breast cancers that are over 5 cm in greatest dimensions, respectively. These dimensions that are derived based on the invasive portion of the breast cancer.
TAC	Docetaxel, doxorubicin and cyclophosphamide with all 3 drugs given at each cycle
TCH	Paclitaxel, carboplatin and trastuzumab
TKI	Tyrosine kinase inhibitor such as neratinib
TNBC	Triple Negative Breast Cancer
Trop-2/EGF-1	A new antibody based immunotherapy target found on many epithelial cell types
VTE	Venothromboembolic disease such as deep vein thrombosis or pulmonary embolism

Introduction

The landscape of invasive breast cancer treatment in America continues to rapidly evolve to reduce the mortality rate of the most common malignancy in women. Despite multi-modality treatment, surgery, radiation therapy, endocrine therapy, immunotherapy and cytotoxic chemotherapy, to prevent local or systemic recurrence of stages I-III breast cancer, women still develop metastatic disease. Despite current treatments patients still die from metastatic disease and succumb to treatment-related complications. Table 1 is a brief list of areas in need of better therapy. This chapter describes the current state of the art in terms of how we got here, discusses the limitations of our current clinical care (Table 2) and takes a look to the future. Agents with specific targets that interdict the growth, spread and survival of metastatic disease as the primary, secondary and tertiary lines of treatment will be the next great step forward in care. Table 3 is but a short list of possible targeted treatments that may exist in the future. Hopefully, this chapter will spark interest into the development of additional novel targeted therapies. Resistance to targeted agents occurs despite well-defined targets and agents to block them but with additional basic scientific research for additional signaling pathways can be identified and new-targeted agents can be brought to clinical fruition. Subsequent chapters will explore resistance to agents designed for actionable targets such as the estrogen receptor, Her-2 neu, fibroblast growth factor receptors and others.

Overview of Surgical Approaches

Understanding the pathobiology of breast cancer is crucial to the successful care of patients. Unfortunately even now a great deal more information is needed in terms of the molecular control of breast cancer initiation, growth, metastasis, survival

Table 1 Areas of need for new understanding and targeted therapies

Treatment of hormone receptor positive breast cancers recurring in the operative field so as to improve OS
Enhancing tamoxifen or aromatase withdrawal responses in metastatic disease
Chemotherapy or other treatments for patients failing to achieve pCR after neoadjuvant chemotherapy
Determining which breast cancers if any will respond to antiangiogenic agents
Determining which TNBC need carboplatin
Blocking resistance to hormone treatment in adjuvant and metastatic disease
Blocking resistance to hormonal therapy after 5 years of hormonal therapy
Blocking resistance to anti-Her-2 neu therapies
Need better treatment for breast cancer recurrence within 6 months of adjuvant therapy
Central nervous system metastatic disease
Liver metastatic disease
Lung/Pleural metastatic disease
Bone metastatic disease
En cuirasse disease
Inflammatory versus non-inflammatory
What to do when next-generation sequencing identifies no target?
Radiation needed or not for 1–3 positive nodes?

outside the breast and successful evasion of the immune system. Lack of understanding of the pathobiology of breast cancer can be seen in of the earliest attempts at treatment.

Locoregional Only

William Stewart Halsted performed the first radical mastectomy at John Hopkins Hospital in 1982. His surgical technique remained as a standard method to treat women with breast cancer until the mid-1970s. The Halsted radical mastectomy involved removal of the breast, underlying minor and major pectoralis muscles and an extensive axillary lymph node dissection. This radical surgery was thought to be necessary in order to achieve the best local control and, therefore, it would prevent metastatic disease as well since breast cancer was thought to first extend to the lymph nodes and then systemically. Despite this aggressive surgical approach with radical mastectomy, local recurrences as well as distant failures were reported and survival of those treated was only marginally improved [1]. The grossly obvious disease was only part of what needed to be targeted.

Table 2 Targeted and less-targeted agents in current clinical use

Agent	Target	Limitations
Tamoxifen	Estrogen receptor blockade	Resistance/VTE-uterine cancer
Toremifene	Estrogen receptor blockade	Resistance/VTE-uterine cancer
Anastrozole	Aromatase inhibitor	Resistance/fractures
Letrozole	Aromatase inhibitor	Resistance/fractures
Exemestane	Aromatase inhibitor	Resistance/fractures
Faslodex	Estrogen receptor down regulation	Resistance
CD4/6	Cyclin inhibitor	Resistance
Bevacizumab	VEGF	Lack of benefit/arterial and venous clots
Herceptin	Erb-2b	Resistance/toxicity to myocardium
Pertuzumab	Erb-2b and 3	Resistance/toxicity
DM-1	Erb-2b and chemotherapy	Resistance/toxicity
Lapatinib	Erb-2b	Resistance
Neritinib	Erb-2b	Resistance
Everolimus	m-TOR	Resistance/toxicity
Chemotherapy	Mostly cells in S phase	Resistance/toxicity
Liposomal or albumin bound drugs	Mostly cells in S phase	Resistance/toxicity
Radiation	Tumor must be visualized	Toxicity/resistance
Surgery	Tumor must be visualized	Complications/incomplete
Embolization	Tumor must be visualized and have a dominant arterial supply	
Photodynamic	Tumor must be localized	
Thermal, Alcohol and Cryoablation	Tumor must be visualized	Toxicity and temperature sinks

Most cases of resistance are due to mutations causing the target to be unaffected by treatment. Drug efflux pumps and changes in the rate of degradation account for most of the rest

Systemic Disease as Well as Locoregional

The Fisher model of breast cancer was recognized as a major turning point in breast cancer care [2]. His seminal work provided evidence that breast cancer was a systemic as well as a local control issue. Dr. Bernard Fisher's research on the biology of tumor metastases has helped provide a rational end to the standard Halsted radical mastectomy. Fisher described breast cancer as a systemic disease at the time of diagnosis. The idea that radical surgery is needed since breast cancer cells must pass through the lymph nodes prior to metastasis was revised. Surgical trials comparing modified radical mastectomy, lumpectomy, and lumpectomy with radiation demonstrated equivalent survival [3]. For example, among these randomized trials including the European Organization of Research and Treatment of Cancer 10,801 trial [4] demonstrated no significant difference in 20-year overall survival, OS, or time to distant metastasis

Table 3 Possible methods or areas to target in breast cancer

Angiogenesis inhibition
Antibody against tumor specific antigen +/− linked to cytotoxic or radioactive agent
Gene transfer to increase immunogenicity
Gene transfer to increase p53 levels
Gene transfer to establish hormone sensitivity
Interfering RNAs to prolong p53 half-life
Deplete DAF, decay acceleration factor, +/− targeted antibody therapy that fixes complement
Hypomethylating agents used to increase immunogenicity or slow progression
Use inhibitors of multiple downstream signaling pathways
Inhibition of lysosomal degradation
Inhibitors of microRNAs
Proteolytic enzymes to strip cell surface proteins so neoantigens are exposed
Inhibitors of metalloproteases
Gap junction blockade
Liposomal delivery of PD-1 to tumor followed by antibody to PD-1
Electrical field inhibition of mitosis
Antibodies to block cancer cell binding in various organs
Electroporation/iontophoresis for accessible lesions
Target cell temperature regulation
Modulation of neutrophil and monocyte interactions with tumor cells

comparing breast conserving surgery plus radiation to modified radical mastectomy alone. The equivalent OS and time to metastasis noted between these experimental arms help point out the critical nature of breast cancer as a systemic as well as a local disease that requires effective systemic as well as local control.

Tumor Size, Lymph Node Metastases and More

While we have gained a better understanding of breast cancer biology it is important to note that not all breast cancers even large ones will spread to local-regional nodes. Among patients with lymph node metastases not all of those patients will succumb to their disease due to systemic metastases (Tables 4 and 5). Data comparing primary tumor size to the proportion of patients with metastases found that 2 cm in diameter tumors had an incidence of metastases of about 25%, tumors of 5 cm had an incidence of 60% and tumors of 10 cm had an incidence of roughly 90% [6]. Another study looking at tumor size compared to the incidence of axillary metastases found that tumors of <0.5–0.9 cm had an incidence of 20%, tumors of 2–2.9 cm had an incidence of 45% and cancers >5 cm had an incidence of 70% [7]. When comparing the number of positive nodes to survival at 5 years it was noted that those with 0 positive nodes group had a 72% survival, 1–3 nodes had a 59–63% survival, 4–10 nodes had a 41–52% survival and 11–>21 had a 22–29% survival [8]. Additional observations by Carter et al. noted breast cancers <0.5—0.9 cm with 0 positive nodes had a

Table 4 Five-year recurrence risks

Tumor size	Node−−	Node++
<2 cm	10–15	40
>2–<5 cm	20–25	55
>5 cm	25–30	70

Adapted from Ref. [5]
Treatment with endocrine therapy may cut the risk of recurrence by half in patients with strong ER positivity. This leaves a lot of patients destined to fail. Additional targets need to be identified that when modified can improve the efficacy of treatment. Chemotherapy is more efficacious in hormone negative breast cancer but mostly in younger patients. Nonselective approaches like chemotherapy have a definite limit to their effectiveness. Many pieces of the puzzle are still missing in terms of our understanding the pathobiology of breast cancer

Table 5 Eight year RFS in patients with breast cancers less than 1 cm [11]

	ER negative		ER positive		
	Surgery	Surgery + Chx	Surgery	Surgery + Endo	Surgery + Endo + Chx
RFS	81%	90%	86%	93%	95%

Certainly these data beg the question could a cyclin inhibitor, targeted agent, boost double or triple therapy in hormone positive breast cancer to as high as 98% given the impressive results with cyclin inhibitors in metastatic breast cancer? Oncotype Dx has found a role for helping clinicians identify those patients with estrogen receptor positive disease who would benefit most from adding chemotherapy to antihormonal therapy
RFS relapse free survival

5 year survival incidence of 98–99%, 1–3 nodes positive the survival was 94–95% and for 4 or more it was 54–59% [7]. Also of note for tumors 2–2.9 cm for 0 nodes it was 92%, for 1–3 nodes it was 83% and for more than 4 nodes it was 63%. It is notable that the survival of 2–2.9 cm tumors with 4 or more positive lymph nodes was better than for <0.5–0.9 cm. Last but not least it was noted tumors >5 cm without nodal involvement had a survival at 5 years of 82%, for 1–3 nodes 73% and for over 4 nodes it was 45% [7]. At ten years of follow up survival continues to decline especially in the group with 4 or more positive nodes. Specifically, Fisher et al. noted that patients with negative nodes had a ten-year survival of 65%, those with 1–3 nodes had a 38% survival and those with 4 or more nodes involved with breast cancer had a 13% survival at 10 years [9]. Tumor size and lymph node status are critical to estimating the risk of the recurrence of disease but additional characteristics of the tumor will need to be factored into consideration as indicated by the observations of Carter et al. [8]. An ongoing area of intense research today is looking at how to use clinical, molecular or a combination of both to determine which patients are at risk to die from metastatic disease [10]. The work being done on smaller breast cancers that are node negative and estrogen sensitive is particularly important since they, in general, respond better to chemotherapy and endocrine but the benefit of chemotherapy while real provides a small absolute benefit. Means of picking out the best candidates for only endocrine treatment or combined therapy would be very helpful to patients and clinicians alike.

Viewing the biology of breast cancer from the opposite end of the spectrum by evaluating the clinical behavior of very small breast cancers considering their hormonal status is also helpful to refine risk assessments. Looking back at the National Surgical Adjuvant Bowel and Breast Cancer Programs, NSABP, trials and culling out small breast cancers of 1 cm or less does highlight both the virulent nature of breast cancer and some features that allow prognostication as to the risk of relapse [11]. These trials had good long term follow up so that the risk of relapse could be better appreciated. Estrogen receptor (ER) positive tumors did respond well to tamoxifen and a benefit of chemotherapy in addition to tamoxifen was also seen. Hormone negative breast cancers had a higher risk of recurrence and a greater relative benefit from chemotherapy. The rate of relapsed disease was notably greater than that appreciated from review of 5 years surveillance epidemiology and end point report Surveillance, epidemiology and end-points, SEER, data (12; see Table 5). Thierault et al. reported data in this similar group of patients that showed results very similar to those of Fisher et al. [11, 13]. However, in the SEER data patients were on average over 10 years older than those studied by Thierault et al. [12, 13]. The data by Fisher et al. was a retrospective analysis over a greater time period than the others [11–13]. Despite these variables all 3 studies noted good survival for patients with small node negative breast cancers that are hormone receptor positive and worse survival for those with hormone receptor negative breast cancer. Considering breast cancer risk of metastases and relapse free survival needs consideration of size of the invasive portion of the tumor, lymphovascular space invasion, lymph node status, histological grade, histological type, hormone receptors, Her-2 neu over-expression, and menopausal status in order to gauge the degree of risk and evaluating the absolute benefits of various adjuvant treatments.

Endocrine Therapy in Breast Cancer

Tamoxifen: A Targeting Treatment Whose Target Was at First Unknown

The endocrine dependent nature of breast cancer growth has been recognized for some time. Beatson used his knowledge from work with sheep to appropriately and luckily guess that oophorectomy in premenopausal women could help treat breast cancer. He achieved this without the information regarding estrogen, estrogen receptors and how estrogen played a role in breast cancer therapy [14]. Oophorectomies, radiation to the ovaries, chemical castration and chemotherapy induced ovarian failure have all been used to treat metastatic breast cancer as well as to provide adjuvant treatment. The initial application of surgical castration and later tamoxifen was done without the knowledge of the cancer's hormone receptor status [15]. Once the hormone receptor status was determined then the application of a means to block estrogen production or action yielded higher response rates simply because the correct breast cancers were targeted. Patients with very high

levels of estrogen receptors by and large respond better to hormonal manipulations than those who have much lower levels. Patients with both estrogen and progesterone receptors positivity do better than patients with only estrogen receptors or just progesterone receptors. Last but not least postmenopausal hormone receptor positive patients do better than their matched premenopausal counterparts. Perhaps this is due, in part, to relative levels of estrogen or the type of breast cancer selected for by lower estrogen levels in postmenopausal patients [16–20]. Certainly, the therapeutic benefit of withdrawal of tamoxifen or aromatase inhibitors in patients with metastatic breast cancer is much more common among older postmenopausal women [21]. Again, this highlights important biological differences in hormonal sensitivity between pre- and post-menopausal related breast cancers.

Understanding the Tests for Hormone Receptors

In view of the key role of hormone sensitivity in a breast cancer it is vital to know the operating characteristics of the hormonal assay. Radioligand assays for estrogen and progesterone were replaced by immunohistochemistry [22]. In general, the modern estrogen receptor assay is at least accurate 95% of the time for the sample tested. This level of accuracy probably helps explain some of the low but notable activity of tamoxifen in hormone receptor negative breast cancer [19]. Items that impact reproducibility are tumor heterogeneity and simple laboratory error [23–27]. These pitfalls are not unique to hormone receptor assays but also include Her-2-neu testing [28] and any future assay developed to determine breast cancer prognosis or probable response to treatment. The importance of hormone receptor status cannot be overstated and, of note, the American Society of Clinical Oncology (ASCO) guidelines indicate that even a 1% positivity for hormone receptors warrants treatment with an antihormonal agent [29].

Antihormonal Treatments Are Still a Major Weapon against Breast Cancer

More treatment options have been added to the armamentarium of breast cancer therapy to further minimize local and more importantly systemic disease recurrence. Identifying estrogen and progesterone receptors, ER+/PR+, in tumor cells led to the informed introduction of endocrine therapy in hormone positive breast cancer in the early 1980s. Data from the Early Breast Cancer Trialists Clinical Group, EBCTCG, meta-analysis showed a 41% RR reduction in distant recurrence for women treated with tamoxifen for 5 years [19]. The duration of adjuvant aromatase inhibitor therapy has been extended to 10 years based indirectly from extending tamoxifen for 10 years and directly from the data from the study adding 5 years of letrozole to 5 years of tamoxifen [30–32]. The use of all adjuvant aromatase inhibitor therapy

for over 5 years has not yet demonstrated its clear and reproducible equivalence to or its superiority to extended tamoxifen or tamoxifen followed by letrozole. Some limitations to the approach using tamoxifen are in patients over 50 years of age include the risks for pulmonary emboli and the cumulative risk of endometrial cancer [19]. Limitations to prolonged aromatase inhibitor trials include arthralgias, osteopenia, osteoporosis, and fractures [33].

While the indications and duration of endocrine therapy in the adjuvant setting are, in part, well established, the use of various endocrine therapies in the neoadjuvant setting is not as clearly defined. Previous systematic reviews showed that tamoxifen can be used in primary endocrine therapy in elderly women over the age 70 who are unfit for surgery [34]. Based in part on these results, endocrine therapies have been used in the neoadjuvant setting. Neoadjuvant endocrine therapy is a viable option for postmenopausal women with hormone positive disease. Aromatase inhibitors, anastrazole, letrozole, and exemestane, were superior to tamoxifen in this setting [35]; however, with a lower pathological complete remission, pCR, rates when compared to neoadjuvant chemotherapy [36]. Data from the IMPACT trial and others showed that all three third- generation aromatase inhibitors, anastrazole, letrozole and exemestane, are acceptable neoadjuvant treatments with equal efficacy for postmenopausal women with luminal A disease [35]. The duration of neoadjuvant endocrine therapy was variable, ranging from 4 to 12 months.

Verify the Hormonal Status of the Patient as Well as the Cancer

Following the serum levels of estrogen to assess the appropriateness of treatment is crucial for patients who are chemically castrated, medically castrated, radiation castrated or even surgically castrated [37]. Proof of menopausal status for amenorrheic patients in their mid-1950s is mandatory before starting aromatase inhibitors, AIs. Patients who are postmenopausal but have non-castrate levels of estrogen might be better served by the use of tamoxifen or possibly even toremifene. If faslodex becomes approved for adjuvant treatment then that would also be a possibility especially since compliance with treatment could be verified. Compliance is a major issue not just for 5-year adjuvant treatment but perhaps more so for extended therapy [32].

Longer Durations of Treatment Are Better?

While endocrine therapy is effective, it is important to note half of all recurrences in hormone receptor positive patients occur after 5 years [19, 38]. This predicted extended adjuvant aromatase inhibitor trials would be positive and probably superior to 10 years of tamoxifen [30, 32, 39, 40]. The Oncotype DX test has been reported to predict the relative benefit of endocrine therapy with or without chemotherapy in patients with relatively small node negative hormone receptor positive

breast cancers [41]. Perhaps additional molecular prognostication tools will predict the timing of recurrence and the benefit of which antihormonal treatments pre and post 5 years of therapy.

Chemotherapy with Antihormonal Therapy Better than Antihormonal Therapy?

The search for models to predict who needs chemotherapy in addition to endocrine therapy as well as those who will be resistant to one or the other or both has been a topic of a great deal of research and remains a current goal for ongoing studies. One of the earliest prognostication models was the Nottingham Index [42]. It used tumor size, number of positive lymph nodes and histological grade to predict survival. Specifically the Nottingham Prognostic Index = (0.2 × tumor size cm) + grade + lymph node stage. Lymph node stage was 0 if no nodes were involved, 1 for 1 to 3 nodes and 2 for 4 or more nodes. Fifteen-year survival was 90% for a score of < or equals 3, 80% for 3.01–3.4, and 50% for 3.41–4.4. 30% for 4.41–5.3 and for over 5.4 it was 8%. It did not use hormone receptor status or Her-2-neu over-expression to predict survival. The clinical model of Ravdin called Adjuvant! uses well characterized clinical criteria for predicting the benefit of endocrine therapy with or without chemotherapy [43]. Its clear advantage over Oncotype DX is its ability to factor in the impact of the patients' general state of health on benefits from treatment as well as being able to be updated with additional clinical data. Intratumoral heterogeneity in terms of estrogen receptors alone has demonstrated considerable amount of variation [23–27]. No data have been reported yet for Oncotype Dx that accounts for the proportion of the whole tumor that was assayed and its effect on the validity and reproducibility of the test. A separate clinical assay uses ER, PR, histological grade and Ki-67. It correlates well with the Oncotype Dx results [44]. Yet another clinical model, the IHC4, also correlates well with the Oncotype Dx data albeit with significantly less cost and a faster turnaround time [45]. None of the currently available models report the tests' range of variation in any patient population. It of course would be helpful to have a matrix of clinical and molecular aspects of the patient and their tumor available to predict the best route to take for treatment of the patient (i.e. sensitivity to what agents, need for extended treatment, primary resistance to current therapies).

The MINDACT peer reviewed report was published in 2016 [46]. This trial tried to determine if clinical versus molecular markers faired better in determining the need for chemotherapy. Patients deemed to be at high risk by Adjuvant! and by Mammaprint receive chemotherapy and endocrine therapy. Patients deemed to be at low risk by both received endocrine therapy alone. Those at high risk by clinical parameters but low risk by Mammaprint who did not receive chemotherapy had only a 5% decrease in overall survival compared to those deemed to be low risk by both tests. Five year overall survival for low risk was 97.4%, high risk was 90.7% and discordant result patients had a 94–95% survival. It is encouraging to see the two tests reaffirm each other when concordance is noted. The trial was not powered

to determine if chemotherapy given to the clinical high risk but low risk Mammaprint signature was of benefit. A slight improvement was noted for those who received chemotherapy but it was not statistically significant. Of note the chemotherapy regimens used were not state of the art by current standards. Certainly high-risk patients by clinical parameters but low risk by Mammaprint whose general health is not good would more likely benefit from just endocrine therapy. Room for improvement in the patient selection and chemotherapy still exist since a low Mammaprint signature does not equate to a low risk when both tests are not congruent.

Additional Targets Are Helpful

The development of aromatase inhibitors and ER down regulators such as faslodex trailed behind the understanding of estrogen production in pre and postmenopausal patients as well as the actionable target's identity. Co-regulatory molecules for the estrogen receptor have been identified as well as pathways intersecting or downstream from the estrogen receptor. Multiple recent trials using exemestane and everolimus and letrozole or faslodex with palbociclib have produced significant improvements in the treatment of patients with metastatic hormone positive breast cancer [47–50]. These impressive results were achieved by a detailed understanding of signaling pathways beyond estrogen. Additional studies defining intersecting signals that can enhance antihormonal manipulations will likely continue to improve progression free survival, PFS, and OS [51]. These studies are currently the best examples of the real clinical power of targeted therapies. Investigations into the presence and role of androgen receptors in breast cancers may also produce new approaches to adjuvant and metastatic breast cancer therapy [52].

(Neo)Adjuvant Chemotherapy for Early Stage Breast Cancer

Chemotherapy for early breast cancer can be delivered with equal efficacy before or after surgical excision of the breast cancer. Frequently the same or very similar chemotherapy drug regimens can also be used for metastatic disease. Certainly the utility of a drug regimen in the neoadjuvant, adjuvant and metastatic scenarios is reassuring as to its benefit. However, the times when adjuvant and metastatic regimens fail to yield relatively equivalent results in scenarios are probably more important for defining the need for further research comparing these disparate situations. The advantage of neoadjuvant chemotherapy is that in addition to improving the odds of breast conservation it provides an in vivo drug sensitivity assay. Clearly, drug sensitive breast cancers are associated with better survival and drug regimens that have the highest rate of pCR appear to be the most promising. The right drugs delivered in the correct fashion to take advantage of growing subpopulations of cancer cells have projected the field of breast cancer oncology to its place today. The

future will most likely be much brighter as the causes of resistance to neoadjuvant chemotherapy are elucidated. Last but not least the cancer cells that metastasize and cause the death of the patient, whether the cancer is treated adjuvantly or neoadjuvantly, will probably hold the most important details since they have escaped chemotherapy and primary therapy. This section while complicated will try to highlight the progress that brought us to this point and some of the apparent questions remaining.

Histology Still Matters

Hormone therapy has proven efficacy only if the tumor expresses appropriate receptors. Adjuvant chemotherapy has proven to decrease the risk of relapse within the first 5 years of the disease regardless of tumor hormonal markers [53, 54]. Typically hormone receptor negative cancers respond better to chemotherapy. Not all histological subtypes of cancer have as high a risk of lymph node metastases or risk of metastatic disease. Specifically mucinous, colloid, typical medullary, adenoid cystic, and tubular subtypes do not behave aggressively [55]. It is very important to note the histology of the tumor in the lymph node in these less virulent breast cancer types. If the histological appearance is that of invasive ductal carcinoma then consider the risk of metastasis according to their more aggressive disease in the lymph node. Recurrence scores have been developed to identify patients with early invasive node-negative estrogen receptor positive breast cancer that might benefit from adding cytotoxic chemotherapy. Patients with invasive ductal carcinoma that were node negative and estrogen receptor positive with a high recurrence score greater or equal to 31 by Oncotype Dx were advised to receive chemotherapy and hormonal therapy [41, 56].

CMF to AC to TC

Modern chemotherapy regimens have evolved via a somewhat stepwise incremental path from single agents to multiagent regimens. Cytoxan, methotrexate and 5-fluorouracil (CMF) were one of the earliest multidrug regimens for breast cancer [57–59]. The next step beyond CMF looked at the benefit of anthracyclines in adjuvant treatment. Of note in the 7 anthracycline-based regimens reviewed that compared with CMF cited an absolute 0–14% better relapse-free survival, RFS, and an absolute 0–10% better overall survival, OS, were noted [60–66]. Adriamycin and cytoxan (AC) when compared to CMF in general produce a relatively reduced the risk of recurrence and death by 12% and 15%, respectively. The ease and shorter treatment course of AC to CMF have also helped results in the less frequent use of CMF type regimens in node negative and in patients with up to 3 positive nodes. The AC combination was also later compared to TC, docetaxel and cyclophosphamide, in randomized trials. Results of these trials indicated nonequivalence in terms of reducing recurrence and death favoring TC [67].

Taxanes

The addition of a sequential taxane (paclitaxel) after four cycles of AC resulted in an even greater disease-free survival, DFS, and OS in CALBG 9344 [68, 69]. Of the 8 studies cited evaluating the benefit of adding taxanes to an anthracycline-based regimen showed an absolute 0–6% improvement in RFS and showed an absolute 0–7% improvement in OS [68–75]. The ECOG1199 compared docetaxel to paclitaxel given weekly for 12 weeks after 4 cycles of AC, with results supporting the use of the weekly paclitaxel regimen [76]. Dose dense-chemotherapy, every 2 weeks, was compared to standard chemotherapy [77]. Better overall survival was reported with dose-dense chemotherapy even in patients with 4 or more positive lymph nodes. Prior studies looking at dose intense therapy with higher doses of cyclophosphamide and doxorubicin did not improve survival over standard dosages but did increase acute and delayed toxicity. Dose-dense AC or epirubicin and Cytoxan (EC) followed by weekly paclitaxel is the preferred regimen for patients with 4 or more positive lymph nodes.

TNBC

Triple negative breast cancer represents a unique entity lacking any currently identified potential therapeutic target. It differs from other subtypes of breast cancer prognostically. However, the same chemotherapy agents are still used with good upfront responses in neoadjuvant therapy albeit with less success in terms of overall survival [78–80]. T1b lesions or larger merit adjuvant treatment since they have a relatively high recurrence rate [81, 82]. Unlike hormone receptor positive tumors the risk of relapse is higher in the first 3 years. The complimentary results of 8541 maximizing the anthracycline dose, 9344 adding a taxane and 9741 maximizing the taxane have resulted in a 23% and 17% relative improvement in OS and DFS, respectively, in this very challenging clinical subset of breast cancers [83].

Pathological complete remission after an anthracycline and taxane-based regimens remains the best surrogate marker for DFS, and OS [84]. Poorer outcomes were found in those patients with residual disease after neoadjuvant chemotherapy. Research into predicting a poor response to chemotherapy and the best adjuvant approach after a less than complete response to neoadjuvant chemotherapy is ongoing. AC followed by docetaxel every 21 days × 4 or paclitaxel every week × 12 are commonly used regimens.

Carboplatin has been used for triple negative breast cancers resulting in a higher rate of pCR in the breast and axilla [85], however, the effect on OS and DFS remains undefined.

The history of how we arrived at our current therapeutic regimens for breast cancer has been outlined in detail in the previous sections. Despite profound improvements in treatment of potentially curable as well as management of more

Table 6 Maximal Proportional Reductions in Odds of Recurrence for ages <50 and ages >50 in Hormone Receptor Negative Breast Cancer [157]

	Relative reduction[a] (%)	At risk[b]	Absolute reduction/100	Patients who die/100[c]
<2−[d]				
−	55	19	11	8
+	48	19	9	10
<2+				
−	55	63	35	28
+	48	63	32	28
>2−				
−	55	31	18	13
+	48	31	14	17
>2+				
−	55	70	39	31
+	48	70	34	36
>5−				
−	55	44	24	20
+	48	44	22	22
>5+				
−	55	82	45	37
+	48	85	40	45

[a]Relative reductions in risk are according to the best available chemotherapy treatments
[b]The at risk is both a percentage as well as the number of patients at risk per 100 similar patients
[c]The absolute reduction and patients who have recurrences are per 100 similar patients
[d]The tumor size is in cm. The less than 2 cm group is 1–2 cm but not the <1 cm tumors. All patients are ER/PR negative. − is less than age 50 and + is over age 50 as indicated under the tumor size. − and + at the right of the tumor size indicates node negative versus 4–9 positive nodes. For tumors 2–5 cm these are averaged estimates. For 4–9 positive these are also pooled estimates
Note Bene: The results for hormone receptor positive patients are very similar. Trastuzumab transformed the most aggressive breast cancers into a more highly curable subset directly by inhibiting the effect of Her-2 neu over-expression. The lack of an actionable target in TNBC highlights the limitations of chemotherapy alone

advanced disease there is great room for improvement. TNBC stands out as a group in most need for identification of an actionable target as well as an agent to inhibit that target. Tables 6 and 7 outline the benefit of adjuvant chemotherapy or adjuvant chemohormonal therapy in breast cancer patients. In both Tables you see room for a great deal of improvement. Nodal involvement is really key to determining the risk of relapse. The impact of the use of more effective chemotherapy is displayed in Table 6. The number of patients per 100 treated is detailed in Table 7. Despite the state of the art chemotherapy many patients relapse and the overwhelming majority of them will die of their disease. It is critical to also consider that these data are based primarily on patients who participated in breast screening programs and were quite healthy apart from their breast cancer. A good many patients seen in practice do not resemble this cohort of study patients and will not do as well as they have done. To say the least there is room for targeted agents with breast cancer control

Table 7 Modern systemic therapy for breast cancer: A relative victory or absolute defeat?

10 year disease free survival sorted by age, tumor size, lymph node status and treatment—all ER negative[a] with age < 50[b,c]												
Nodes	<1 cm			1–2 cm			2–5 cm			>5 cm		
	X	AC	ACP	X	AC	ACP	X	AC	ACP	X	AC	ACP
0[d]	90	93	95	81	87	90	63–75	74–83	80–87	56	69	75
1–3	60	72	78	56	69	76	42–50	57–64	66–72	37	53	62
4–9[e]	41	57	64	37	43	62	26–34	42–50	52–60	18	34	45
>10[f]	22	38	49	19	35	46	14–17	29–33	40–44	13	28	39

X surgery only; *AC* adriamycin + cytoxan; *ACP* AC + paclitaxel; *TC* taxotere + cytoxan; *TAC* TC + adriamycin; *ACD* AC + docetaxel

Dose dense is q_2 weeks AC followed by q_2 weeks docetaxel or weekly paclitaxel

[a]For ER positive patients results with tamoxifen with or without chemo are similar with tamoxifen yielding a 50% relative reduction in recurrence and adding chemo improves this by another relative10–15%

[b]For patients over 50 the success rates are even lower

[c]Adapted from Ref. [43] and Loprinzi et al. [157]

[d]In general for small node negative tumors chemotherapy does add benefit albeit small in absolute numbers. This is also true in ER positive tumors treated with tamoxifen and chemotherapy. Death due to treatment for breast cancer managed in clinical trials is about 2%. Many of patients seen off clinical trials are not physically or emotionally well enough for the clinical trial and will not derive the same amount of benefit. OncotypeDX and the Adjuvant! Program help pick who is most likely to benefit from a specific therapy

[e]There is a marked break between 1–3 nodes involved to 4 or more involved. For 0–3 nodes involved ACP > TC > AC > CMF in terms of benefit. Over 3 nodes really only TAC, ACP, ACD or DOSE DENSE ACP/D should be used. The relative benefit of chemotherapy is quite high but the absolute benefit is smaller. The worse the situation the greater absolute benefit is observed. For patients with 4 or more nodes the chance for victory is about 50% or less even with best chemotherapy. There is ample room for improvement with new treatments like targeted agents. Look at Herceptin results in those with Her-2-neu over expressing breast cancers for an idea of how a targeted agent can dramatically improve survival

[f]Even in very large tumors with over 10 nodes positive some patients live 10 years without recurrence without receiving systemic therapy

rates much greater than seen with chemotherapy alone and that can be tolerated by even less healthy patients.

Her-2 Neu

Contrary to the lack of a defined target in TNBC, a major advance in therapy of metastatic and adjuvant treatment of breast cancer was the recognition of a cell surface protein Her-2 neu. Cancers that over-express this protein are more likely to spread to regional lymph nodes and more likely to metastasize. However, use of antibodies specific to the cell surface portion of this transmembrane protein turned the tide of battle making this aggressive subtype of breast become highly amenable to treatment. Addition of a second anti-Her-2 neu antibody, pertuzumab also improves response to treatment in that there is now a lower rate of primary

resistance to therapy. Unfortunately, the development of resistance to this therapy is a very adverse event. Development of agents to overcome this point of resistance are very critical and will be outlined in detail elsewhere. The current state of the clinical art is outlined in this section.

The most striking independent prognostic and therapeutic indicator other than ER/PR status is over expression of the transmembrane protein Her-2-neu found in 15–20% of breast cancers [86]. Both immunohistochemistry and fluorescent in situ hybridization (FISH), dual probe methods, are used to determine the Her-2 status. There were a 38% and 34% reduced relative risk of recurrence and death, respectively, when trastuzumab was added to chemotherapy. Multiple trials looked at differences in DFS and OS whether trastuzumab was given sequentially after paclitaxel or concurrently with the initiation of paclitaxel. No major difference was seen with 48% and 39% relative improvement in DFS and OS, respectively [87, 88]. However, the use of concurrent treatment has been supported by the NCCTG N9831 study [87]. No difference in progression free survival, PFS, or OS was observed when comparing 1 versus 2 years of trastuzumab was demonstrated in the HERA trial [88]. Shorter duration of therapy for 6 months was proven inferior to 1-year duration [89]. Considering the 5% risk of cardiotoxicity, other non-anthracyclines regimens were studied (BCIRG 006) including the TCH regimen consisting of docetaxel, carboplatin, and trastuzumab. To date the anthracycline-containing regimens remain the preferred regimens except in select patients such as those with significant cardiac risk factors and a lower risk of disease recurrence where the TCH regimen can be used [85]. Trastuzumab is the only anti-Her-2 therapy approved to date in the adjuvant setting with one year of therapy being the standard. Addition of other Her-2 blocking agents to trastuzumab such as lapatinib did not show any significant additional benefit [90].

Neoadjuvant chemotherapy is as efficacious as adjuvant chemotherapy. It also has the advantage of converting some inoperable breast cancers other than inflammatory into operable breast cancers. When a pathological complete remission is obtained it also allows for the greater reassurance of freedom from relapse and probable cure [91, 92]. Most adjuvant chemotherapy regimens can also be used in the neoadjuvant setting. Taxane-based regimens are preferred [84, 91, 92]. The introduction of dual antibody blockade of Her-2-neu, trastuzumab with pertuzumab, has further improved OS in patients in the metastatic setting and for those receiving neoadjuvant treatment [93]. The use of the chemically coupled trastuzumab to a cytotoxic agent has also improved OS in the metastatic setting [94, 95]. Dual Her-2-neu blockade using trastuzumab and pertuzumab has not caused increased cardiotoxicity. Perhaps the use of agents that block down stream signaling from Her-2-neu will increase survival without increasing toxicity since this combination of targeted agents has not resulted in a 100% cure rate. Various regimens containing Her-2 directed therapy were also studied in the neoadjuvant setting. These have included trastuzumab and lapatinib combined with anthracyclines or pertuzumab-containing regimens, and higher pCR rates were observed [93]. Trastuzumab containing regimens should be given for all Her-2 positive breast cancers treated preoperatively and then continued after surgery to a total of 1 year [88, 89]. In addition, data from the NeoSphere trial showed that addition of pertuzumab to trastuzumab and

docetaxel and in other various regimens are beneficial for those with tumors ≥T2, ≥N2 that are Her-2 positive [92]. DFS and progression free survival, PFS at a 5-years follow up supported the primary contention that a complete pathological response accurately predicts these key endpoints.

No Clear Cut Champion between TAC and Dose Dense Chemotherapy

The other most recent notable advance in adjuvant or neoadjuvant therapy has been the demonstration that dose dense, not dose intense, chemotherapy is superior to the standard schedule. AC every 2 weeks for 4 cycles followed by paclitaxel weekly for 12 weeks or followed by 4 cycles of every 2-week paclitaxel at 175 mg/m^2 or docetxel of 100 mg/m^2 every 2 weeks for 4 cycles provides superior outcomes to every 3-week regimens. Certainly, the non-anthracycline portions of the regimen can be given with trastuzumab [84, 87–89] as well as pertuzumab [92].

While good data for dose dense chemotherapy has been provided other regimens such as TAC (docetaxel, doxorubicin and cyclophosphamide) are routinely used [96]. No prospective trial has pitted TAC versus dose dense AC followed by T with or without trastuzumab. The traztuzumab data not only provide a potent regimen for Her-2-neu over expressing breast cancers but it indicates duration of therapy as well as its specific targeting are key. Passive immunotherapy requires much more time to exert its effects than chemotherapy. This may reflect a basic difference in the biology of Her-2-neu over expressing breast cancers as well as the mechanism of action of trastuzumab. Data looking using doxorubicin or epirubicin indicate for all intents and purposes the two anthracyclines are interchangeable [97]. Epirubicin appears to have less cardiotoxcity but as much or more mucosal toxicity when compared with doxorubicin.

Stage IIB and III Breast Cancer

Preoperative chemotherapy followed by surgery and radiation is the best approach for patients with large tumors in which resection will render a poor cosmetic result, have lymphedema on presentation, multiple or extensive nodal involvement on presentation or if they have chest wall involvement. Neoadjuvant chemotherapy allows more opportunities for breast conservation, and can minimize the extent of axillary node dissection [84]. The same chemotherapy regimens used in the adjuvant setting can be used preoperatively [97–102]. Patients with stage IIB (T3 N0), stage III, extracapsular lymph node extension of cancer or those with 4 or more positive nodes benefit from post-mastectomy radiation therapy as well as those who had breast conservation. The role of postoperative radiation is not as well established for patients with less than 4 positive lymph nodes [103]. Lymph node dissection is necessary if any of the sentinel lymph nodes, SLN, are positive after neoadjuvant

chemotherapy. Whether radiation therapy can replace the need for axillary node dissection for those with positive SLN or those rendered negative after neoadjuvant chemotherapy remains an area of research [104, 105]. It is currently recommended to complete axillary node dissection after neoadjuvant chemotherapy if a positive lymph node was present at the time of diagnosis or a SLN was positive after completion of chemotherapy [106].

Inflammatory Breast Cancer

Inflammatory breast cancer represents a rare but highly aggressive disease [107–110]. Therefore, it should be treated with a trimodality approach with neoadjuvant chemotherapy, surgery and radiation. Neoadjuvant chemotherapy similar to those regimens used with non- inflammatory cancer is reasonable but they must be used emergently to render the cancer operable and to avoid systemic relapse. Radiation may be used pre-surgery if there is an inadequate response to chemotherapy [111]. Endocrine and Her-2 targeted therapy should be given to those in a similar fashion to other breast cancer subtypes. Breast conserving therapy and SLN are contraindicated in order to maximize local control and to avoid systemic disease and loco-regional recurrence. This rare but highly deadly variant of breast cancer does not yet have unique actionable targets with specific inhibitors identified.

Management of Locally Recurrent Disease

Loco-regional and distant recurrences remain the main treatment failures despite improved combined treatment modalities and advances in various endocrine and targeted therapies. Loco-regional recurrence is defined as cancer recurrence in the ipsilateral breast following breast-conserving therapy, chest wall recurrence following mastectomy or regional node recurrence. The latter is associated with shorter DFS and OS as compared to the others with higher risks of development of metastatic disease after loco-regional recurrence [111]. The first 5 years represent the highest risk of recurrence of the original tumor; however, relapses at 10 years and beyond are possible. Repeat biopsy at the time of first recurrence is mandatory as tumor characteristics can change. Ipsilateral breast tumor recurrence is treated with total mastectomy with axillary node dissection at level I and II if not done previously. Whereas chest wall recurrence following mastectomy is treated with radiation therapy after surgical excision with negative margins. Data from the CALOR trial [112] advocate for the use of adjuvant chemotherapy after completion of loco regional treatment for recurrence, since 5- year DFS was improved with chemotherapy especially for triple negative breast cancer, TNBC. Adjuvant endocrine therapy failed to improve OS. Perhaps the microenvironment around the recurrent hormone sensitive breast cancer has also changed enough to thwart an OS benefit from antihormonal therapy.

Metastatic Disease

In General

Metastatic breast cancer treatment is dictated by disease manifestations, which reflect the extent and burden of the disease. It is imperative to know the tumor characteristics, hormone status, Her-2, and Ki-67%, since a wide range of therapeutics options exist, therefore, biopsy proof of disease and biomarkers are required. Furthermore, defining the extent of tumor by proper staging will allow a personalized treatment approach in most cases. Hormone therapy, chemotherapy and targeted therapy such as anti-Her-2 neu agents, should all be used in a manner to slow progression of the disease and prolong survival without compromising the patient's quality of life [113].

Endocrine Manipulations

In the absence of any visceral crisis endocrine therapy should be used as first line treatment in hormone positive cancers. Switching to chemotherapy after failure of three sequential endocrine therapies is appropriate. Defining menopausal status is crucial before the initiation of endocrine therapy. Young premenopausal women should be treated aggressively with a combined modality in order to achieve better ovarian suppression. LHRH, luteinizing hormone releasing hormone, agonists are proven to be as effective as surgical ovarian ablation [114]. The addition of tamoxifen to ovarian ablation results in a higher reduction of disease progression and risk of death by a relative 30 and 22%, respectively [115]. Third generation AIs, letrozole, anastrazole and exemestane have been used with equal success in postmenopausal women [116–118]. A better toxicity profile was noted with the nonsteroidal AIs, letrozole and anastrozole. Emerging data indicate that fulvestrant is superior to anastrazole in the first line setting provided it is used at the correct dose [119, 120]. Switching between aromatase inhibitors (AIs) rarely can be an acceptable approach at relapse on an AI since cross-resistance to those endocrine agents is the rule not the exception [50].

The mTOR, mammalian inhibitor of rapamycin, pathway plays an essential role in the resistance to endocrine therapy [121]. Based on this discovery, everolimus, an mTOR inhibitor, was approved to be used in combination with endocrine therapy, examestane, in second line treatment of hormone receptor positive breast cancer in postmenopausal women [48, 122]. Stomatitis and pneumonitis were the two most aggravating and dangerous adverse reactions, respectively. In 2015, pablociclib, a cyclin dependent kinase 4 and 6 inhibitor, which blocks progression of the cell cycle from G1 to S phase, was approved in combination with letrozole [123] or with faslodex [49]. Recently, the federal drug administration approved the use of palbociclib in combination with letrozole as first line treatment in metastatic disease [124]. Ribociclib is another cyclin-dependent kinase 4 and 6 inhibitor recently approved in

first line setting in metastatic breast cancers with positive hormone receptors and negative Her-2 [125].

One example of where the fine-tuning therapy based on breast cancer subtype based on molecular profiles, basal, luminal A, Luminal B, and Her-2-neu, might improve therapy in luminal B hormone sensitive breast cancer where the prognosis is poorer yet they are treated like luminal A hormone sensitive breast cancers [110]. The duration of therapy is not defined either and is solely guided by disease response to treatment and cumulative toxicity. More effort and research are needed toward a personalized treatment based on certain tumor and patient characteristics that might predict a better response to certain chemotherapy or hormonal agents as opposed to others.

Chemotherapy

As mentioned earlier, chemotherapy should be initiated in the setting of visceral crisis, triple negative breast cancers or progression after three or more sequential endocrine therapies. No standardized treatment is available for all patients. However, single agents were proven non-inferior to combination chemotherapy but with fewer side effects. In a visceral crisis combination chemotherapy will be needed since a response is needed as soon as possible [113]. As in the adjuvant setting, anthracyclines and taxanes remain the most studied and effective chemotherapy in the metastatic setting. Doxorubicin, pegylated doxorubicin, paclitaxel and nab-paclitaxel are very commonly used drugs. Other drugs such as eribulin and ixabepilone, are used especially upon the emergence of taxane resistance [126]. Several other agents are used as well such as capecitabine, gemcitabine and carboplatin especially in the setting of breast cancer-1 or 2 (BRCA)-mutated or TNBC patients [127]. None of these treatment options is disease specific and most of the treatment decisions are made according to previous treatment responses, current disease status and the rate of progression.

Her-2 Neu

The use of Her-2 directed therapy, trastuzumab and pertuzumab or trastuzumab, emantasine, together and in combination with chemotherapy, docetaxel or paclitaxel, has resulted in significantly higher PFS and better OS. They are, therefore, considered the preferred first line treatments for metastatic Her-2 positive breast cancer [93–95]. ER/PR+ breast cancers with positive results for Her-2 neu expression that received early administration of Her-2 targeted therapy had better OS [128]. Other agents are available to treat Her-2 positive breast cancer including lapatinib, a dual epidermal growth factor-1, EGFR -1 and Her-2 TKI, tyrosine kinase inhibitor, and T-DM1, an antibody–drug conjugate of trastuzumab and

chemotherapy DM1 that causes microtubule inhibition [94, 129]. Lapatinib can be used in combination with capecitabine after progression on trastuzumab [130]. This combination was compared to T-DM1 and the differences in PFS and OS were both highly in favor of T-DM1 as well as less toxicity with T-DM1. Adding pertuzumab to T-DM1 showed no benefit compared to T-DM1 alone as first line therapy [95]. Continuation of trastuzumab beyond disease progression is beneficial in terms of the response of the next agent added [131]. The combining of Her-2-neu and endocrine therapy in hormone positive Her-2 disease is recommended resulting in a significant improvement in PFS compared to endocrine therapy alone [128]. Targeting the estrogen receptor and targeting Her-2 over expression as well as the use of downstream signaling inhibitors with anti-hormonal agents certainly offer clear direction that more targeted agents will yield positive clinical results.

Treatment Options on the Horizon Depend on Finding New Actionable Targets

Maximize Benefit to Toxicity

Considerable work has been done to improve the toxicity to benefit ratio of several chemotherapeutic agents used for metastatic disease. Liposomal and albumin nanoparticle delivery of doxorubicin and paclitaxel, respectively, have improved the toxicity to benefit ratio [132–134]. This benefit was achieved by changing the drugs pharmacokinetics in that more drug was delivered to cancer cells by using a liposomal or albumin nanoparticle delivery system. Capecitabine was developed to take advantage of differences in enzyme metabolism between normal and malignant tissues. This drug takes an advantage from pharmacodynamics but it might be improved by increasing the amount of drug reaching the cancer [135, 136]. Most likely better-targeted agents will also require help with their pharmacokinetics. Combinations of better delivery systems with better-targeted agents will most likely make the greatest advances.

New Targets Based on Cell Behavior

Experimental work with breast cancer models in the laboratory setting has demonstrated that metastases will also metastasize [137–139]. In fact there has been demonstrated to be a constant shedding of malignant cells and evidence for tumor cells migrating from one metastatic lesion to the other and back to the primary lesion. These observations pose multiple clinical questions such as: (1) Would constant round the clock chemotherapy be better than intermittent pulses for tumor kill and stopping progression? (2) Could well-timed aphaeresis be used to stop or slow this constant flow of malignant cells? (3) Would proteolytic agents even such as

asparaginase interrupt this flow and improve clinical outcomes by breaking up the tumor cell clusters? (4) Does it only take one mutated cell now with drug resistance to go around providing this clone to the rest of the metastatic lesions? (5) Should non-aggressive and non-drug resistant clones with a suicide gene be injected to stop tumor progression (i.e. increase the susceptible cells at the expense of the resistant cells)?

Get All the Metastases if Possible?

Clinical data has accumulated that indicates patients with breast cancer who have a limited number of bone only metastases do well with surgery or radiation therapy to sterilize their metastases when used with either systemic chemotherapy or endocrine therapy [140]. Perhaps interdicting the process of additional metastases from the various metastatic lesions will provide prolonged PFS and OS [141]. Certainly clinical work with resection of a limited number of liver metastases in colon cancer patients whose other disease has been controlled supports the idea eradicating metastatic disease [142]. A similar clinical scenario exists in patients with non-small cell lung cancer when the patient has a resectable brain lesion and their thoracic disease is controlled by surgery or chemoradiotherapy [143]. Prostate cancer has similar examples [144, 145]. The current trial exploring the benefits of definitive treatment of 4 or less breast cancer metastatic lesions; hopefully, will provide good clinical information to guide the use of aggressive treatment of all known metastatic lesions in breast cancer patients so as to improve their quality as well as quantity of life [146]. It may be found that the pathways operative in metastases from metastases will be markedly different from the primary tumor. Targeted therapy for these patients will be critical since options now are limited and the patients' ability to tolerate side effects is markedly reduced by prior cytotoxic agents or radiation therapy.

Detecting Treatment Failure Early Might Be Helpful?

Considering the failure rates and recurrence risks despite the best available multimodality treatment approaches, researchers continue to explore options for more effective therapy. Detecting circulating tumor cells has proved to be an adverse prognostic factor in early and metastatic disease [147, 148]. Perhaps these circulating cells will provide the best samples to test for targeted therapies or to help elucidate more aspects of epithelial-mesenchymal transition. The presence of circulating tumors cells has not been prognostically as clearly linked to estrogen status as would be predicted from other means of predicting the risk of metastases or death. Perhaps elucidating the reasons for this lack of correlation will provide data to improve the assay as well as identify a target against the cancer.

Other Targets

Targeting vascular endothelial growth factor (VEGF) with bevacizumab failed to demonstrate an improved OS in metastatic breast cancer; therefore, it was abandoned by the FDA [149]. Hopefully, other angiogenesis inhibitors will prove beneficial. Poly adenosine monophosphate ribose polymerase (PARP) inhibitors agents were also studied in the treatment of BRCA1/2 breast cancer with proven clinical efficacy; however, not approved yet pending more studies [150]. The BRACAness, impaired DNA repair, of a particular breast cancer may prove to be an exploitable target outside of the classic BRCA 1/2 mutated breast cancer. Work on tissue infiltrating lymphocytes hopefully will provide key information that will facilitate active and passive immunotherapy [151]. Immunotherapy treatment, blocking, programmed death −1 receptor or programmed death ligand-1, PD-1 or PD-L-1, is being tested in multiple trials using different agents and awaiting early results [151]. Several other ongoing trials are exploring different pathways such as a targeting the AKT/PI3K, protein kinase b/phosphatidylinositol 3-kinase pathway [152], androgen receptor antagonists such as enzalutamide [153], and Trop-2/EGP-1, a pan-epithelial cancer antibody [154]. These agents as well as inducible nitric oxide reductase and fibroblast growth factor inhibitors will be reviewed in detail elsewhere. Next generation sequencing has been able to identify targets not obvious by other assays; however, sometimes no target can be identified [155]. Patients without a specific target can now receive either ipilumumab or nivolumab [156]. This technology may have to be used to explore tumor and microenvironment interactions that can be interrupted. Sifting for new targets may prove fruitful by using comparative genomic sequencing between normal and malignant tissue as well as comparing progressive disease to the initial cancer. New targets will mean new targeted agents can follow. Most likely these newly identified targets and inhibitors will likely provide new ways of overcoming resistance like what has been reported for antiestrogen and palbociclib combinations. Unfortunately, the need for these agents is urgent and enormous (Table 1).

Conclusion: The End of the Beginning Requires Learning from the Past

Current and future research into targets for therapy of breast cancer as well as mechanisms of resistance owe their foundations to the vast amounts of basic and clinical research done in the preceding 100 plus years. The understanding that breast cancer is both a locoregional and systemic illness is a key point that forms the basis for the type of procedures used for local control and the need for adjuvant therapy. Cytotoxic chemotherapy has a relative preference for cells actively dividing. It has an established role in the adjuvant and palliative treatment of breast cancer. Efforts to improve the pharmacokinetics of these drugs has achieved some success in

decreasing drug toxicity as well as efficacy in some cases. The use of chemotherapy to attack replicating breast cancer cells based on growth kinetics has been the driving force behind the successful development of dose dense rather than dose intense adjuvant chemotherapy. These clever developments can be thwarted by multiple pathways in the breast cancer cells. Hence the pursuit of more specific targets and therapies.

Anti-hormonal therapies were the first to target a specific pathway. The various ant-hormonal agents such as tamoxifen or toremifine may block estrogen binding to its receptor or like LHRH agonists reduce estrogen production by the ovaries or like aromatase inhibitors block its production in the adrenal glands and adipose tissue, and in the case of fulvestrant down regulate the estrogen receptor. Mutations in the estrogen receptor, coregulatory molecules and parallel pathways can cause resistance to endocrine therapies. Blocking these mechanisms of resistance has been achieved with everolimus and palbociclib. These results are clear proof of principle that blocking multiple signaling pathways can overcome resistance. Resistance to these drug combinations will be identified and most likely will involve similar mechanisms of resistance like that seen with the various well established clinical anti-hormonal agents. Undoubtedly further work will identify microenvironmental mechanisms that will be aiding and abetting resistance to anti-hormonal therapies.

The development of trastuzumab to target cancer cells overexpressing Her-2 neu provides another vivid example of the clinical success that can be achieved by blocking a specific molecule and pathway. The addition of pertuzumab to trastuzumab overcomes primary resistance to this targeted therapy. Neither agent can achieve therapeutic success against truncated mutations of Her-2 neu. Certainly inhibitors of downstream pathways will be identified as potent agents to overcome resistance to this very potent agents. Small molecule inhibitors of the intracellular signaling portion of this transmembrane receptor such as neritanib have been developed and are clinically useful. Blocking additional pathways used by Her-2 neu transmembrane signaling will provide another means to overcome resistance to this class of agents.

TNBC stands out as an ominous clinical entity in that while it initially responds well to chemotherapy the long term results are suboptimal. The lack of a specific target limits our arsenal to cytotoxic chemotherapy. Identification of unique targetable features of this class of breast cancer are urgently needed. TNBC stands out as a stark and grim reminder of what happened before specific targets and their inhibitors were developed.

Data pointing to actionable targets in breast cancer such as fibroblast growth factor receptors, polyadenosine ribose polymerase inhibitors, and immunological approaches will aide overcoming resistance to targeted therapies. It is critical to view these approaches based on growth kinetics of small groups of breast cancer cells that make up micrometastases as well as the migration of malignant cells. Failure to do so will be met with defeat since lack of understanding of the pathobiology of breast cancer has proven repeatedly to be the rate limiting process in progress against this disease. Pharmacokinetics should be modified to help limit toxicity and improve efficacy. Combinations of agents with multiple independent mechanisms

of blocking resistance to targeted agents will ultimately be the standard of care in oncology clinics in the future. Circulating tumor cells or circulating break down products of breast cancer cells will possibly be useful to verify sensitivity to targeted agents and when use of agents to block resistance to these drugs should be initiated before clinical relapse or progression occurs. A greater cure rate of primary episodes of Stages I-III breast cancer and very prolonged good quality of life for patients with metastatic breast cancer will be realized by overcoming resistance to targeted therapies.

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Resistance to HER2-Targeted Therapy

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Abstract HER2 is a member of the *ErbB*/HER family of receptor tyrosine kinases that promotes the proliferation of a subset of human breast cancers. HER2 is over-expressed in 20–25% of breast cancers and high expression is associated with poor clinical outcomes if not treated with an HER2-targeted drug**.** Although HER2 does not have a known extracellular ligand, HER2 is the preferred heterodimerization partner for the other HER-family receptors and HER2-containing heterodimers have increased affinity for ligands that bind to those heterodimer partners. The downstream effect is strong signal transduction and tumor cell proliferation. Even in the absence of a ligand, HER2 over-expression by itself can drive tumorigenesis and tumor growth. Given its elevated expression and oncogenic activity, its preferred status as a HER-family signaling partner, and the enhanced activity of HER2-containing heterodimers, HER2 is a valuable pharmacological target for the treatment of $HER2^+$ breast cancer. HER2-targeted agents fall into three general categories: therapeutic antibodies, antibody-drug conjugates, and tyrosine kinase inhibitors. The resistance mechanisms associated with each of these classes of drugs will be reviewed here.

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AUC	Area under the curve
CDK	Cyclin dependent kinase

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CML	Chronic myelogenous leukemia
CYP	Cytochrome P450
DFS	Disease-free survival
EGF	Epidermal growth factor
ER	Estrogen receptor
ERK	Extracellular signal regulated kinase
FAK	Focal adhesion kinase
FDA	United States Food and Drug Administration
HER1	Human *ErbB* receptor 1 also known as EGF receptor
HER2	Human *ErbB* receptor 2
HER3	Human *ErbB* receptor 3
HGF	Hepatocyte growth factor
HR	Hazard ratio
HRG	Heregulin
IGF	Insulin-like growth factor
IGF-1R	IGF-1 receptor
MEK	ERK kinase
MET	Receptor tyrosine kinase encoded by c-*MET* gene, also known as hepatocyte growth factor receptor
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
MUC4	mucin-4
OS	Overall survival
pCR	Pathological complete response
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Gene encoding the p110α catalytic subunit of PI3K
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKA	Protein kinase A
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RTK	Receptor tyrosine kinase
STAT3	Signal transducer and activator of transcription 3
TDM	Therapeutic drug monitoring
T-DM1	Trastuzumab-emtansine antibody drug conjugate
TGF-α	Transforming growth factor-α
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor

HER2 as a Therapeutic Target

The human *HER* (*ErbB*) gene family codes for four receptor tyrosine kinases (RTKs): HER1 (EGFR, ErbB-1), HER2 (Neu, ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). As typical RTKs, the receptors consist of an extracellular ligand binding domain (except for HER2, which has no known ligand), a single transmembrane domain, and an intracellular tyrosine kinase domain (except for HER3, which does not have intrinsic kinase activity) (see Fig. 1). Growth factor ligand binding leads to a series of conformational changes that result in homodimer and heterodimer activation and ensuing phosphorylation at specific tyrosine residues that reside in the intracellular tail region of each receptor. This, in turn, leads to binding of SH2-containing docking proteins, the links between activated receptors and their downstream signaling pathways, eventually producing enhanced proliferation, migration, angiogenesis, and survival [1].

HER2 was first described as an oncoprotein when the rat homolog (encoded by *neu*) was identified as a 185 kDa receptor in a group of chemically-induced neuroblastomas and glioblastomas and shown to confer loss of contact inhibition when expressed in mouse fibroblasts [2]. In humans, HER2 is over-expressed in 20–25% of breast cancers and high expression is associated with poor clinical outcomes if not treated with an HER2-targeted drug [3]. Cell-surface HER2 exists in a constitutively active configuration that allows it to homodimerize and stimulate downstream signaling even without a known ligand [4]. At the same time, HER2 is the preferred heterodimerization partner for the other HER family receptors and HER2-containing heterodimers have increased affinity for the respective partner's ligands, resulting in strong downstream signaling activity by those heterodimers. HER2/HER3 heterodimers are the most active signaling complex in the HER family and the most effective

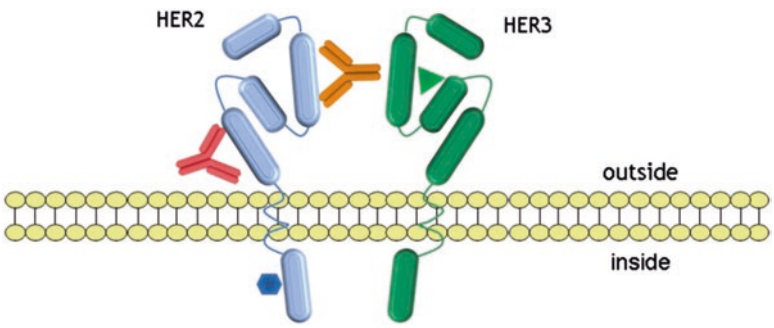

Fig. 1 Diagrammatic representation of the HER2 receptor and its interaction with trastuzumab (red antibody), pertuzumab (orange antibody), and tyrosine kinase inhibitors (blue hexagon). The diagram illustrates trastuzumab's inability (due to its binding location) to prevent HER2 dimerization with HER3 (and HER1) compared to pertuzumab's inhibition of ligand-mediated heterodimerization. The natural HER3 ligand, heregulin, is represented by the green triangle. The oblong segments within each receptor represent folded domains and the yellow balls connected by lines represent the plasma membrane of the cell

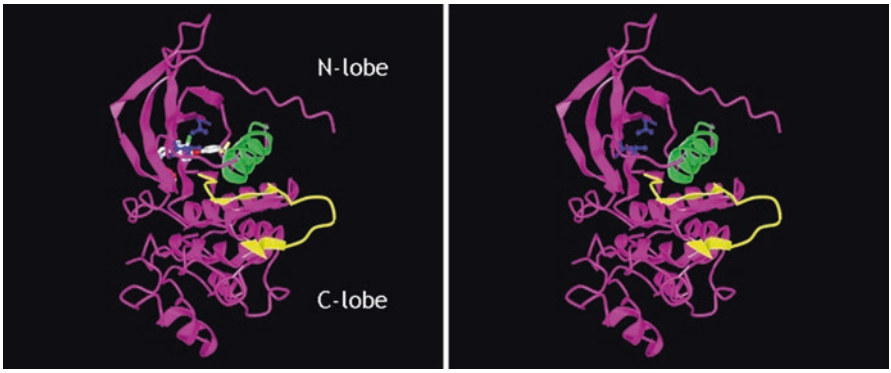

Fig. 2 Molecular model of the HER2 kinase domain based on the crystal structure of the domain in complex with SYR127063 (pdb ID 3PPO) [137]. SY127063 is a pyrrolo[3,2-*d*]pyrimidine-based selective TKI of HER2, which binds HER2 in an active conformation. SY127063 is shown in CPK coloring format (left panel); the panel on the right has the TKI ligand hidden. The green in both panels shows the C-helix; the yellow shows the A-loop; the blue shows the side chains of amino acid residues, L755 and T798, which are known to affect lapatinib resistance when they are mutated (see text)

activator of phosphatidylinositol 3-kinase (PI3K)/AKT, the crucial signal transduction pathway in HER2-positive breast cancer [5, 6]. Given its elevated expression and oncogenic activity, its preferred status as an HER family signaling partner, and the enhanced activity of HER2-containing heterodimers, HER2 represents an attractive pharmacological target for the treatment of HER2-positive ($HER2^+$) breast cancer. HER2-targeted agents fall into three general categories: therapeutic antibodies, antibody-drug conjugates, and tyrosine kinase inhibitors (TKIs). The resistance mechanisms associated with each of these classes of drugs will be reviewed here. Figure 2 shows a diagrammatic representation of the most significant molecular mechanisms described herein, using trastuzumab (antibody inhibitor) and lapatinib (TKI) as the reference drugs. Table 1 provides a summary of molecular mechanisms with literature citations.

Antibody Inhibitors

Trastuzumab (Herceptin)

Early studies showed that monoclonal antibodies directed at the extracellular domain of HER2 can dramatically inhibit proliferation of $HER2^+$ breast cancer cells in culture and reduce their growth as xenografts in animal models [7–10]. Using $HER2^+$ breast cancer cell lines, later studies showed that a humanized form of an anti-HER2 monoclonal antibody enhances the anti-tumor effect of cytotoxic chemotherapy drugs cisplatin, paclitaxel, and doxorubicin [9, 11].

Table 1 Molecular mechanisms of resistance

Resistance mechanism[a]		Antibodies (trastuzumab)[b]	TKI (lapatinib)[b]	Targeted combination drugs[c]
Steric hindrance	p95	[25–28, 274]		Lapatinib [229, 230]
	Δ16HER-2	[29, 30]		
	MUC4	[31–33]		
	Kinase domain mutation		[146]	
Alternative receptors	HER1 & HER3	[36–42]	[147, 148, 153]	Pertuzumab [74, 101–106, 130] Other antibodies [108–110] T-DM1 [120–129] Lapatinib [144, 189, 225, 226] Irreversible TKIs [208–224]
	IGF-1R	[44–47]		IGF-1R antibodies [233–235] IGF-1R TKIs [232, 234, 236] IGF-1R/IR TKIs [234, 237, 238]
	VEGFR	[60–64]		Bevacizumab [64, 243, 244] Pazopanib [242]
	MET, AXL	[52, 55]	[154, 155, 159]	Foretinib [239–241]
Downstream effectors	PI3K/PTEN	[66–69, 73–83]	[164, 166–170]	PI3K/mTOR inhibitors [217, 247, 260, 261, 275] PI3K inhibitors [262] Akt inhibitors [265–268]
	mTOR		[171–173]	mTORC1 inhibitors [246, 247, 254, 255] mTORC1/2 inhibitors [172, 245, 256]
	Src	[38, 87, 88]	[174–178]	
	$p27^{kip\ a}$	[91]		
	PKA	[92, 93]		
	t-Darpp	[36, 92, 94–96]	[276]	
	Estrogen receptor		[159, 165, 186–189]	ER antagonists [269] Aromatase inhibitors [270, 271]

[a]Shown are the most prominent resistance mechanisms

[b]Literature citations relevant to each mechanism as it pertains to trastuzumab and lapatinib, the lead HER2-targeted antibody and TKI, respectively

[c]Targeted drugs or drug classes being developed to combat trastuzumab and/or lapatinib resistance mechanisms, typically used in combination with the primary drug, with corresponding literature citations

From these observations emerged the development of trastuzumab (Genentech, Inc.), a humanized, recombinant IgG1 monoclonal antibody with high affinity to the extracellular domain of HER2. Following a successful clinical trial that showed an increase in both time to disease progression and response rate [12], trastuzumab was approved by the U.S. Food and Drug Adminisration (FDA) for use, in combination with the microtubule inhibitor paclitaxel, for first-line treatment of HER2$^+$ metastatic breast cancer. Trastuzumab is also recommended for use in the adjuvant setting for early breast cancer, either as monotherapy or in combination with chemotherapy, and a number of trials point to benefit when trastuzumab is added to neoadjuvant chemotherapy as well [13–16].

The mechanism by which trastuzumab acts in patients is still not entirely known, but it most likely has a combination of effects subsequent to HER2 binding. The most well-documented mechanisms are antibody-dependent cell-mediated cytotoxicity (ADCC); inhibition of cleavage of the extracellular domain of HER2, which prevents the formation of a p95 truncated form of HER2 that is constitutively active (and resistant to antibody binding); and inhibition of downstream signal transduction (predominantly through blockage of the PI3K/AKT pathway), which in turn disrupts normal cell cycle control, survival/apoptosis responses, and DNA repair [5]. These effects on apoptosis and DNA repair may be particularly important in patients who are treated with conventional cytotoxic chemotherapy or radiation along with trastuzumab, possibly accounting for synergistic effects of the combination therapies. Trastuzumab also appears to inhibit angiogenesis as a downstream effect of signal transduction inhibition, by lowering levels of pro-angiogenic factors such as VEGF, and upregulating the anti-angiogenic factor thrombospondin-1 (see [17] for a recent review of trastuzumab mechanisms).

Despite significant impact on outcomes for HER2$^+$ breast cancers, inherent and acquired resistance to trastuzumab remain a considerable clinical barrier. Approximately 75–85% of patients fail to respond to trastuzumab monotherapy due to inherent resistance [18–20]. Response rates increase to 50–75% when trastuzumab is combined with conventional chemotherapy [12, 18, 21], but most patients who respond to monotherapy or combination therapies will nevertheless experience disease progression and resistance within 1 year [12, 15, 22]. The mechanisms of resistance are varied, but the bulk of findings from *in vitro* and pre-clinical model systems indicate that sustained activation of the primary downstream signaling pathway, PI3K/AKT, is crucial. The molecular mechanisms for achieving such sustained signaling can be divided into three categories: (1) Steric effects that prevent HER2 inhibition – i.e., modification to the trastuzumab binding site due to changes in the HER2 structure or masking of its extracellular binding site (2) Activation of alternative receptors – i.e., compensation for blocking of HER2 signaling by activation of other receptor tyrosine kinases and (3) Modification to downstream effectors – i.e., alteration to effectors downstream of HER2, allowing for constitutive activation of PI3K/AKT and other pro-survival pathways. Each of these will be discussed next.

Steric Effects

Elimination or masking of the extracellular region of HER2 can prevent trastuzumab from binding to the receptor and, thus, promotes resistance. An amino-terminally truncated form of HER2, called p95HER2, results from the alternative translation of HER2 mRNA and/or proteolytic shedding of the extracellular domain of full-length HER2 [23, 24]. This truncated variant has constitutive tyrosine kinase activity and it appears to confer resistance to non-trastuzumab treatment. A retrospective study of specimens from breast cancer patients treated with non-trastuzumab regimens found a reduced time for disease free survival (DFS) for patients with high p95HER2 expression levels (median of 32 months) vs. patients with low p95HER2 expression levels (median of 139 months) ($p < 0.0001$) [25].

p95HER2 can heterodimerize with HER3, but not HER1, and trastuzumab is ineffective against this receptor complex [26] and p95HER2 generally [27]. A retrospective examination of metastatic tumors from patients treated with trastuzumab found a significant inverse relationship between p95HER2 expression and responsiveness to trastuzumab [27]. Of nine patients with p95HER2 expression, only one (11%) showed a partial response to trastuzumab therapy, whereas 19/37 (51%) of patients with tumors expressing full-length HER2 (and not p95HER2) exhibited complete or partial response ($p = 0.029$). In a separate study, a specific antibody targeting p95HER2 was used to quantify its expression in 93 specimens from patients with trastuzumab-treated metastatic breast cancer. A correlation was found between high p95HER2 levels and shorter progression-free survival (PFS) (hazard ratio (HR) = 1.9; $p < 0.05$) and lower overall survival (OS) (HR = 2.2; $p \leq 0.01$) [28].

A second variant, Δ16HER-2, is created by a splice variant of HER2 mRNA that encodes a receptor with an altered conformation in the extracellular domain. Δ16HER-2 forms stable homodimers that are highly oncogenic and resistant to trastuzumab binding and growth inhibition, with possible coupling to Src kinase as an important mediator of Δ16HER-2 efficacy [29, 30]. Δ16HER-2 has been detected in 52% of $HER2^+$ primary breast cancers and 89% of Δ16HER-2^+ patients present with lymph node-positive disease [30]. The association between Δ16HER-2 and patient response to trastuzumab has not been reported.

A steric effect on trastuzumab binding can also be achieved by over-expression of non-receptor proteins such as Mucin-4 (MUC4), an O-glycosylated membrane protein that binds to HER2 [31]. MUC4 over-expression in breast cancer cell lines has been shown to decrease the accessibility of trastuzumab to HER2 through partial masking of its extracellular domain [31, 32]. A recent retrospective analysis of 78 $HER2^+$ patients who had received adjuvant trastuzumab therapy found that $MUC4^+$ tumors are associated with significantly shorter DFS in univariate analysis (HR = 4.40; $p = 0.018$) and MUC4 is an independent predictor of poor DFS in multivariate analysis after adjusting for stage and nodal status (HR = 5.43; $p = 0.008$) [33].

Activation of Alternative Receptors

Another mechanism for sustained PI3K/AKT signaling in the face of trastuzumab is through compensatory activation of alternative RTKs, either other members of the HER family or non-HER receptors that signal through the same or parallel pathways (see [34] for a recent review).

HER1 and HER3

Perhaps the most likely compensatory receptors for HER2 inhibition are family members HER1 and HER3, both of which are seen to be up-regulated in different models of trastuzumab resistance [35–38]. These receptors can function either as HER1/HER1 homodimers or as HER1/HER3 heterodimers but there is also evidence to suggest that HER1/HER2 and HER2/HER3 heterodimer levels are an important determinant of both primary (de novo) and acquired trastuzumab resistance [39]. Increased HER1 and HER3 levels in resistant cells are often accompanied by increased levels of their specific ligands, resulting in increased signaling through these receptors and increased sensitivity to their respective HER inhibitors (tyrosine kinase inhibitors or antibodies). This is true in cultured cell lines (*in vitro* selection) and in xenografts (*in vivo* selection) [35, 37, 40]. Several studies have shown a synergistic or enhanced effect of HER1 inhibition in combination with trastuzumab on trastuzumab-resistant breast cancer cell lines [35, 36, 41], further suggesting that compensatory signaling through HER1 is part of the resistance mechanism. Additional evidence comes from the efficacy of HER2-targeted drugs that also inhibit HER1 or prevent ligand-dependent HER2/HER3 signaling, which will both be discussed later.

The implication from the observations about HER1 and HER3 compensatory signaling is that relative HER family expression levels (not just HER2), and possibly their dimerization patterns, could be important in determining the primary response to trastuzumab and HER-targeted combinations, as well as the response to these agents in the relapse/resistance setting. Clinical data on this point are scarce. In the primary setting, only HER2 is used for therapeutic decision-making, and clinical trials testing newer HER2-targeted antibodies or combinations of trastuzumab with small molecule HER inhibitors (both of which should prevent HER-family compensatory activity) generally do not consider different HER expression levels or dimerization patterns as part of the trial design. In an analysis of biomarkers in the TRYPHAENA trial – which looked at trastuzumab plus pertuzumab (see below), sequential or simultaneous with chemotherapy, in the neoadjuvant setting – high levels of membrane-bound HER1 correlated with lower pathological complete response (pCR) rate ($p = 0.0157$ by chi-squared test) only when patients from all arms of the study were pooled [42]. The conclusion from this small study was that HER1 is not a suitable biomarker for patient selection at this time, but larger studies should be conducted before ruling out the importance of HER1 and HER3 as predictors of trastuzumab response.

Insulin-Like Growth Factor-1 Receptor

The insulin-like growth factor-1 receptor (IGF-1R) is another alternative RTK that can be activated to overcome HER2 inhibition. IGF-1R is not a native dimerization partner of HER2, but its signaling cascade shares several key features with HER2, most notably the PI3K/AKT pathway. IGF-1R over-expression commonly occurs in breast tumors [43] and in trastuzumab-resistant cancer cells [38, 44]. Early indication of a connection to trastuzumab resistance came from a study of MCF7 cells (which naturally express high levels of IGF-1R) transfected with HER2 cDNA. These cells show trastuzumab resistance in the presence but not the absence of the IGF-1R ligand, IGF-1 [45]. This same study showed that exogenous over-expression of IGF-1R in SK-Br-3 cells (which naturally express low levels of IGF-1R and are HER2$^+$) is sufficient to confer trastuzumab resistance and that resistance can be abrogated by adding IGFBP3, a protein that binds IGF-1 and sequesters it from IGF-1R [45]. SK-Br-3 cells selected for trastuzumab resistance do not have elevated IGF-1R, but they do display a IGF-1R/HER2 dimer that is not seen in parental SK-Br-3 cells [46]. In these cells, IGF-1R activation with IGF-1 results in HER2 phosphorylation/activation and specific IGF-1R tyrosine kinase inhibitors reduce the HER2 activation state only in the resistant cells. Taken together, these studies suggest that up-regulation or dysregulation of the IGF-1R signaling could be a mechanism for conferring trastuzumab resistance.

The cross-talk between IGF-1R and HER2 in trastuzumab-resistant cells in culture prompted investigation of the predictive value of IGF-1R expression for therapeutic response. However, at least four different studies have found no correlation between IGF-1R or phospho-IGF-1R and survival or trastuzumab response/resistance in treatment-naïve patients (see [47]). It is possible that IGF-1R is more important as a mechanism of acquired resistance, rather than being a determinant of initial response, but there have been no reports to date on possible changes in IGF-1R levels or activity after trastuzumab therapy and the possible contribution of IGF-1R to the acquired resistance phenotype.

MET

The mesenchymal-epithelial transition (MET) RTK and its ligand, hepatocyte growth factor (HGF), are often over-expressed in breast cancer and MET over-expression is an independent predictor of poor prognosis in breast cancer [48–52]. A subset of HER2$^+$ breast cancers is positive for MET over-expression and there is evidence that co-expression might contribute to a more invasive phenotype [53, 54]. Similar to the IGF-1R story, ligand-mediated activation of MET confers resistance to trastuzumab in HER2$^+$ cell lines and MET inhibition sensitizes them to the drug. MET activation results in increased signaling through AKT in the presence of trastuzumab, suggesting that MET signaling is able to compensate for HER2 inhibition as a mechanism for MET's involvement in drug resistance [52]. In a retrospective analysis of 130 HER2$^+$ metastatic breast cancers, increased c-*MET* and *HGF* gene copy number, as determined by fluorescence in situ hybridization (FISH),

were observed in about 25% of cases. c-*MET* FISH-positivity (n = 36) was associated with higher trastuzumab failure rate (p = 0.0001) and shorter time to progression (HR = 1.74; p = 0.001), compared to c-*MET* FISH-negative cases. *HGF* FISH-positivity (n = 33) was associated with higher trastuzumab failure rate (p = 0.007), compared with *HGF* FISH-negative cases [55].

VEGFR

The angiogenesis pathway stimulated by vascular endothelial growth factor (VEGF) and its receptor (VEGFR) is strongly implicated in HER2$^+$ breast cancer growth and trastuzumab's mechanism of action. HER2 expression and signaling are associated with high VEGF expression in cell lines [56]. Clinically, the majority of HER2$^+$ breast cancers exhibit VEGF over-expression and co-expression of HER2 and VEGF predicts worse clinical outcomes in patients with primary breast cancer [57, 58]. As mentioned earlier, one mechanism of trastuzumab action appears to be an inhibition of angiogenesis mediated by reduced levels of VEGF and VEGFR signaling [17, 59].

Further evidence of VEGF-mediated trastuzumab resistance comes from inhibitor studies. Cell line models of inherent and acquired trastuzumab resistance have high VEGF expression. Bevacizumab, a monoclonal antibody targeted to VEGF, or sorafinib, a relatively non-specific inhibitor of VEGFR kinase activity, can reverse the resistance phenotype in these cell line models, both *in vitro* and as xenografts [60–62]. Sunitinib, another non-specific inhibitor of VEGFR kinase, has shown activity in patients with HER2$^+$ breast cancer who had previously received trastuzumab [63]. In a phase I dose-escalation study of bevacizumab in combination with trastuzumab and the dual HER1/HER2 inhibitor lapatinib (see below for a further discussion of lapatinib), 50% of the 26 breast cancer patients had partial response, complete response or stable disease for ≥6 months. This was true even for the patients who had received therapy with trastuzumab and/or lapatinib within the previous year [64].

Modification to Downstream Effectors

The same effect achieved through activation of parallel RTKs to compensate for HER2 inhibition can be achieved by modifying downstream effectors of their common signaling pathways in a way that allows for signal transduction even when HER2 and other receptors are inhibited. There are several key regulators of HER2 signaling that are associated with trastuzumab resistance.

PI3K and PTEN

PI3K activates the AKT signaling pathway by phosphorylating the signaling lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) and converting it to phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which in turn phosphorylates and activates AKT (for a

review, see [65]). The PI3K family consists of multiple members divided into three main classes. HER2 exerts its oncogenic function primarily through the p110α catalytic isoform encoded by the *PIK3CA* gene [66–68]. Activating mutations in *PIK3CA*, including "hotspot" mutations in exons 9 and 20, are found in approximately 20% of HER2$^+$ tumors, and there is preclinical evidence linking such mutations to trastuzumab resistance [66].

Phosphatase and tensin homolog (PTEN) is a lipid phosphatase that dephosphorylates PIP_3 and is thus an inhibitor of the PI3K/AKT pathway [65]. Activating mutations in PI3K or loss of PTEN function result in hyperactive PI3K/AKT signaling that should counteract HER2 inhibition by trastuzumab. PTEN activity is crucial to AKT dephosphorylation following treatment with trastuzumab. Trastuzumab resistance can be achieved in cell culture and in mouse xenografts by antisense-mediated inhibition of PTEN and the resistance phenotype can be rescued by inhibition of PI3K [69]. Moreover, a large-scale RNA interference screen performed on the HER2$^+$ BT474 cell line found that expression of a shRNA targeted to PTEN leads to a decreased growth inhibitory effect of trastuzumab, which can be reversed by the expression of constitutively active PI3K [66]. PTEN protein is lost or low in approximately 40% of HER2$^+$ breast cancers [70, 71].

The contribution of PI3K/PTEN alterations to clinical trastuzumab resistance has been difficult to tease out. Activating mutations in *PIK3CA* or loss of PTEN function would each result in increased PI3K/AKT signaling, so they are largely mutually exclusive occurrences in cancer patients [72]. Attempts to correlate *PIK3CA* mutation or PTEN loss with patient response or outcomes have produced varying results, most likely due to small sample sizes, different treatment backgrounds and tumor status of the patients, and different study designs. Several retrospective analyses of specimens from HER2$^+$ patients treated with trastuzumab-based regimens have shown correlations between PTEN loss and poor trastuzumab response or worse patient outcomes [66, 69, 73]. *PIK3CA* mutations by themselves are not associated with patient outcomes, but the combination of PTEN loss and *PIK3CA* mutation (i.e., PI3K pathway activation) is even more significantly associated with shorter PFS than PTEN loss alone [66]. Studies analyzing PTEN and *PIK3CA* status in patients presenting with metastatic breast cancer and treated with first-line trastuzumab-based regimens have generally reported associations between *PIK3CA* mutation and/or PTEN loss and shorter time to progression or survival, to varying degrees of statistical significance [74–76].

In the adjuvant setting, there might be a trend towards an association between PTEN positivity and metastasis-free survival [77], but *PIK3CA* mutation does not appear to be predictive of response or outcome [77–79]. Perhaps the most pertinent results come from a set of neoadjuvant trials in which trastuzumab is compared with lapatinib or the combination of trastuzumab + lapatinib, a small molecule inhibitor of HER2 and HER1 tyrosine kinase activity (see section "Small-Molecule Inhibitors" below). Data from the trastuzumab arms of those trials showed that low PTEN or *PIK3CA* mutations are associated with lower pCR rate [80–82], but statistical significance is achieved only when data for low PTEN and *PIK3CA* mutation are combined [80]. *PIK3CA* mutations alone are significantly associated with pCR rate when data from multiple treatment arms (trastuzumab, lapatinib, trastuzumab +

lapatinib) and trials are combined [81, 83]. A similar finding emerged from the analysis of metastatic breast cancer patients treated with first-line trastuzumab or trastuzumab + pertuzumab, an antibody that prevents HER2 dimerization with other HER-family receptors and IGF-1R (see below). *PIK3CA* mutations are associated with lower PFS with either treatment, but statistical significance is achieved only when data from both treatments are combined [74].

Taken together, there is growing evidence to suggest a mechanistic role for the PI3K pathway in trastuzumab response/resistance, with perhaps the greatest effect when multiple HER receptors are inhibited by trastuzumab in combination with other HER-targeted agents. The clinical value of *PIK3CA* or PTEN as predictive biomarkers has yet to be definitively established, however.

Src

The Src tyrosine kinase lies downstream of multiple RTKs, including HER-family receptors, and it is a common node in the PI3K/AKT, ERK, STAT3, and FAK pathways, thereby playing a role in major cellular functions such as proliferation, survival, angiogenesis, motility and adhesion [84, 85]. Its role in breast cancer has recently been reviewed [86]. Src is activated in a number of cell line models of acquired trastuzumab resistance, most likely downstream of RTK compensatory signaling as described earlier [38, 87], and when resistance is artificially created by PTEN knockdown [38]. In one recent model, trastuzumab resistance conferred by physical contact between breast cancer cells and mesenchymal stem cells appears to occur by Src activation [88]. Over-expression of either wild type Src or a constitutively active Src mutant is sufficient to confer trastuzumab resistance, whereas expression of a dominant-negative form of Src leads to enhanced trastuzumab sensitivity. Saracatinib, a small molecule inhibitor of Src, reverses trastuzumab resistance associated with RTK activation, PTEN knockdown, and constitutive Src activation. In retrospective analyses of primary breast tumors from patients treated with trastuzumab, high levels of Y416-phosphorylated (active) Src are found to be associated with low clinical response rate and lower OS compared to tumors with low levels of phosphorylated Src [38, 87].

$p27^{kip1}$

As noted earlier, one of the downstream effects of trastuzumab is G_1 cell-cycle arrest. This observation has been correlated to a dose-dependent effect of trastuzumab on accumulation of the CDK2 inhibitor $p27^{kip1}$ by mechanisms that involve multiple signaling pathways known to be impacted by trastuzumab binding (including PI3K/AKT) [89, 90]. Moreover, $HER2^+$ breast cancer cell lines selected for trastuzumab resistance express lower levels of $p27^{kip1}$ and exogenous expression of $p27^{kip1}$ in resistant cells renders them once again susceptible to growth inhibition by trastuzumab [91]. This indicates an important role for $p27^{kip1}$ in trastuzumab-mediated cell

cycle arrest and resistance and raises interesting questions about whether $p27^{kip1}$ might be used as a predictive or prognostic biomarker and whether one or more mechanism of trastuzumab-mediated $p27^{kip1}$ accumulation can be exploited therapeutically (see section "Implications for New Targets and Drug Combinations").

Protein Kinase a (PKA) and t-Darpp

Microarray analysis of trastuzumab-resistant cell lines has revealed significant differences, relative to trastuzumab-sensitive parental cells, in the expression of several genes involved in the regulation of PKA signaling [92]. These same trastuzumab-resistant cells have increased PKA activity (as measured by CREB DNA binding activity) and enforced down-regulation of a PKA regulatory subunit that normally keeps PKA catalytic activity in check is sufficient to confer increased PKA activity and partial trastuzumab resistance [92]. PKA activation was further implicated in trastuzumab resistance in a screen that examined the effect of exogenously expressing a panel of kinases on breast cancer cell proliferation in the presence of a HER2-targeted shRNA [93]. This study suggested that PKA activation does not result in increased signaling through the PI3K or ERK pathways, but rather confers survival through inactivation of the proapoptotic protein BAD.

Another gene involved in PKA regulation is *PPP1R1B*, which codes for two transcriptional variants, Darpp-32 and its truncated isoform known as t-Darpp. Several studies have identified t-Darpp as being up-regulated in trastuzumab-resistant breast cancer cell lines and over-expression of exogenous t-Darpp is sufficient to confer trastuzumab resistance [92, 94–96]. Cells that over-express exogenous t-Darpp have elevated PKA activity and trastuzumab-resistant PI3K/AKT signaling [95]. The mechanism by which t-Darpp activates PKA and/or PI3K/AKT signaling and confers trastuzumab resistance is not entirely known, but models involving stabilization of HER1 signaling, inhibition of apoptosis, and a direct effect on the PKA holoenzyme have all been proposed [36,94; D. Theile, manuscript in preparation]. Regardless of the mechanism, t-Darpp may ultimately represent a resistance-specific factor and new target for overcoming or preventing trastuzumab resistance.

Pertuzumab

The breakthrough in targeted HER2 therapy achieved by trastuzumab and the ensuing resistance to its growth inhibitory effects encouraged the development of a second generation of HER2-targeted drugs. Pertuzumab (Genentech, Inc.) is one of many second-generation antibody inhibitors of HER2. Unlike trastuzumab, which only disrupts the ligand-independent homodimerization of HER2, pertuzumab binds to the extracellular dimerization domain of the receptor and eliminates dimerization with other HER-family receptors, as well as IGF-1R [46, 97–99].

As discussed above, a key driver of trastuzumab resistance is increased signaling by heterodimerization with HER3, HER1 and IGF-1R. Pertuzumab's ability to prevent such heterodimerization has made it a candidate for combination therapy along with trastuzumab. Preclinical studies showed a synergistic effect of combining trastuzumab with pertuzumab in BT474 breast cancer cells [99] and in xenografts of HER2⁺ breast cancer cells in mice [100]. The same xenograft study also demonstrated that a combination of the two drugs can inhibit the growth of tumors following acquired resistance to trastuzumab [100].

In early clinical trials, pertuzumab showed great potential as a second-line therapy in patients who progressed on trastuzumab regimens, and results are even more impressive when trastuzumab and pertuzumab are used in combination as second-line therapy [101, 102]. In 2013, pertuzumab was approved by the FDA for first-line treatment of metastatic, HER2⁺ breast cancer in combination with trastuzumab + docetaxel. This approval was granted following the CLEOPATRA phase III clinical trial in which patients received either standard first-line treatment with trastuzumab + docetaxel (control arm) or a combination of pertuzumab + trastuzumab + docetaxel. The pertuzumab arm of the study resulted in a PFS of 18.5 months and 80.2% objective response (OR) rate, compared to 12.4 months PFS and 69.3% OR rate in the control arm ($p < 0.001$) [103]. A follow-up a year later also demonstrated significant improvement in OS in patients treated with pertuzumab + trastuzumab + docetaxel [104]. Pertuzumab + trastuzumab + docetaxel also showed significantly improved pCR rate ($p = 0.0141$) and trends towards better 5-year PFS and DFS rates over trastuzumab + docetaxel in neoadjuvant treatment of HER2+ breast cancer patients in the NeoSphere trial [105, 106].

Taken together, the data on pertuzumab suggest that this antibody holds great promise for treating trastuzumab-resistant disease or perhaps preventing a major mechanism of resistance. The most common resistance mechanisms acquired by tumors following treatment with trastuzumab involve activation of alternative signaling pathways in order to maintain activity in the PI3K/AKT pathway. Pertuzumab has the ability to overcome or prevent many of these mechanisms by denying HER2 heterodimerization with other receptors. This idea is supported by biomarker studies alluded to earlier, in which *PIK3CA* mutations, which act downstream of receptor dimerization, were found to be associated with poorer response to pertuzumab/trastuzumab regimens in the CLEOPATRA trial [74], consistent with earlier trends seen in smaller trials, NeoSphere and TRYPHAENA [42, 107].

Other Antibodies

There are two new HER2-targeted antibodies currently in clinical trials for treatment of HER2⁺ cancers, with limited information on their efficacy and mechanisms of resistance.

Margetuximab

Margetuximab (MGAH22, MacroGenics, Inc.) is a HER2-targeted antibody with a Fc-domain (non-targeting end of the antibody) engineered to bind to and activate immune effector cells and induce ADCC. Preclinical trials have shown it can induce ADCC better than HER2-targeted antibodies with wild type Fc domains [108]. Phase I clinical trials concluded that margetuximab treatment is tolerable at all doses examined and shows promising activity in $HER2^+$ tumors, including breast cancer [109]. Margetuximab efficacy is being examined in SOPHIA (clinicaltrials.gov identifier NCT02492711), a phase III trial testing margetuximab in combination with chemotherapy compared to trastuzumab + chemotherapy for third-line metastatic breast cancer, with no outcome data yet available [110].

FS102

FS102 (F-Star Biotechnology, Ltd. in partnership with Bristol-Myers Squibb) is a HER2-targeted Fc fragment with an antigen binding domain that recognizes a unique site on the HER2 receptor and induces programmed cell death and marked HER2 internalization and degradation. Preclinical studies have shown that SK-Br-3 cells exposed to FS102 go through apoptosis [111], as opposed to the G1 phase cell cycle arrest that trastuzumab causes in these cells. Such studies have also demonstrated complete tumor regression, apoptosis induction and reduction of HER2 levels in patient-derived xenograft models of breast and gastric cancer. The effect of FS102 is superior to the effects of trastuzumab or trastuzumab + pertuzumab studied in parallel and FS102 is able to inhibit the growth of xenografts that progress on trastuzumab or trastuzumab + pertuzumab, suggesting non-overlapping mechanisms of resistance to these agents [111]. FS102 is being tested in a dose-escalating phase I clinical trial for treatment of $HER2^+$ solid tumors (NCT02286219).

Induction of apoptosis overcomes a serious flaw in the G1 arrest mechanism mediated by trastuzumab. Cells arrested in G1 remain viable and can escape cell cycle arrest by activating alternative signaling pathways or the HER2 signaling pathway further downstream. Although not well studied, resistance to FS102, in contrast, would likely arise through mechanisms of avoidance -- either through modifications to the HER2 receptor or masking of the binding site -- or through mechanisms of apoptosis inhibition. This, along with the preclinical studies with patient-derived xenografts [111], raises the possibility that trastuzumab resistance might not result in cross-resistance to FS102, thus allowing for FS102 as second-line therapy or even in combination with standard first-line therapies.

Antibody-Drug Conjugates

T-DM1

T-DM1 (Genentech, Inc.) is the prototypical antibody-drug conjugate that targets HER2. The antibody component is trastuzumab and the drug component is emtansine (DM1), a small molecule that binds tubulin and prevents microtubule assembly, thus inducing mitotic arrest and cell death in dividing cells. DM1 is derived from maytansine, which was originally found to be highly potent but with unacceptable systemic toxicity due to lack of specificity to tumor cells [112–115]. DM1 was specifically designed to be used as a conjugate with trastuzumab, which allows for more focused drug activity in the cytoplasm of target cells following HER2-mediated endocytosis [116–118].

In the original pre-clinical studies of T-DM1, it was found to be a potent inhibitor of growth in an array of $HER2^+$ cells, regardless of whether those cells respond to trastuzumab alone and by a mechanism that results in cell death. In $HER2^+$ xenograft models, T-DM1 was shown to inhibit tumor growth and promote tumor regression [119]. Following these initial studies, a multitude of phase I/II clinical trials in patients previously treated with trastuzumab reported significantly reduced toxicity, compared to DM1, with levels tolerable beyond the known clinical dose. These trials also found an overall response rate of at least 40%, indicating the potential of T-DM1 as second-line therapy of $HER2^+$ breast cancer [120–125].

The success of these trials led to EMILIA, a phase III trial testing the efficacy and safety of T-DM1 in patients with locally-advanced or metastatic $HER2^+$ breast cancer previously treated with trastuzumab + taxanes. The trial included 991 patients divided into two arms, one in which patients received a common second-line therapy of lapatinib + capecitabine and the other in which patients received T-DM1 as second-line therapy. The study demonstrated improvements in both OS (30.9 months with T-DM1 vs. 25.1 months in the control arm) and PFS (9.6 months with T-DM1 vs. 6.4 months in the control arm) while also exhibiting reduced toxicity and adverse effects for patients [126, 127].

The EMILIA trial led to FDA approval of T-DM1 in February, 2013, for the treatment of metastatic $HER2^+$ breast cancer. A more recent phase III trial named TH3RESA compared the efficacy of T-DM1 to treatment of physician choice in similar second-line therapy and reconfirmed the improved PFS, OS and tolerability of T-DM1 compared to current treatment options, thus solidifying its position as the standard second-line therapy for $HER2^+$ breast cancer patients [128].

The MARIANNA trial examined the efficacy of T-DM1 alone and T-DM1 + pertuzumab in first-line therapy of $HER2^+$ breast cancer, compared with the current standard of care of trastuzumab + taxane. Although the results did show reduced toxicity of T-DM1, it failed to show superiority in either PFS or OS to the current standard regimen [129]. Taking in consideration the results of the CLEOPATRA trials discussed above, which show superiority of pertuzumab to trastuzumab + taxane therapy, T-DM1 is unlikely to be used as first-line standard of care for $HER2^+$ breast cancer [130].

The overall response rate to T-DM1 is 80% in patients with HER2$^+$ breast cancer who progress on prior HER2-directed therapy [121]. This suggests that primary resistance to T-DM1 is relatively infrequent, but all of the clinical trials discussed above indicate that patients eventually cease to respond to T-DM1 therapy due to acquired resistance. Since T-DM1 is a relatively new drug, little is known about the mechanisms involved in acquired resistance. However, we can speculate on some likely resistance factors, based on what is known about the molecular mechanism of T-DM1 action:

a) Reduced binding and internalization. Since T-DM1 is dependent on binding to HER2 and receptor-mediated endocytosis to enter the cytoplasm, any changes to HER2 that prevent trastuzumab from binding to the extracellular region would result in reduced sensitivity to T-DM1 as well. As discussed earlier, mechanisms that affect binding to HER2 include expression of the p95HER2 or Δ16HER-2 isoforms and over-expression of MUC4 that result in masking of the trastuzumab binding site. In addition, the internalization of HER2 following trastuzumab binding is a regulated process that depends on clathrin activity (see [131] for a review on HER2 trafficking), suggesting that inhibition or down-regulation of clathrin-dependent endocytosis would also lower intracellular levels of DM1.

b) Reduced lysosomal processing. Following internalization of the T-DM1/HER2 complex into endosomes, DM1 needs to be released from trastuzumab by lysosomal degradation and accumulate in the cytoplasm at a concentration that meets the threshold needed to promote cell death. Changes to endosomal trafficking, which are known to occur following endocytosis-mediated therapy [132, 133], could result in increased re-shuttling of the complex to the plasma membrane and decreased lysosomal trafficking. At the same time, modification to the lysosomal degradation machinery could result in decreased degradation of T-DM1. Both mechanisms would result in reduced cytoplasmic concentrations of DM1.

c) Reduced intracellular activity. Once in the cytoplasm, DM1 binds to tubulin and inhibits microtubule polymerization. Any modifications to tubulin or to enzymes involved in the microtubule dynamics could impact the efficacy of the drug [112, 134]. DM1 is also a substrate for the efflux pump P-glycoprotein encoded by the *MDR*1 gene [135]. *MDR*1 over-expression may result in reduced sensitivity to DM1 as is the case for several other drugs that are exported from the cell via efflux pumps [136].

Small-Molecule Inhibitors

Pharmacological and clinical activity of antibody-based drugs mainly relies on their extracellular interaction with HER2. An alternative approach is to inhibit the kinase moiety located intracellularly, especially under certain conditions of trastuzumab resistance. The kinase domains of the HER family are structurally quite similar to other kinases [137, 138]. They contain two large lobes (called the N-lobe and the C-lobe), with the actual kinase activity site located in the cleft (called the hinge

region) between the two lobes. The kinase activity site harbors an ATP-binding pocket, a flexible substrate binding site, and two regulatory regions called the activation loop (A-loop) and the C-helix (see Fig. 2). In the inactive conformation of the kinase domain, the activation loop hinders the binding of substrates. Upon activation, the loop is structurally altered leading to an open substrate binding site.

In the early 1990s, natural compounds such as erbstatin and synthetic biosimilars like the 'tyrphostins' were shown to inhibit HER-family function, but they were found to interact with the substrate binding pocket [139] and had rather poor HER kinase selectivity [140]. Subsequent enzymological studies of HER1 determined that an intermediate ternary complex forms during catalysis, in which the kinase domain interacts with the γ-phosphate of ATP and a tyrosyl hydroxyl and tyrosyl aromatic ring of the peptide substrate (the target of the RTK phosphorylating activity) [141]. This information was used to search a database of predicted three-dimensional structures for compounds that mimic these interactions. The goal was to identify compounds that act similarly to and compete with ATP but do not lead to receptor phosphorylation. From this, the 4-anilino-quinazolines emerged as low nanomolar ATP-competitive inhibitors of HER1 [142]. Because the kinase domains of the four HER receptors show a high degree of identity (59%–81%), inhibitors that selectively inhibit only one of the four potential kinase activities are hard to design [143]. Structure-activity relationship studies determined that substitutions on the 4-anilino ring of the 4-anilino-quinazolines play a role in selectivity, with large substitutions being correlated with increased affinity for HER2 [142]. At least four other classes of bicyclic compounds – pyridopyrimidines, pyrrolopyrimidines, pyrrolotriazines, and cyanoquinolines – have been developed as HER kinase inhibitors. Although less is known about their structure-activity relationships, they appear to follow the same principles for target binding as the quinazolines. In the following sections we will review the dominant small molecule drugs targeting HER2 and other HER family members.

Lapatinib (See Fig. 3)

Lapatinib targets HER2 and HER1 and was the first TKI approved by the FDA for the treatment of patients with metastatic $HER2^+$ breast cancer. Numerous clinical trials evaluated its efficacy in various patient cohorts ($HER2^+$ or $HER2^-$; early or advanced breast cancer; pretreated or therapy-naïve). Taken together, these studies (recently reviewed in [144]) showed that (1) monotherapy with lapatinib is effective in heavily pre-treated $HER2^+$ but not $HER2^-$ patients; (2) lapatinib shows some, but rather minor, clinical efficacy in trastuzumab-resistant advanced or metastatic breast cancer; (3) lapatinib enhances the efficacy of other concurrently administered cytotoxic drugs that target different sites within the cell (e.g. taxanes, capecitabine etc); (4) when directly compared to mono-ty of molecular mechanisms with literature citationst cancer without prior therapy, lapatinib is equally effective as trastuzumab; but (5) trials evaluating lapatinib + chemotherapy vs. trastuzumab + chemotherapy in $HER2^+$ advanced breast cancer revealed that the latter is superior. Based on these

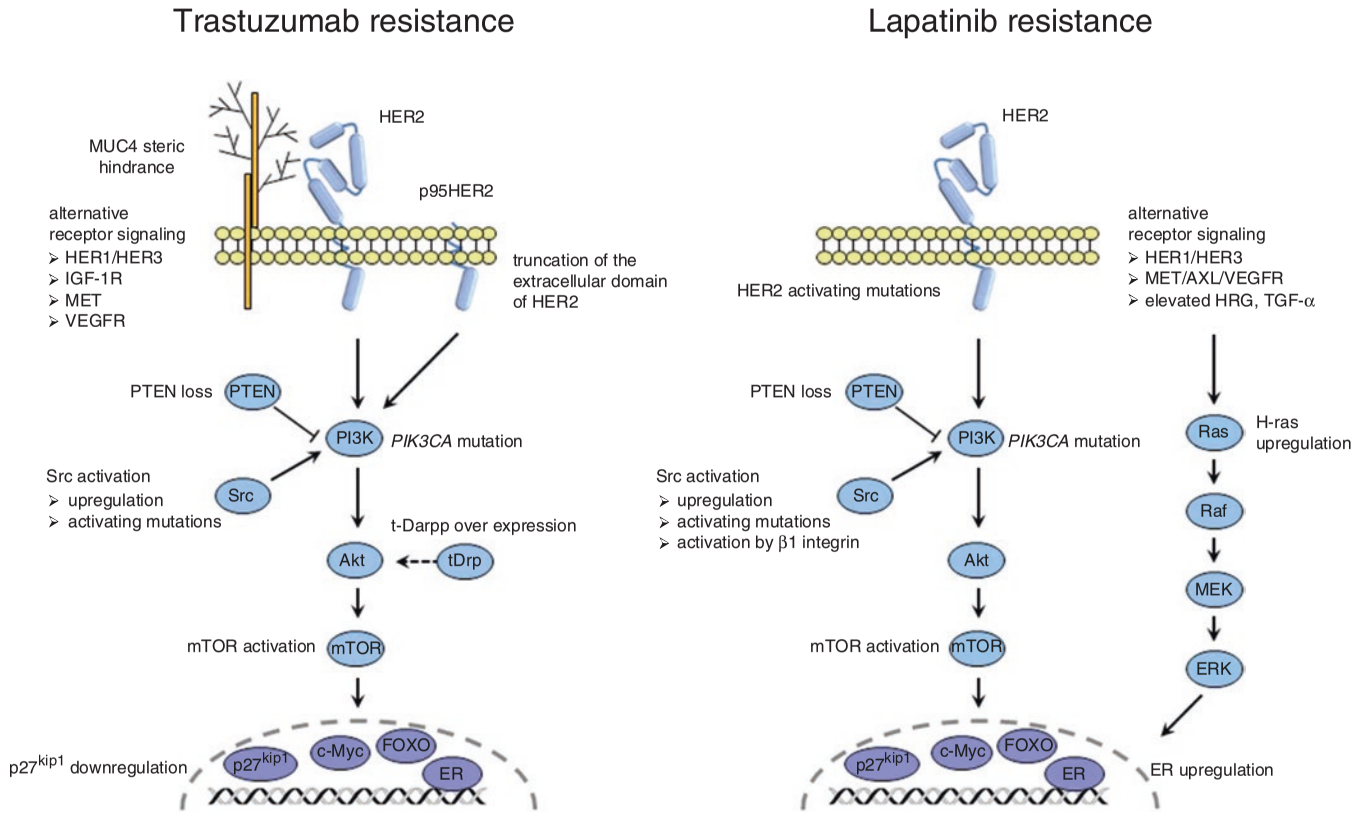

Fig. 3 Schematic representation of the most significant molecular resistance mechanisms to trastuzumab (left) and lapatinib (right). Additional information and corresponding reference citations can be found in the text. Arrowheads (→) and bars (--) attached to solid lines indicate known activation or inhibition effects, respectively, on the indicated pathway or protein. The dotted-line arrow from t-Darpp indicates an indirect mechanism of activation when t-Darpp is over-expressed

clinical trials, lapatinib is currently mostly used in combination with capecitabine for palliation of patients who were previously treated first-line with a combination of trastuzumab, an anthracycline (doxorubicin or epirubicin) and a taxane (docetaxel or paclitaxel).

Thus, although lapatinib is a potent inhibitor of HER2 kinase activity, its clinical impact, over and above that of trastuzumab, is limited. This is in part due to the compensatory nature of HER3 upregulation and its heterodimerization with HER2, leading to activation of PI3K/AKT signaling despite HER2 inhibition (see below). As a consequence, supra-therapeutic concentrations would be needed for a complete inhibition of HER2/HER3 signaling by lapatinib. Such high concentrations are not tolerable given the severe dermatologic and gastro-intestinal toxicities that result from HER1 and HER2 inhibition by lapatinib. Dividing doses into two applications per day, in contrast to once daily dosing, has been suggested to increase total drug exposure while minimizing toxicity. Even 7000 mg per day can be safely administered in this manner without evidence of dose-limiting toxicity and there is preliminary indication of therapeutic efficacy [145].

Given these findings and the considerable amount of pre-clinical work done with lapatinib, it is worth exploring the mechanisms of lapatinib resistance that probably represent mechanisms that will be shared by other less-studied TKIs as well. We describe molecular mechanisms of resistance, as we did in Sections "Antibody Inhibitors" and "Antibody-Drug Conjugates", and also discuss pharmacological factors that affect drug efficacy in the clinical setting.

Steric Effects

Since lapatinib targets the kinase domains of HER1 and HER2, mutations that render the kinases constitutively active or prevent TKIs from binding are likely mechanisms of resistance. Experimental approaches screening for HER2 mutations associated with lapatinib resistance have revealed amino acid substitutions at 16 different HER2 residues, with 12 mutated amino acids mapping to the kinase domain [146]. Mutations with the highest impact on lapatinib efficacy cluster in the N-lobe and hinge region. L755S and T798I mutations are associated with considerable lapatinib resistance. Notably, a T790 M mutation in HER1 also confers resistance to lapatinib at a level that is comparable to the T798I mutation (the analogous site) in HER2. The L755S and T798I amino acid substitutions most likely lead to steric hindrance of the lapatinib-receptor interaction and diminish the structural flexibility of the drug binding site. As a result, the inactive conformation of the kinase domain required for lapatinib binding becomes energetically unfavorable and, thus, is less likely to form. Interestingly, EXEL-7647, an experimental TKI that targets both inactive and active receptor confirmations, potently inhibits receptor function and downstream signaling by wild type but also mutant forms of HER2 [146].

Activation of Alternative Receptors

There is a large body of evidence indicating that alternative receptor pathways are switched on to compensate for lapatinib's inhibitory effect on HER2 signaling. The most salient of these are discussed here.

HER1 and HER3

As with trastuzumab, the most effective means of compensating for HER2 inhibition by lapatinib most likely comes from the HER family of receptors themselves and several lines of evidence support this idea. Apart from the receptors themselves, over-expression of their respective ligands can mediate lapatinib resistance. Using a comprehensive set of protein- and DNA-based methodologies, one study showed that lapatinib-resistant cells have up-regulated expression of the HER3 ligand heregulin (HRG) and that this functions in an autocrine fashion to stimulate signal transduction [147]. The observation in this system is that HER2 is inhibited by lapatinib, but HER1 phosphorylation is only partially inhibited in the lapatinib-resistant cells. This slightly sustained HER1 signaling is apparently sufficient to detour signaling to a HRG-driven HER1/HER3 pathway. Gefitinib and erlotinib, two HER1-specific TKIs, cannot overcome HRG-HER3-mediated activation of HER1, nor reverse lapatinib resistance in this model system, whereas neratinib, an irreversible pan-HER TKI (see below), can overcome lapatinib resistance [147]. This same report suggests the clinical relevance of HRG expression. In a study of 204 $HER2^+$ breast cancers, HRG mRNA levels were found to correlate significantly with risk of recurrence ($p = 0.0036$) and there is a statistically significant association between high HRG expression and decreased DFS. High HRG expressers have a median DFS of 2.84 years and intermediate + low expressers have a median DFS of 10.04 years ($p = 0.0034$) [147].

Other HER ligands have been implicated in lapatinib resistance. For example, exposing SK-Br-3 cells to transforming growth factor-α (TGF-α) increases phosphorylation of HER1 and its downstream targets and decreases the sensitivity of these cells to lapatinib [148]. Notably, high serum levels of TGF-α and amphiregulin, another HER1 ligand, predict poor response to gefitinib in patients with advanced non-small cell lung cancer [149]. Thus, it is possible that high TGF-α levels could also associate with poorer outcome in breast cancer patients treated with lapatinib. Indeed, in a study of 64 patients treated with lapatinib + capecitabine, the response rate was found to be higher in individuals with low serum TGF-α compared to those with high levels ($p = 0.001$). There was a trend towards shorter time-to-progression in patients with high serum TGF-α compared to low TGF-α ($p = 0.067$) [148].

Other means of activating HER1 have also been implicated in resistance. For example, heparanase has been suggested to modulate lapatinib efficacy by promoting signaling through HER1. Heparanase is an endoglycosidase that cleaves heparin sulfate to biologically active fragments that promote proliferation or

angiogenesis, and this activity has been implicated in metastatic potential, mostly to the brain [150, 151]. Heparanase can also affect HER1 phosphorylation by a mechanism that appears to be independent from its enzymatic activity [152], suggesting that heparanase could play a role in lapatinib resistance. Such a role was demonstrated using a potent inhibitor of heparanase activity, Roneparstat, and a cell line that over-expresses HER1 and HER2 and was selected for lapatinib resistance. These cells have elevated heparanase levels, activity and secretion, compared with lapatinib-sensitive parental cells, and elevated signaling through HER1, FAK and ERK1/2 pathways, even in the presence of lapatinib. Roneparstat inhibits HER1 phosphorylation and downstream signaling through FAK and ERK1/2, thereby reversing the lapatinib resistance phenotype, both *in vitro* and in mice [153]. This study again underlines the importance of alternative signaling/survival pathways that cancer cells use to withstand HER2 or HER1 inhibition by lapatinib. The clinical significance of heparanase has been determined and a variety of heparanase inhibitors are being studied as therapeutic agents [150, 151], but a link between heparanase and clinical lapatinib resistance has not yet been established.

MET and AXL

MET, described earlier in the context of trastuzumab resistance, also appears to affect response to lapatinib. MET's ability to mediate lapatinib resistance was demonstrated in gastric cancer cells. Exposure of these cells to HGF induces MET phosphorylation and leads to ERK and AKT signaling and lapatinib resistance, whereas down-regulation or selective inhibition of MET re-sensitizes resistant cells to lapatinib [154]. Although these data pertain to $HER2^+$ gastric cancer cells, it seems probable that the same mechanism of lapatinib resistance also occurs in breast cancer, where MET and HER2 are frequently co-expressed and appear to compensate for each other in activating downstream signaling [155].

AXL is an RTK that exhibits tumorigenic potential, most likely related to its kinase domain that can be activated independent of ligand binding by simple over-expression [156–158]. AXL over-expression is associated with poor prognosis of numerous human cancers including tumors of the breast, colon, esophagus, thyroid, ovaries, stomach, kidney, brain, or lung [159]. Increased AXL expression might potentially play a role in resistance to a c-KIT TKI, imatinib, in gastrointestinal stromal tumors that express c-KIT [160] and in chemotherapy resistance in acute myeloid leukemia [161], lung cancer [162] and ovarian cancer [163]. In addition, when $HER2^+$ breast cancer cells (BT474) are exposed to lapatinib for long periods of time, surviving lapatinib-resistant subclones significantly over-express AXL. Subsequent inhibition of AXL expression by RNAi or function by treatment with foretinib (a multi-kinase inhibitor of AXL, MET, and VEGFR) restores lapatinib sensitivity in this model, whereas more specific MET and VEGFR inhibitors do not [159]. Interestingly, AXL expression can also be diminished and lapatinib sensitivity restored when cells are deprived of estrogen or treated with the

estrogen receptor (ER) antagonist fulvestrant, indicating that the ER pathway stimulates AXL expression and in turn promotes lapatinib resistance. Indeed, up-regulation or enhancement of the ER pathway is considered a redundant survival mechanism contributing to lapatinib resistance [165] (see below).

Modification to Downstream Effectors

PI3K and PTEN

Mutations in downstream mediators of HER2 signaling can play a significant role in lapatinib resistance. For instance, PTEN loss and/or *PIK3CA* mutations not only mediate trastuzumab resistance, as described earlier, but they also cause lapatinib resistance. The E545K and H1047R amino acid substitutions in PI3K have repeatedly been associated with lapatinib resistance, both *in vitro* and in animal models [166, 167]. Notably, expression of the H1047R mutant markedly up-regulates HRG, discussed earlier as a HER3 ligand that contributes to lapatinib resistance, and corresponding cells have elevated phospho-HER3 levels [168]. However, such cells actually maintain cell growth and AKT activation through a pathway that does not completely depend on HER3 [168]. Only combined inhibition of PI3K and HER2 with BEZ235 (see Section "Implications for New Targets and Drug Combinations") and lapatinib, respectively, completely inhibits growth of cells with PTEN deficiency or *PIK3CA* mutation [166, 168].

The role of increased PI3K activity in lapatinib's clinical efficacy is not entirely clear. On the one hand, activation of the PI3K pathway (through activating *PIK3CA* mutations or PTEN loss) was found to be associated with lower lapatinib efficacy in patients with metastatic breast cancer treated with lapatinib + capecitabine. Clinical benefit rate was 36% and OR rate was 9% in patients with PI3K activation (n = 22), compared to 61% and 31%, respectively, in PI3K non-activated tumors (n = 35) [164]. On the other hand, PTEN status was not associated with response in a phase II trial of lapatinib monotherapy in patients with recurrent $HER2^+$ inflammatory breast cancer [169, 170]. The potential involvement of PI3K activation in lapatinib response has nevertheless prompted the idea that combined inhibition of PI3K and HER2 could be a preferred approach against cancers that contain both *HER2* gene amplification and *PIK3CA* activating mutations (see Section 5).

mTOR, the primary downstream mediator of PI3K/AKT signaling, can be a marker of enhanced signaling through this pathway due to alternative RTK activation or PI3K/PTEN mutation, and mTOR thereby becomes a potential target for overcoming HER2-targeted resistance (see Section 5). But there also appears to be a role for mTOR in lapatinib resistance that is independent of upstream modulators of mTOR activity. This was shown originally in a SK-Br-3 cell line selected for lapatinib resistance [171] and recently confirmed in an AU565 cell lines selected for lapatinib resistance [172], and it may also be the case in a MCF7-based model of acquired lapatinib resistance [173]. The resistant SK-Br-3 and AU565 cells show sustained activation of mTOR that does not depend on signaling through upstream

RTKs or PI3K/AKT, or modulation of other known PI3K/AKT-independent regulators of mTOR activity (Erk, IKKβ, AMPK, RHEB, GSK-3β, and PRAS40, among others). Importantly, lapatinib resistance in these systems is reversible with pharmacological inhibitors of mTOR [171, 172]. The mechanism by which mTOR is constitutively activated in these lapatinib-resistant cells is not known but it does not appear to involve mutation of mTOR or its known regulators [172].

Src

In several examples of breast cancer cell lines selected for lapatinib resistance, HER2 auto-phosphorylation is inhibited by lapatinib but considerable signaling via the PI3K/AKT axis is nevertheless sustained through a mechanism that involves up-regulated Src and Src family kinases [174, 175]. In such cells, Src inhibition by saracatinib abolishes PI3K/AKT signaling and re-sensitizes cells and their respective tumor xenografts to lapatinib. The importance of Src for lapatinib resistance has been confirmed in breast cancer and esophageal cancer cell lines and xenografts [176, 177]. On a molecular level, certain activating mutations in Src can mediate lapatinib resistance. In one model system, breast cancer cells selected for lapatinib resistance carry an E527K mutation in Src and ectopic expression of this mutant is sufficient to confer lapatinib resistance in previously lapatinib-sensitive cells [178].

Besides constitutive activation through mutation, Src can be activated via signals from integrin proteins such as β1-integrin [179], a mechanoreceptor that promotes breast cancer initiation and progression and may be coupled to the HER1/HER2 pathway when breast cancer cells are grown in 3-dimensional systems [180]. A role for β1-integrin in lapatinib resistance was demonstrated by diminishing β1-integrin activity (anti-β1 antibody or RNAi), with subsequent restoration of lapatinib efficacy, although a mechanistic connection to Src was not made in this study [181].

Ras

The Ras family of small GTP binding proteins represents another well-known mechanism of malignant transformation that might also mediate lapatinib resistance. Mutations in Ras can affect its GTP binding property and thus lead to constitutively active Ras proteins, eventually leading to tumorigenesis through activation of MEK and ERK (see [182]). Although Ras mutations are rarely seen in breast cancer, elevated Ras signaling is frequently observed in $HER2^+$ breast cancers [183, 184]. Ras might be a mediator of lapatinib resistance given the high likelihood that the MEK/ERK axis is an alternative signaling pathway when HER2/PI3K/AKT signaling is disrupted [86]. Over-expression of either a wild type H-Ras or an oncogenic H-Ras allele (Ras G12 V) is sufficient to reduce lapatinib efficacy in two different $HER2^+$ cell lines and resistance can be reversed by a MEK inhibitor (U0126) [185].

Estrogen Receptor

Signaling through the ER pathway as a mechanism of acquired lapatinib resistance has been observed both in tissue culture and in mice. Unlike trastuzumab, lapatinib causes up-regulation of ER and progesterone receptor (PR) expression and activity. In breast cancer cell lines (UACC-812 and BT474), ER mRNA levels are increased 6-fold and PR levels are increased 15-fold by lapatinib. This up-regulation is also reflected at the protein level. Fulvestrant, an ER-targeted agent that blocks estrogen binding and causes ER degradation, accordingly suppresses elevated ER levels induced by lapatinib and re-sensitizes lapatinib-resistant cells and xenografts to lapatinib. Alternative approaches to deplete cells of estrogen (estrogen-free cell culture media) confirm ER signaling as a mediator of de novo or acquired lapatinib resistance [159, 186].

Molecularly, the mechanism by which ER signaling can lead to lapatinib resistance has not been directly demonstrated, but it seems reasonable to suggest that this occurs through ER's effect on genes that promote the cell cycle, proliferation and anti-apoptosis [187]. In BT474 cells, 24-hour exposure to lapatinib leads to increased ER signaling (increased levels of ER-target genes such as PR and bcl-2) that appears to be mediated by induction of FOXO3a (a transcription factor that promotes ER expression and is inactivated by PI3K/AKT signaling) in response to the acute inhibitory effect of lapatinib on PI3K/AKT activity [165]. These same molecular changes (increased FOXO3a and enhanced ER signaling activity) are seen in BT474 cells selected for lapatinib resistance and in early-stage breast tumors after 14 days of neoadjuvant lapatinib therapy [165].

Hormone receptor status (ER and PR) seems to be of general clinical relevance in the context of lapatinib response. In a retrospective analysis of $HER2^+$ metastatic breast cancer patients treated with lapatinib + paclitaxel, event-free survival in women with $HER2^+$ and concurrently ER^+/PR^+ tumors was only 5.7 months, compared to a previous study showing 8.3 months for women with $HER2^+$ but ER^-/PR^- tumors [188]. Moreover, the pCR rate during neoadjuvant treatment with lapatinib is clearly lower in patients with ER^+/PR^+ cancers (16.1%) than in women with ER^-/PR^- tumors (33.7%). This does not seem to be restricted to lapatinib, as the same negative influence of ER/PR expression is seen with trastuzumab alone and with the combination of trastuzumab + lapatinib [189].

Pharmacological Factors of Resistance

Several clinical pharmacological factors can also contribute to ineffectiveness of HER2-targeting small molecules such as lapatinib. Although the exposure-response relationship for lapatinib is not entirely clear, preliminary data from clinical trials suggest that there is a threshold level for therapeutic efficacy. In a phase I dose-escalation study evaluating women with advanced $HER2^+$ breast cancer, a relationship between lapatinib plasma concentration and clinical response or biological activity (transient tumor reduction during lapatinib therapy) was reported [145]. The

most striking responses were seen in patients who achieve a maximum lapatinib plasma concentration (C_{max}) approaching 10,000 ng/ml, whereas patients with lapatinib C_{max} levels of 3500 ng/ml all had progressive disease at 2 months after the start of treatment [145]. This suggests a dose-response relationship, although definite conclusions cannot be drawn because C_{max} does not reliably indicate drug exposure. A trial evaluating heavily pretreated patients with $HER1^+$ and/or $HER2^+$ metastatic cancers (breast, colorectal, head and neck, lung, and ovarian cancers) demonstrated that steady-state trough levels (C_{min}) of lapatinib below 1000 ng/ml are associated with non-response [190], again suggesting a threshold level of lapatinib exposure that needs to be met for minimum clinical efficacy and consistent with the idea that ineffectiveness can arise from under-exposure (plasma concentrations below efficient levels). The factors that might limit plasma concentrations are discussed next.

Non-adherence

Non-adherence to orally administered anti-cancer drugs is frequently observed and well documented. Non-adherence occurs when drug doses are skipped, additional doses are taken, or doses are taken in the wrong quantity or at the wrong time [191]. Incidence of non-adherence among patients taking orally administered anti-cancer drugs range from 0% to 84% [191, 192], depending on the definition of non-adherence, the tool used to measure non-adherence, and the type of therapy (therapy complexity; patterns and kind of adverse drug effects).

Although studies evaluating non-adherence and associated outcomes among breast cancer patients on lapatinib have not been reported, some indication of the importance of non-adherence for efficacy of the general class of TKIs comes from trials with chronic myeloid leukemia (CML) patients given imatinib [193]. For instance, in a study of 87 patients with chronic-phase CML treated with imatinib for 6 years, adherence rates monitored during a three-month period significantly ($p < 0.001$) correlated with the 6-year probability of a 1000-fold reduction in BCR-ABL (imatinib target) transcripts. Such a reduction is considered to be a major molecular response and an important predictor of overall survival. Multivariate analysis additionally identified adherence as an independent predictor for major molecular response (relative risk, 11.7; $p = 0.001$). Moreover, no molecular responses were observed when adherence was below 80% ($p < 0.001$). It was concluded that in patients with CML treated with imatinib, poor adherence might be the predominant reason for inability to obtain adequate molecular responses [194]. Educational or behavioral approaches are recommended to ensure high therapy adherence of patients treated with oral TKIs [191].

Pharmacokinetics

Pharmacokinetic disturbances can also lead to underexposure and thus potentially mediate clinical resistance to TKIs. TKIs show high inter-individual variability in pharmacokinetics and are expected to exhibit considerable pre-systemic clearance

(first-pass effect) and, thus, poor oral bioavailability [195, 196]. Data on absolute bioavailability is scarce, however, given the lack of drug formulations suitable for intravenous delivery for most TKIs. All TKIs (including lapatinib) are extensively metabolized by the cytochrome P-450 isoenzyme 3A4 (CYP3A4) [197]. Given the high propensity for CYP3A4 to be induced or inhibited by co-medications, drug-drug interactions with concomitantly administered therapeutics or over-the-counter drugs are very likely. Since under-exposure is most relevant for clinical non-responsiveness, drug-drug interactions with inducers of lapatinib disposition are highlighted here. Anti-epileptic drugs substantially lower exposure to lapatinib. For example, when patients with recurrent glioblastoma multiforme are treated with lapatinib, its apparent oral clearance increases by about ten-fold when patients are also treated with CYP3A4-inducing anti-epileptics such as phenytoin or carbamazepine [198]. The latter has been confirmed to lower lapatinib exposure in a study examining the effect of carbamazepine titrated up to 200 mg (BID) over 20 days on a single 250 mg dose of lapatinib. Carbamazepine decreases lapatinib area under the time-concentration curve (AUC) by 72%, C_{max} by 59% and absorption rate by 28%, but has no effect on drug half-life [199]. This suggests that the major site of interaction is the intestine, with only minor effects on hepatic drug metabolism. A likely explanation is that carbamazepine also induces P-glycoprotein, the product of the *MDR*1 gene that is a drug efflux pump and a known transporter of lapatinib [200]. Assuming dose-linear pharmacokinetics, the magnitude of carbamazepine-lapatinib interaction (72% AUC decrease) might also be critical when clinical doses of lapatinib (>1250 mg per day) are administered.

The aqueous solubility of lapatinib declines when pH is >4 [201, 202], suggesting that acid-reducing drugs might also lower absorption, exposure and subsequent efficacy of lapatinib. When women with metastatic $HER2^+$ breast cancer are treated with 1250 mg lapatinib once daily in the morning with or without esomeprazole (a proton pump inhibitor) before bed time, AUC of lapatinib is significantly decreased by 26% (ranging from 6% to 49%) in the patients receiving esomeprazole [202]. Although the magnitude of this effect might have only minor clinical relevance, the time point of dosing should be considered when co-administration of acid-reducing drugs is needed. For example, concurrent dosing might avoid this interaction because the stomach (where the drugs are most likely to interact) has a pH <4, where lapatinib is most soluble.

Herbal drugs are commonly consumed by cancer patients to increase quality of life or to manage adverse effects of therapy. A survey of 2000 cancer patients reported that 49% of breast cancer patients consume herbal drugs [203]. The most relevant herbal drug for lapatinib is St. John's Wort, which has substantial impact on CYP3A4- and CYP2C9-mediated metabolism and P-glycoprotein-mediated drug transport. St. John's Wort can lower exposure of co-administered drugs by up to 70%. A considerable interaction between St. John's Wort and imatinib has been demonstrated, and interactions with other TKIs are likely, but current evidence is limited [204].

Given the high inter-individual variability of TKI pharmacokinetics and the suggested concentration threshold for efficacy, adjustment of drug dosing accord-

ing to therapeutic drug monitoring (TDM) leading to optimization of lapatinib pharmacotherapy seems appropriate in some circumstances. Indeed, TDM has been suggested for a subset of TKIs, albeit with different levels of evidence supporting its routine clinical application. Although there is good evidence for the meaningful role of imatinib TDM, for other TKIs, including lapatinib, data is insufficient to incorporate TDM into clinical routine. The use of TDM during targeted therapy regimens might best be reserved for situations pertaining to lack of therapeutic response, unexpected toxicity, anticipated drug-drug interactions or concerns over treatment adherence. In the future, concentration–effect relationships should be evaluated in more detail. For example, performing randomized trials comparing classic dosing with pharmacokinetics-guided adaptive dosing will help to establish target plasma concentrations and eventually individualize pharmacotherapy to maximize efficacy and prevent toxicity [205].

Other Small Molecule Inhibitors

Because significant mechanisms of TKI resistance arise from pathway switching and over-expression of targeted receptors and their ligands (leading to autoactivation), a long-lasting inhibition of the RTK kinase activity is desired. Irreversible TKIs are considered advantageous compared to reversible inhibitors in this regard [206]. Several irreversible inhibitors of HER2 are currently under investigation for their clinical efficacy, although little information is so far available on mechanisms of resistance to these newer agents. Following is a summary of findings with three irreversible inhibitors that have particular relevance to HER2$^+$ breast cancer.

Neratinib

Neratinib (HKI-272, Puma Biotechnology) is an irreversible, pan-HER (HER1, −2, −4) TKI [207–209]. In pre-clinical studies, neratinib was shown to overcome trastuzumab resistance, both in cell culture and in xenograft models [210]. In the clinic, neratinib has shown substantial activity in patients with advanced HER2$^+$ breast cancer (78% 16-week PFS rate, median PFS of 39.6 weeks, OR rate of 56% in a cohort of 64 patients who had not received prior trastuzumab therapy). Response rates are lower for patients who previously received trastuzumab (16-week PFS of 59%, median PFS of 22.3 months, 24% OR rate among a cohort of 63 patients) [211]. This perhaps suggests a trastuzumab resistance mechanism in these patients that is downstream of HER2 and thus shared by neratinib. This low response was also evident in a phase II randomized trial of HER2$^+$ breast cancer patients with locally advanced or metastatic disease and prior trastuzumab therapy that compared lapatinib + capecitabine (n = 116) with neratinib (n = 117) as second-line therapy. Median OS for the neratinib arm was 19.7 months vs. 23.6 months for the lapatinib + capecitabine arm and clinical benefit rate was lower with neratinib (44% versus 64%; p = 0.003) [212]. In a phase III trial comparing neratinib

(n = 1420) with placebo (n = 1420) in patients with HER2$^+$ breast cancer who had previously received neoadjuvant or adjuvant trastuzumab, neratinib was associated with improved two-year invasive DFS (HR = 0.67, p = 0.0091). There was a greater benefit to patients with ER$^+$/PR$^+$ tumors (HR = 0.51, p = 0.0013 relative to placebo) than patients with ER$^-$/PR$^-$ tumors (HR = 0.93, p = 0.74) ($p_{interaction}$ = 0.054) [213]. OS data from this trial have not yet been reported.

Canertinib

Canertinib (CI-1033, Pfizer) is a 4-anilinoquinazoline that irreversibly inhibits HER1, HER2 and HER4 through interaction with the ATP binding site of the respective receptor kinase domains, leading to inhibition of receptor auto-phosphorylation when cell lines are stimulated with EGF (HER1) or HRG (HER2, HER3, HER4) [214–216]. Canertinib inhibits tumor growth in xenograft models and it was the first pan-HER inhibitor to undergo clinical trial. Canertinib resistance appears to occur by sustained PI3K/AKT/mTOR signaling, with the dual PI3K/mTOR inhibitor BEZ235 able to overcome the resistance phenotype [217]. In a randomized, phase II, dose-finding study of patients with measurable, progressive or recurrent metastatic breast cancer, HER2$^+$ patients who received 450 mg canertinib once daily for 14 days every 21 days had a response rate of 18.8% and one-year OS rate of 86.7%. The drug was well tolerated only at the 50 mg dose, however, thus potentially limiting the clinical utility of this drug [218].

Afatinib

Afatinib (BIBW-2992, Boehringer Ingelheim) also binds covalently to HER1, HER2 and HER4 and inhibits signaling through all HER-family homodimers and heterodimers [86, 216, 219]. Afatinib is currently approved for first-line treatment of metastatic non-small cell lung carcinomas that contain HER1 mutations known to sensitize the receptor to TKI inhibition [219]. In the HER2$^+$ breast cancer setting, afatinib has shown modest clinical benefit in phase II studies of women with metastatic disease who had progressed during or after trastuzumab and/or lapatinib therapy [220, 221]. The DAFNE phase II trial looked at neoadjuvant afatinib + trastuzumab + conventional chemotherapy in previously untreated HER2$^+$ patients (n = 65) [222]. The overall pCR rate was 49.2%, below the target rate of 55% that would have advanced the regimen to phase III trials. ER$^-$/PR$^-$ patients (n = 19; pCR = 63.2%) performed slightly better than ER$^+$/PR$^+$ patients (n = 46; pCR = 43.5%), a difference that did not achieve statistical significance (p = 0.153), and there was no statistical difference between *PIK3CA*-wild type (n = 48; pCR = 54.2%) and *PIK3CA*-mutant (n = 13; pCR = 38.5%) patients (p = 0.363). Statistical significance in these cases might have been compromised by the small number of patients in the study. In a smaller neoadjuvant trial comparing afatinib (n = 10) to trastuzumab (n = 11) and lapatinib (n = 8), the objective response was seen in 8 of the afatinib-treated patients, comparable to lapatinib (6 objective

responses) and better than trastuzumab (4 objective responses), but the study was terminated early due to slow enrollment [223]. Finally, in a phase III study of patients with HER2$^+$ metastatic breast cancer who had progressed on trastuzumab (n = 508), afatinib + vinorelbine (a tubulin-targeted drug) did not improve PFS or OS and was less tolerable than trastuzumab + vinorelbine [224]. Hormone receptor status did not affect outcomes in either treatment arm.

Implications for New Targets and Drug Combinations

Understanding mechanisms of resistance raises the possibility of using corresponding mechanism-based inhibitors either to prevent the initial development of resistance or to restore HER2-targeted drug efficacy once resistance has emerged. Only a relatively small number of such mechanism-based inhibitors have made it into clinical phases of development and most of these newer agents are being tested in combination with trastuzumab, either as front-line combination therapy or in patients who have progressed from prior trastuzumab therapy. This is because of pre-clinical and clinical evidence that trastuzumab-resistant cell lines and tumors continue to be dependent on HER2 signaling and that combining trastuzumab with other targeted agents is clinically beneficial in patients who have progressed on trastuzumab [35, 225–228]. The possibility of combining three or more targeted agents is also being explored. The following sections describe the mechanism-based targets and corresponding drugs that are the most promising in the setting of HER2$^+$ breast cancer. Several other proteins discussed earlier as resistance mechanisms are not included in this section either because corresponding drugs have not yet advanced clinically or because such drugs have not shown enough clinical benefit to warrant further development for treating HER2$^+$ breast cancer.

Targeting Alternative Receptors

HER1 and HER3

Pan-specific agents that target HER2 plus HER1 and/or HER3 have been discussed earlier in the context of possible resistance mechanisms pertaining to these multi-receptor agents. The trials testing combinations of such drugs are too numerous to summarize in the current chapter, but the predominant drugs to date have been pertuzumab and lapatinib. Pertuzumab showed clear clinical benefit when added to trastuzumab, both in patients who had progressed on prior trastuzumab and in trastuzumab-naïve patients [101, 103, 104], leading to FDA approval for use in combination with trastuzumab to treat HER2$^+$ breast cancer in the neoadjuvant and metastatic settings. Likewise, lapatinib + trastuzumab was shown to be beneficial in patients who had progressed on trastuzumab [189, 225, 226] and it is notably effective in patients with p95HER2, a key resistance factor for trastuzumab [229, 230].

Lapatinib, in combination with capecitabine, was approved in 2007 for the treatment of advanced HER2+ breast cancer.

IGF-1R

IGF-1R can confer trastuzumab resistance but it does not appear to be involved in lapatinib resistance. Recent reviews of development and clinical trials of IGF-1R inhibitors can be found in [231–235]. These inhibitors include monoclonal antibodies and TKIs, the most notable of which in the context of breast cancer and trastuzumab resistance are cixutumumab (IMC-A12, ImClone) and BMS-754807 (Bristol-Myers Squibb). Cixutumumab is a monoclonal antibody with high selectivity and affinity for IGF-1R. It has undergone phase II clinical trial (NCT00684983) in combination with capecitabine + lapatinib in HER2+ metastatic breast cancer patients who had progressed on trastuzumab-based regimens [235]. BMS-754807 is a selective, non ATP-competitive IGF-1R TKI that shows strong synergy with trastuzumab in inhibiting proliferation of a HER2+ breast cancer cell line and with lapatinib in inhibiting a lung cancer cell line [236]. A phase I/II trial (NCT00788333) evaluated BMS-754807 in combination with trastuzumab in patients with HER2+ metastatic breast cancer. Results from NCT00684983 and NCT00788333 are still pending.

A newer TKI, KW-2450 (Kyowa Kakko Kirin Co. Ltd.), inhibits IGF-1R and insulin receptor kinases with similar efficacy *in vitro* [237]. It showed promise in an initial phase I trial (NCT00921336), with four of 10 evaluable patients having stable disease as best response [238]. However, a follow-on phase I/II trial (NCT01199367) to evaluate KW-2450 in combination with lapatinib + letrozole in patients with advanced or metastatic HER2+ breast cancer was terminated in phase I because a well-tolerated dose permitting further phase II study was not identified.

MET, AXL, VEGFR

MET, AXL and VEGFR are RTKs that have all been implicated in trastuzumab and/or lapatinib resistance, as described earlier. Foretinib (GSK1363089, GlaxoSmithKlein), which inhibits all three receptors, has proven effective *in vitro* (see above) [159]. In phase I and II trials, efficacy was highly variable, ranging from stable disease as best response [239, 240] to 43% of head and neck cancer patients experiencing tumor shrinkage [241]. Foretinib was considered to be of potential clinical value, especially when combined with other (HER-targeting) drugs [241]. Pazopanib, another multi-kinase inhibitor that targets VEGFR, PDGFR and FGFR, has shown clinical activity in combination with lapatinib [242]. In this study, patients with HER2+ breast cancer who had not previously received trastuzumab or chemotherapy were treated with pazopanib + lapatinib (n = 69) or lapatinib alone (n = 72). The 12-week response rate was 36.2% for the combination treatment arm vs. 22.2% for the lapatinib treatment arm but the week-12 progressive disease rates, PFS and OS rates were not statistically different between the two arms [242].

In an attempt to target the VEGFR pathway more specifically, several clinical trials have evaluated the efficacy of bevacizumab, a VEGF-targeted monoclonal antibody, in combination with lapatinib or trastuzumab. A phase II study of lapatinib + bevacizumab in HER2⁺ metastatic breast cancer patients (n = 52, 90% of whom had received prior trastuzumab therapy) found a 12-week PFS rate of 69.2% and median PFS of 24.7 weeks. The conclusion from this trial was that lapatinib + bevacizumab is active in patients with HER2⁺ breast tumors [243]. Efficacy of lapatinib + bevacizumab was even higher when combined with trastuzumab: half of the 23 patients in the study showed complete response, partial response or stable disease for more than 6 months. This relatively high rate of stable disease was not negatively influenced by prior treatment with any of the study drugs [64].

AVEREL (NCT00391092) is a large (n = 424) phase III trial testing the addition of bevacizumab to first-line trastuzumab + docetaxel therapy in patients with locally recurrent or metastatic HER2⁺ breast cancer who had not received prior therapy for their metastatic disease. At a median follow-up of 26 months, there was a trend towards longer PFS (16.5 months in the bevacizumab arm vs. 13.7 months in the non-bevacizumab arm; HR = 0.82) that did not reach statistical significance (p = 0.0775) [244]. Further analysis of this trial's outcomes is pending.

Targeting Downstream Effectors

PI3K/AKT/ mTOR

As described earlier, the PI3K/AKT pathway is an attractive target to inhibit cancer cell growth or to restore efficacy in HER2⁺ breast cancers that have become resistant to HER2-targeted drugs. mTOR is the primary downstream effector of PI3K/AKT signaling and is thought to be a key target for short-circuiting multiple mechanisms of HER2-related drug resistance [245–248]. mTOR is the catalytic subunit of mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2), both of which act downstream of PI3K/AKT to regulate cell growth. mTORC1, the target of the original mTOR inhibitor known as rapamycin, phosphorylates S6 K1 ribosomal protein and 4EBP1 translation initiation factor to promote mRNA translation. mTORC2 phosphorylates AKT and controls the cytoskeletal network, and it is critical for cancer survival and progression [249–252]. mTORC1 and mTORC2 are both valid targets in the context of breast cancer and HER2-based therapies [253]. Inhibitors of PI3K, AKT, mTOR and dual PI3K/mTOR inhibitors have all been explored clinically and are discussed here in the context of HER2⁺ breast cancer.

mTOR Inhibitors

The most advanced mTOR-targeted drug is everolimus (RAD001, Novartis Pharmaceuticals), which functions as an allosteric inhibitor of mTOR in the context of mTORC1. Everolimus inhibits the growth of HER2⁺ breast cancer cell lines and

enhances the effectiveness of trastuzumab and lapatinib in cell lines and human xenografts in mice [246, 247]. Two phase III clinical trials, Bolero-1 and Bolero-3, have demonstrated clear clinical benefit of combining everolimus with trastuzumab, most particularly for patients with ER^-/PR^- tumors. In Bolero-3 (n = 569) the addition of everolimus to trastuzumab + vinorelbine prolongs PFS in patients with trastuzumab-resistant advanced $HER2^+$ breast cancer [254]. Median PFS in the everolimus group was 7.00 months compared with 5.78 months in the placebo group (HR = 0.78; p = 0.0067). The benefit is seen in ER^+/PR^+ and ER^-/PR^- patients but was more pronounced in the ER^-/PR^- group (HR = 0.65 in the ER^-/PR^- group; HR = 0.93 in the ER^+/PR^+ group). Bolero-1 (n = 719) studied the effect of adding everolimus (vs. placebo) to trastuzumab + paclitaxel as first-line treatment for advanced $HER2^+$ breast cancer [255]. The addition of everolimus had only a modest effect on PFS in the entire population of patients (median PFS of 14.95 months in the everolimus group vs. 14.49 months in the placebo group) that was not statistically significant (HR = 0.89; p = 0.1166). The benefit of everolimus was much more profound in the ER^-/PR^- sub-population -- median PFS of 20.27 months for the everolimus group versus 13.08 months for the placebo group (HR = 0.66; p = 0.0049). Taken together, the findings from these two studies suggest that mTOR is most important as a mechanism of trastuzumab resistance when the ER/PR pathway is not available as an alternative signaling pathway, and that the use of mTOR-targeted drugs should be confined to this group of patients.

There are several newer mTOR inhibitors that compete with ATP binding to mTOR and thereby inhibit both mTORC1 and mTORC2. These include AZD8055 (AstraZeneca), AZD2014 (AstraZeneca), TAK-228 (developed by Millenium, also known as MLN01 and INK-228), CC-223 (Celgene), and the recently described MTI-31 (Chinese Academy of Sciences), some of which have been shown to restore sensitivity to trastuzumab or lapatinib in cell line models of resistance [172, 245]. A phase I clinical trial of CC-223 identified PR or stable disease in two of three patients with advanced breast cancer (unknown HER2 status) [256]. Nevertheless, mTOR inhibitors by themselves might be of relatively minor clinical value in $HER2^+$ breast cancer.

PI3K/mTOR Inhibitors

Most PI3K inhibitors are actually dual inhibitors of PI3K and mTOR. The dual inhibition is both for the purpose of short-circuiting multiple mechanisms of PI3K/AKT/mTOR activation and for the apparent need to inhibit feedback up-regulation of PI3K signaling when mTOR is blocked [247, 257–259]. Most of these dual PI3K/mTOR inhibitors are still early in their clinical testing, but we describe several here because of the importance of the PI3K/mTOR pathway and the promise shown for this class of drugs overall. We also include brief discussion of a pan-PI3K inhibitor that is not in the dual PI3K/mTOR class because of its potential relevance to $HER2^+$ breast cancer.

BEZ235 (Novartis Pharmaceuticals) is an early example of a dual PI3K/mTOR inhibitor. Pre-clinical studies with this agent indicated that BEZ235 is a potent inhibitor of breast cancer cell lines across a spectrum of molecular sub-types, including lines with inherent and acquired trastuzumab resistance. Growth inhibition is via growth arrest and apoptosis. The drug also inhibits tumor progression in xenograft models, both as a single agent and even more effectively in combination with trastuzumab [247]. Although several early-phase trials of BEZ235 have been conducted, no results from trials with HER2$^+$ patients have been reported and clinical efficacy with BEZ235 appears to be limited to disease stabilization. Although BEZ235 is considered to be of minor clinical value and is no longer in clinical development, it nevertheless represents an important advance in targeted efforts to manage trastuzumab (and other drug) resistance resulting from PI3K/AKT activation.

SAR245409 (Sanofi) is another PI3K/mTOR inhibitor. When it was combined with erlotinib, an HER1-targeted TKI, in a phase I trial with 46 patients (mostly lung cancer), stable disease (in 37% of patients) was the best response. Analysis of pharmacodynamic markers in serial skin samples showed that the maximum inhibition of PI3K, HER1 and MAPK pathways was only between 37% and 75%. Based on these results, it was speculated that other dosing regimens leading to higher systemic drug exposure might be needed to achieve better clinical efficacy [260].

PKI-587 (PF-05212384, Pfizer) is a dual PI3K/mTOR inhibitor that was evaluated in a phase I trial with 77 solid tumor patients, including four with breast cancer [261]. Treatment with PKI-587 lowered pAKT levels in post-treatment biopsies compared with paired pre-treatment biopsies, as well as other PI3K/mTOR pathway markers to varying degrees. Two patients experienced objective PR and 27 exhibited stable disease, with eight patients having stable disease for >6 months. PKI-587 is currently under investigation in phase II trials in endometrial and non-small cell lung cancer patients and should perhaps be tested in breast cancer settings as well.

Buparlisib (BKM120, Novartis Pharmaceuticals) is a pan-class 1A PI3K inhibitor that was combined with carboplatin + paclitaxel in a phase I trial that included patients with solid tumors [262]. Of 25 patients with measurable disease, five exhibited objective responses (one CR, four PR) and the three patients with known loss of PTEN expression all benefited from the treatment. Dose-limiting toxicities included paclitaxel-typical uncomplicated neutropenia. It was concluded from this trial that addition of buparlisib to carboplatin + paclitaxel is safe and might increase the efficacy of this cytotoxic combination therapy, especially in tumors with loss of PTEN expression. NeoPHOEBE is a phase II trial (NCT01816594) investigating the effect of adding buparlisib to trastuzumab + paclitaxel for the neoadjuvant treatment of HER2$^+$ primary breast cancer. Results are still pending.

AKT Inhibitors

AKT is a serine-threonine kinase that lies downstream of PI3K and upstream of mTORC1. AKT phosphorylation results in a cascade of downstream molecular events that ultimately result in cell proliferation and survival, and the turning off

of AKT phosphorylation is an essential element of effective HER2 inhibition mediated by the HER2-targeted drugs described in this chapter. Initial enthusiasm for development of AKT-targeted drugs was perhaps dampened by the discovery that AKT inhibition causes release of a negative feedback loop on expression and phosphorylation of multiple upstream RTKs, including IGF-1R and the HER family of receptors [263, 264]. The end result in the context of $HER2^+$ breast cancer is increased levels of ligand-activated HER1, HER3 and HER4, and increased dimerization with and signaling through HER2. Importantly, trastuzumab and lapatinib are both capable of reversing the feedback effect and the combination of AKT inhibition with HER2 inhibition is effective in reducing the growth of $HER2^+$ cells in culture and as xenografts in mice [263, 264]. Based on this, a few AKT-targeted drugs are in development for use in combination with HER2 targeting.

The most advanced AKT inhibitor is MK-2206 (Merck), which shows potent and selective inhibition of AKT and anti-proliferation activity both *in vitro* and *in vivo* against $HER2^+$ cells and xenografts [265, 266]. Its growth inhibitory effect is enhanced in cells with PTEN loss or *PIK3CA* mutation [266], which perhaps are more dependent on PI3K/AKT signaling for proliferation. Phase I clinical trials tested MK-2206 in combination with trastuzumab [267] or trastuzumab + lapatinib [268] in patients who had progressed on HER2-targeted therapy. Results showed that the combinations are safe and demonstrate some clinical activity in a pre-treated patient population, thus warranting further investigation. Ongoing clinical trials include NCT01042379 (a phase II trial testing MK-2206 with or without trastuzumab, along with several other targeted drugs, using a tumor's molecular profile to assign each patient to a selected protocol) and NCT01277757 (a phase II trial testing MK-2206 in patients with stage III or stage IV breast cancer who have failed prior therapy and have tumors with a *PIK3CA* mutation, an AKT mutation, and/or PTEN loss/PTEN mutation).

ER Pathway

Given the role of ER in lapatinib resistance described earlier, combining anti-HER2 drugs with therapeutics targeting the ER pathway (ER modulators such as tamoxifen or fulvestrant; aromatase inhibitors such as anastrozole, letrozole or exemestane) might be a promising approach in $HER2^+/ER^+$ cancers. In one such trial, adding lapatinib to fulvestrant did not improve PFS or OS, but patients with $ER^+/PR^+/HER2^+$ and $ER^+/PR^+/HER2^-$ tumors were all included in the trial, meaning that outcomes might have been affected by the non-expression of one of the main targets of lapatinib (HER2) [269]. In an earlier trial, letrozole showed a beneficial effect in combination with lapatinib (enhanced PFS and clinical benefit rate) in patients with metastatic breast cancer that is both ER^+/PR^+ and $HER2^+$ [270]. Thus, the combination of lapatinib and letrozole seems to be a rational and clinically effective strategy to improve outcomes in patients with $HER2^+$, ER^+/PR^+ breast cancer and potentially to combat ER-mediated resistance against HER2-directed therapies [271]. Additional discussion of ER pathway inhibitors and resistance can be found in the chapter on "Resistance to Endocrine Therapy".

Concluding Remarks

This review chapter has focused on molecular mechanisms of resistance, but clinical resistance is ultimately a manifestation of a selection process that happens at the cellular level based on the phenotype(s) conferred by an accumulation of molecular changes in a population of malignant cells. In our case, the molecular changes include mutations and gene expression or activity alterations that compensate for the therapy-mediated inactivation of HER2. In a pre-treatment cancer, intratumor heterogeneity -- the varied molecular landscape that results from genomic instability in all cancers -- hinders the efficacy of therapeutics that are targeted to a single oncogenic driver (such as HER2). Even when a targeted therapy is initially effective, a subset of cells in the population with the greatest growth advantage – due to their particular combination of pre-existing and adaptive molecular changes – will emerge as therapy-resistant disease. This, too, is heterogeneous in nature, due to the multiple molecular mechanisms that can promote survival [272, 273].

Proper analysis of inherent and adaptive heterogeneity will likely be required to develop effective patient-specific therapeutics. To be most effective, such analysis should occur at multiple time points to account for ongoing selection/evolution as patients undergo treatment [273]. This is becoming increasingly feasible with the development of fast and inexpensive sequencing techniques, even at the single cell level. Thus we are poised to make further advances in cancer therapy based on molecular characterizations, a concept now known as precision medicine. Indeed, knowledge about particular molecular mechanisms of resistance described in this chapter – and our ability to detect such mechanisms – has led to the identification of new targets and corresponding new drugs to prevent or counteract resistance in patients with $HER2^+$ breast cancer. Agents like pertuzumab and lapatinib arose from an understanding that HER1 and HER3 are significant compensatory signaling mechanisms that contribute to trastuzumab resistance. Given the central role of common downstream nodes in multiple signaling pathways, proteins such as PI3K/AKT and mTOR represent the next generation of targets for combating resistance.

We still have much to learn about the best combinations to use in the clinic and whether to deploy the combinations concurrently as a means of preventing the emergence of one or more resistance mechanism, or sequentially as a way to overcome acquired resistance. It can be argued that both approaches will be necessary, depending on the molecular details of an individual cancer at the time of diagnosis and the likely mechanisms of resistance that will emerge as an adaptation to first-line therapy. The presence of PI3K/AKT aberrations in a primary tumor, for example, might argue for concurrent treatment with a HER2-targeted drug and a PI3K/AKT/mTOR inhibitor. On the other hand, this first-line approach might simply lead to the emergence of other, unknown resistance mechanisms against which we have no therapeutic options. Given the evolutionary nature of resistance, it has been suggested that it might be more effective to take a sequential approach, in an attempt to anticipate the evolution of a cancer and maintain it in a state that is treatable with available drugs to known adaptive mechanisms of resistance [273]. In this latter

case, it will be important to customize second- and third-line therapies to the molecular features of resistance in each individual and to optimize timing of the sequential deliveries. The solutions to these therapeutic problems will only come from further understanding of mechanisms of drug action and resistance that we discern from laboratory studies and of drugs' clinical activity, tolerability and pharmacokinetic interactions that we discern from clinical trials. The future of precision medicine will thus depend on a better knowledge of the evolution of resistance mechanisms and knowing how to apply that information to the selection and administration of targeted drugs.

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Endocrine Resistance and Breast Cancer Stem Cells: The Inflammatory Connection that Could Lead to New and Improved Therapy Outcomes

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Abstract Breast cancer is the most commonly diagnosed cancer among American women and claims over 40,000 lives each year. Nearly 75% of breast tumors express estrogen receptor (ER) and will be treated with endocrine therapy, such as tamoxifen or aromatase inhibitors. Interfering with ER action via endocrine therapies has been a mainstay of breast cancer treatment for more than a century. But despite its proven success, the onset of endocrine resistance limits its usefulness. It is estimated that up to 50% of ER+ tumors fail to respond to endocrine therapy and eventually recur as aggressive, metastatic cancers. Therapy failure and aggressive tumor phenotypes have been attributed to the presence of a therapy-resistant population of cells within the tumor called breast cancer stem cells (CSCs). Breast CSCs are a small subpopulation of highly tumorigenic cells with stem-like features that sit at the apex of the hierarchy to drive tumor initiation, growth, and progression. Like their normal counterparts, being able to self-renew and differentiate are two hallmarks of CSCs, which in turn drive tumorigenesis, contribute to heterogeneity, and are the seeds of recurrent tumors and distant metastasis. Thereby, targeting breast CSCs will sensitize resistant, aggressive tumors to therapy, and prevent future recurrence and metastasis. Given that the inflammatory nuclear factor κB (NFκB) pathway plays an essential role in regulating breast CSCs, NFκB pathway inhibition can be exploited to eradicate CSCs and potentially overcome endocrine resistance.

Abbreviations

AF1, 2	Function domain 1 and 2
AI	Aromatase inhibitor
ALDH1	Aldehyde dehydrogenase 1

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AP-1	Activator protein 1
CSC	Cancer stem cell
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen response element
HER2	Human epidermal growth factor receptor 2
IGF1R	Insulin-like growth factor receptor
LBD	Ligand-binding domain
NFκB	Nuclear factor kappa B
SERD	Selective estrogen receptor downregulator
SERM	Selective estrogen receptor modulator
SP-1	Specificity protein 1

Introduction

Globally, breast cancer represents a substantial burden in terms of incidence, mortality and economic cost, and unfortunately this burden is on the rise. In the United States it is estimated one in eight women will develop breast cancer in their lifetime. According to the American Cancer Society, in 2016 over 246,000 of new breast cancer cases will be diagnosed, making breast cancer the most common cancer among American women. As a result, this year alone, over 40,000 women will succumb to the disease and die, making breast cancer the second deadliest after lung cancer. Despite ongoing efforts and advances in treating breast cancer, major clinical challenges still remain. Contributing to these challenges is the fact that breast cancer is a heterogeneous disease comprised of different clinical, histopathological, and molecular subtypes. Broadly speaking, breast cancer is classified into four main intrinsic subtypes: luminal A, luminal B, HER2-enriched, and basal-like/triple negative breast cancers [1, 2]. Approximately 75% of breast cancers express estrogen receptor α (ER), which remains the most important prognostic factor in breast cancer. Decades of basic and clinical research have established the fundamental role of ER and its estrogen ligands in normal mammary gland development, but also in the etiology and progression of breast cancer.

ER+ tumors are typically hormone-dependent – they are fueled by estrogens for growth and proliferation. ER expression generally predicts a good outcome, and one reason for that is because ER is an excellent and well-validated therapeutic target, and represents the first example of molecular targeted therapy in cancer. Drugs targeting ER in breast cancer are known as endocrine therapy. ER+ tumors are of luminal subtypes – either luminal A, the more differentiated and endocrine-therapy sensitive, or luminal B, the more aggressive and relatively more endocrine-resistant [1–3]. This points to the fact that breast cancer heterogeneity, even among ER+ tumors, manifests itself also through a heterogeneous response to endocrine therapy. Indeed, despite the well-established effectiveness of endocrine therapies, almost 50% of ER+ of breast cancers fail endocrine therapy, and eventually recur as aggressive, therapy-resistant or metastatic tumors. At this stage therapy options remain limited and this accounts

for the majority of breast cancer-related mortality. Therefore, the development of effective therapeutic strategies for women with ER+ breast tumors that fail endocrine therapy requires a fundamental understanding of the mechanisms and pathways contributing and driving resistance that still remain largely unknown. This overview explores some of the basic principles that have emerged in understanding the rise of endocrine resistance in ER+ tumors, including the likely contribution of breast cancer stem cells (CSCs) via the enhanced pro-inflammatory nuclear factor kappa B (NFκB) signaling. In turn, targeting inflammation and CSCs hold great potential and may represent the next frontier to overcome endocrine resistance.

Estrogen Receptor and Its Signaling Pathways

There are two different forms of estrogen receptors, known as estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Estrogen receptors belong to the nuclear receptor super family that comprises classical steroid receptors. ERα is the first steroid receptor to be discovered by Jensen in 1958 [4], and almost 20 years later another ER, named ERβ, was discovered by Gustafsson [5]. ERα and ERβ are encoded by the *ESR1* and *ESR2* genes respectively, which are located on different chromosomes. Although both ERs act as transcription factors or are capable of initiating extranuclear-initiated kinase signaling cascades, they have tissue specific expressions and have distinct functions. In breast tumors, ERα expression dominates. Instead, the presence of ERβ is reported to modulate the effects of ERα [6]. However, there is conflicting data concerning their relative co-expressions and their associations with established prognostic variables, endocrine responsiveness, or survival. Because of the breast cancer focus, throughout the text ER refers to ERα unless otherwise specified.

ER consists of two transcriptional activation domains: the N-terminal ligand-independent activation function domain (AF-1), and the C-terminal ligand-dependent (AF-2) domain. The ligand-binding domain (LBD) of ER resides in its C-terminal region, and the DNA-binding and hinge domains are positioned in the central core of the protein [7]. Upon ligand binding, ER dimerizes with another receptor monomer, and in turn the ER dimer-ligand complex attracts a complex machinery of co-regulators to a specific DNA sequence called the estrogen response element (ERE) to regulate gene transcriptional activation or repression [8, 9]. This constitutes the classical ER genomic activity. The transcriptional activity of ER is fine-tuned by the expression and availability of numerous co-regulators, several of which have been implicated in breast cancer or in endocrine resistance [10]. The ER-ligand complex can also function by tethering to other transcription factors, such as activator protein 1 (AP-1) and specificity protein 1 (SP-1) at their specific sites on DNA, to mediate ERE-independent signaling by regulating the transcriptional activity of these factors and their responsive genes [11]. Furthermore, ER activity is also regulated by membrane receptor tyrosine kinases, including HER2 [12], epidermal growth factor receptor (EGFR) [13], and insulin-like growth factor

receptor (IGF1R) [14]. These membrane kinases activate signaling pathways that eventually result in phosphorylation of ER as well as its co-activators and co-repressors at multiple sites to influence their specific functions [14, 15]. This activation of ER by growth factor receptor signaling is sometimes referred to as non-classical ligand-independent receptor activation, which is known to contribute significantly to endocrine therapy resistance [14]. Lastly, studies also show that ER works by rapid non-genomic, non-transcriptional mechanisms. Low levels of ER have been found outside the nucleus in the membrane, cytoplasm, or even mitochondria, although the exact location and biological impact for this receptor remain controversial [16]. Overall, ER signaling is a complex network with multiple levels of regulation, fine-tuning capabilities, redundancy, and evolvability. This intricate ER signaling network either at individual branches or collectively is hacked and exploited by cancer to evade endocrine therapy.

Classes of Endocrine Therapy Drugs

Endocrine therapy, which interferes with ER action has been a mainstay of breast cancer treatment for more than a century. These agents classify as: Selective Estrogen Receptor Modulators (SERMs), Selective Estrogen Receptor Downregulators (SERDs) and Aromatase Inhibitors (AIs).

SERMs were the first to be developed. Antagonizing ER binding during the 1970s led to the first, and to date most successful, targeted cancer therapy tamoxifen [17]. The SERM concept emerged afterwards from a series of preclinical and clinical studies, which revealed that the "antiestrogen" tamoxifen actually exhibited substantial ER agonist activity in bone and in the uterus [18]. The antagonist effects of tamoxifen in breast tissue stem from its ability to bind to the ligand-binding domain of the ER, and effectively block the potential for estrogen stimulation. Tamoxifen binding prevents critical ER conformational changes that are required for the association of co-activators. However, because of its agonistic activity in the uterus, tamoxifen treatment is associated with an increased risk of endometrial cancer. Other SERMs, such as raloxifene, toremifene, lasofoxifene, bazedoxifene and arzoxifene have been developed with the goal of reducing some of the deleterious effects of tamoxifen.

SERDs are a class of endocrine therapy drugs that affect ER stability (degradation) and cause downregulation. The main drug in this class, fulvestrant (ICI 182,780), binds to ERα with 100-fold greater affinity than does tamoxifen and in so doing, inhibits receptor dimerization and abrogates estrogen signaling [19]. Fulvestrant is referred to as a "pure" antiestrogen because it is exclusively a pharmacological antagonist in all tissues. Both laboratory and clinical studies have shown a decrease in overall ER protein levels in response to fulvestrant treatment [20]. Theoretically, a strategy of completely or nearly completely destroying the ER may lead to a more effective inhibition of highly ER-dependent tumors. Fulvestrant has emerged as a valuable endocrine modality for patients with metastatic ER+ breast cancer, but tolerability and side effects may be limiting. New SERDs are already in clinical trials and include the orally active ARN-810/GDC-810 agent.

AIs, such as anastrozole, exemestane, and letrozole, represent another class of endocrine therapy drugs that inhibit the action of aromatase, an enzyme necessary for the conversion of androgens to estrogens [21]. Clinically, AIs have had success as a second line of therapy for post-menopausal patients who have progressed after tamoxifen treatment.

Mechanisms of Endocrine Resistance

In cancer cells, resistance to a variety of drugs, in the context of response and time, can be classified largely into two basic patterns of drug failure. First, breast tumors that show no response to first line endocrine therapies represent de novo or intrinsic resistance. Second, tumors that show a good initial response but then re-grow or recur on endocrine therapy reflect acquired or adaptive resistance. It remains unclear how these two types of resistance are related, or if they are exclusive in breast cancer. Nonetheless, some of the underlying molecular mechanisms are shared between the two types of resistance. Tamoxifen represents the archetype endocrine therapy drug – it is the most widely used drug in breast cancer supported by extensive clinical data. Many of tamoxifen's mechanisms of resistance apply to other classes of endocrine therapy drugs as well, although the lack of clinical cross-resistance indicates that some resistance mechanisms are independent [22].

For de novo or intrinsic resistance to tamoxifen, one major mechanism is the reduction or lack of ERα expression. Loss of ER expression has been observed in ~15–20% of patients with metastatic disease [23]. A second intrinsic mechanism is observed in patients unable to convert tamoxifen to its active metabolite, endoxifen, and are consequently less responsive to tamoxifen [24]. By contrast, a plethora of mechanisms have been postulated to account for acquired resistance following prolonged exposure to tamoxifen, some of which may also account for intrinsic resistance. One such mechanism of acquired resistance is based on the pharmacologic tolerance principle, which postulates that the emergence of drug resistance is due to reduced intracellular drug concentrations as a result of decreased influx, increased efflux, or altered intracellular drug metabolism. For example, altered expression or activity of the tamoxifen-metabolizing enzyme, CYP2D6, even within tumor cells, has been implicated in tamoxifen's loss of potency [24, 25]. Because antagonizing ER is central to endocrine therapy, other factors that perturb ER signaling, in turn will interfere with tamoxifen's ability to block ER. To summarize, ER-related mechanisms that contribute to endocrine therapy failure are: (i) ER expression loss over time [26], (ii) ER crosstalk with other growth factor signaling and survival pathways [12–14], (iii) co-activator/co-repressor availability for ER complex formation and chromatin remodeling/accessibility [27], (iv) kinase pathways that phosphorylate ER or other ER posttranslational modifications [28, 29], (v) kinase pathways that phosphorylate other ER-accessory proteins to modify their activity [15], (vi) non-genomic membrane-initiated ER signaling [16, 30], and (vi) more recently gain-of-function mutations in the *ESR1* gene discovered in patients with ER+ metastatic disease [31].

The majority, if not all of the above-described mechanisms illustrate how a genetic or epigenetic event confers resistance or an escape mechanism to endocrine therapy, a clonal evolution path to resistance. One recently proposed alternative route for acquiring therapy resistance is via a drug-tolerant persister cell [32, 33]. An increasing body of evidence suggests that small subpopulations of cancer cells can evade strong selective drug pressure by entering a persister state of negligible growth. Furthermore, findings point to a likely overlap between the reversibly drug-tolerant cancer cell subpopulation and stem-like cells or cancer stem cells [32]. This drug-tolerant state has been hypothesized to be part of an initial strategy towards eventual acquisition of bona fide drug-resistance mechanisms. The relevance of 'persister cells' has yet to be determined in endocrine resistance. However, the observation that ER+ luminal B tumors, which respond poorly to tamoxifen, recur after a prolonged period of dormancy [2, 34], would suggest a likely link. The idea that a small subpopulation of cells within the tumor is phenotypically different and is intrinsically more refractory to standard cancer therapy, is also central to the paradigm-shifting cancer stem cell (CSC) model. According to the CSC model, tumors are hierarchically organized and at the apex of the hierarchy are cells that display stem-like properties. CSCs are also highly tumorigenic, hence also known as tumor-initiating cells. Similar to normal stem cells, breast CSCs can also self-renew and differentiate [35, 36]. The self-renewal process drives tumor initiation and growth, while differentiation helps to generate the bulk tumor cells and maintain tumor cell heterogeneity. Therefore, breast CSCs are at the center of therapy resistance, tumor heterogeneity metastasis and tumor recurrence – all deadly aspects of breast cancer disease [37–42].

Breast CSCs Identification and Isolation

The first evidence of a hierarchical organization in a solid tumor was provided in the seminal work by Clarke and colleagues in breast cancer [37]. Indeed, the highly heterogeneous nature of breast cancer disease is analogous and reminiscent of the hierarchical organization of the normal mammary epithelium. Clarke and colleagues showed how a small population of breast CSCs were identified by virtue of their expression of the cell surface markers ESA+, CD24−, and CD44+. As few as 100 cells bearing this phenotype were capable of establishing tumors in immune-deficient mice. Furthermore, these cells recapitulated the cell type heterogeneity of the initial tumor [37]. A historical perspective of breast CSCs isolation is reviewed by Wicha and colleagues [43]. Since then, the identification and isolation of CSCs in a number of cancers has led to a paradigm shift of how cancers form, progress, relapse and metastasize. Despite the fact that not all aspects of the CSCs model are fully delineated or understood, the existence of breast CSCs is unanimously accepted. Regardless of the cellular origin of CSCs (rising from a stem cell/progenitor undergoing mutation or a more differentiated cell acquiring stem-like properties), an 'operational' definition of breast CSCs based on their tumor-initiation,

self-renewal/differentiation, and intrinsic therapy resistance has significant ramifications on how breast cancer treatment should be approached.

As mentioned above, to identify, isolate and purify breast CSCs the well-established breast CD44+CD24− immunophenotype can be used [37], but its usefulness is limited by the fact that this is not a universal marker. Alternative methods to identify breast CSCs are: (i) the side population technique, which is based on the abilities of stem cells to exclude vital dyes via transmembrane transporters [44], and (ii) the ALDEFLUOR assay, which is based on the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1) [45]. A superior method to assess stemness in vitro is the mammosphere assay. Mammosphere formation is a functional assay because it exploits the unique property of stem-like/progenitor cells to survive and grow in serum-free suspension, while more differentiated cells undergo anoikis and die in these conditions [35, 36].

CSC markers together with gene-expression profiling were used to assess the CSCs content of various breast cancer subtypes; the basal/claudin-low/triple-negative breast cancers display the highest enrichment, followed by the HER2-subtype [46]. This correlates with the poor patient outcome of these two subtypes. On the other side of the spectrum, luminal A tumors, which have the best prognosis, display the lowest proportion of cells expressing CSC markers. These tumors also display the highest proportion of ER+ cells. Luminal B tumors, express still a lower proportion of cells with CSC markers than basal and HER2 tumors, but slightly higher than luminal A. Yet, this minor difference cannot account for the vastly different aggressiveness, risk of recurrence and endocrine resistant phenotype observed in luminal B tumors compared to luminal A. To reconcile this discrepancy, one hypothesis is that currently used markers to identify and isolate breast CSCs are better suited to the basal and HER2 subtype rather than the luminal tumors.

ER Status in Breast CSCs and Implication to Endocrine Resistance

The common feature that sets apart luminal A and B tumors from other breast tumors is ER expression. But the dogma in the field is that breast CSCs are ER–. One corollary of this is that CSCs will not be eliminated by endocrine therapy, hence contribute to resistance. The idea that CSCs are ER– comes primarily from analysis of adult stem cells of the normal mammary gland [47, 48]. Whether breast CSCs are also ER– is less certain. When probing for ER expression status in breast CSCs, one study showed that 20–25% of CD44+CD24–ESA+ cells express ER [49], another study indicated that ER was expressed but in “low abundance” in CD44+ cells [50], whereas a third study showed that the highly tumorigenic CD44+CK5+ progenitor population is ER– [51]. Moreover, the exact role of ER in regulating the breast CSC population remains unclear. Some studies have shown that although breast CSCs are thought to be ER–, estrogen can expand the CSC population via paracrine signaling from non-CSCs, while others have shown that

estrogen withdrawal or tamoxifen regulates this cell population [49, 51–54]. Just recently, single cell profiling of functionally enriched CSC pools of ER+ breast cancers identified at least 3 different clusters – all three express varying levels of ER [55]. The functional consequence of this remains unknown.

Attributing endocrine-resistance to CSCs because they are ER– is overly simplistic and most likely incorrect. The connection between CSCs and endocrine resistance is supported by mounting evidence showing that: (i) tamoxifen resistant cells display elevated CSC markers [53, 56], and (ii) introducing a stem-like factor or activating CSC pathways can render cells resistant to tamoxifen [57, 58]. However, conclusive data on the exact role of CSCs in driving endocrine resistance are still lacking. Addressing this question is paramount and may provide new targeting strategies to overcome endocrine resistance. To accomplish this, it also requires the development of improved markers capable of unequivocally discriminating CSCs in ER+ tumors, and understand the role ER plays in maintaining and propagating CSCs.

Targeting CSCs' Inflammatory Roots to Overcome Therapy Resistance

Multiple mechanisms, genetic, epigenetic or biochemical have been described in regulating maintenance and propagation of breast CSCs. To summarize, some of these factors or deregulated pathways implicated in CSCs include: (i) self-renewal, canonical developmental, embryonic pathways such as Wnt/β-catenin, Hedgehog, Hippo, Notch, TGFβ, etc. [59]; (ii) epigenetic, chromatin modifiers, transcription factors such as Nanog, Oct4, Sox2, Snail, Slug, Twist, Sox9, EZH2, Suz12 etc. [60]; (iii) microRNAs such as Let7, miR30, miR200, etc. [61], (iv) oncogenes such as *HER2, mTOR, c-Myc, BRCA1,* etc. [59]; (v) metabolic reprograming and energy expenditure, and (iv) pro-inflammatory and microenvironment niche signals, such as the NFκB pathway, the cyclooxygenase/prostaglandin axis, Jak/Stat, cytokine, and chemokine cues [62]. For therapeutic purposes, inhibiting CSCs features such as self-renewal and differentiation as a way to block the tumor-regenerating effects of CSCs is desirable, but trying not to harm the normal cells as a collateral effect, is not always easy. Furthermore, some of the above mentioned pathways and factors are not optimal druggable targets.

More recently, activation of the multiple pro-inflammatory pathways, including the NFκB pathway has been shown to regulate survival and propagation of breast CSCs [63–65]. Therefore, NFκB pathway inhibition can be exploited to eradicate breast CSCs. NFκB pathway inhibition can simultaneously contribute to anti-tumor activity by blocking other NFκB-dependent tumor promoting mechanisms. Significant evidence also suggests that the NFκB pathway is critical to endocrine resistance. Intriguingly, NFκB may serve as a key determinant linking CSCs and endocrine resistance. Studies have shown that a deregulated, or constitutively active NFκB pathway is associated with hormone-independence, and both chemo and

endocrine therapy failure [66–68] . More specifically: (i) overexpression of an NFκB family member induced aromatase inhibitor resistance in cell lines and its expression was increased in recurrent resistant breast tumors, as compared to the primary tumors before treatment [68], (ii) significantly higher basal NFκB activity and expression were detected in raloxifene resistant cells [69], and (iii) high DNA binding activity of NFκB subunits identifies a high-risk subset of ER-positive primary breast cancers destined for early relapse despite adjuvant endocrine therapy with tamoxifen [66]. Furthermore, studies have shown that repressing NFκB activity can restore sensitivity to ER antagonists in cell-based models of resistance [70, 71]. Together, these findings support a critical role for NFκB in the development of endocrine resistant ER+ breast tumors.

Whether the NFκB pathway is required and sufficient to mechanistically link breast CSCs to endocrine resistance remains unknown. However, because of the central role NFκB plays in both CSC properties and endocrine resistance, it is conceivable that NFκB inhibitors can be used to simultaneously target both. Although an anti-CSC drug has yet to be approved, compounds with anti-CSC activity have been identified in vitro and tested in preclinical models. Inhibition of NFκB activity is proposed to contribute to the anti-CSC activity reported for metformin [72] and parthenolide [73, 74]. Likewise, curcumin, piperine, and sulforaphane, like parthenolide, are electrophiles known to inhibit NFκB activity, and all three agents have reported anti-CSC actions [75–77]. We have demonstrated how the anti-inflammatory drug dimethyl fumarate and a novel ‘super anti-inflammatory’ aspirin-fumarate chimeric drug, both can effectively inhibit the NFκB pathway and block breast CSC properties [78, 79]. Furthermore, the enhanced anti-CSC activity of the aspirin-fumarate chimeric drug, which is capable of targeting both the NFκB and cyclooxygenase-prostaglandin cascade, supports the concept of multiple anti-inflammatory components in one hybrid drug as an effective new strategy to specifically target breast CSCs. The anti-CSC activities were observed in cells representing all the different breast cancer subtypes. The value of these agents at overcoming endocrine resistance remains to be determined.

Conclusions

Multiple mechanisms contribute to the either de novo or acquired endocrine resistance in breast cancer. Significant evidence supports the idea that CSCs may be the primary culprits to mediate endocrine resistance. Better understanding of fundamental CSC biology, more specifically: (i) determining the role ER plays in CSCs, and (ii) defining improved CSC markers for luminal tumors, may provide the conclusive evidence for CSCs’ contribution to endocrine resistance. In turn, this may propel the anti-CSC strategy into a clinically useful reality, and improve prognosis for patients with aggressive therapy-resistant ER+ breast tumors.

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EGFR Resistance

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Abstract In recent years, the management of breast cancer has been revolutionized by the discovery of targeted therapies. Some of these emerging agents stop cell growth by inhibiting epidermal growth factor receptors (EGFRs). Targeting the EGFR has been intensely pursued in the breast cancer, with mixed results and challenges with respect to treatment resistance emerging over time. The resistance to EGFR inhibitors is now well recognized due its high prevalence in lung cancer. However, in breast cancer the mechanism of resistance is not yet fully understood. This review provides an overview of the known mechanisms that lead to EGFR inhibitor resistance in breast cancer.

Abbreviations

AKT	v-akt murine thymoma viral oncogene homolog
Ca++	Calcium
CAMK	Calcium/calmodulin-dependent protein kinase
DAG	Diacylglycerol
EGFR	Epidermal growth factor receptor
EGFR TK inhibition	Epidermal growth factor tyrosine kinase inhibition
ELK1	ELK1, member of ETS oncogene family
FOS	FBJ murine osteosarcoma viral oncogene homolog
GRB2	Growth factor receptor-bound protein 2

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IP3	Inositol 1,4,5-trisphosphate
JAK1	Janus kinase 1
JUN	Jun proto-oncogene, AP-1 Transcription Factor Subunit
MAP 2K	Mitogen-activated protein kinase kinase
MAP 2K7	Mitogen-activated protein kinase kinase 7
MAP 3K7	Mitogen-activated protein kinase kinase kinase 7, E3 ubiquitin protein ligase
MAPK	Mitogen-activated protein kinase
MAPK8	Mitogen-activated protein kinase 8
MYC	Mitogen-activated protein kinase 8
NFKB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
Pip2	Phospholipid phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3)
PLCG	Phospholipase C, gamma
Prkc	Protein kinase C
RAF1	Rapidly accelerated fibrosarcome-1 proto-oncogene, serine/threonine kinase
RAS	Rat sarcoma
SHC	SHC (Src homology 2 domain containing) transforming protein
SOS1	Son of sevenless homolog 1 (Drosophila)
SP1	Specificity protein 1
STAT	Signal transducer and activator of transcription

Introduction

Breast cancer is one of the most common malignancies among women, accounting for approximately 2.4 million new cases diagnosed in 2015 (most recent data available) [1]. Survival of patients with breast cancer depends not only on tumor stage but also on the biological factors which represent the tumor aggressiveness, such as the estrogen or progesterone receptor status and the human epidermal growth factor receptor (HER) 2 status. Epidermal growth factor receptor (EGFR) gene amplification is emerging as a new biological factor that may affect breast cancer mortality. Although the EGFR gene amplification is infrequent in breast cancers, accounting for 0.8–14% of all tumors, it is associated with more aggressive tumors. EGFR overexpression is present in all subtypes of breast cancer, however, it is more frequently seen in triple-negative breast cancer (TNBC) and inflammatory breast cancer [7, 8]. In these tumors, EGFR is overexpressed in at least 50% of TNBC and 30% of inflammatory cases [2–5]. EGFR overexpression in these cases is associated with poor clinical outcomes, as patients generally present with poorly differentiated large tumors [2, 3]. These types of breast cancer are deemed the most aggressive of the breast cancer subtypes for which there are no specific targeted therapies. Thus, EGFR has potential as a therapeutic target in these cancers.

Enhanced understanding of the molecular targets involved in the pathogenesis of tumor cell growth has led to the clinical development of several novel targeted agents. Among these targets, are members of the EGFR/ErbB family, and include EGFR (also known as human epidermal receptor 1 (HER1)), HER2, HER3, and HER4 [6]. Of these, HER2 is the most well well-known target in breast cancer, and treatment for patients with HER2 overexpressing tumors is well established. Currently, treatment for EGFR targets is emerging as a promising option, as several drugs are being investigated as potential EGFR inhibitors.

Presently, lapatinib, a dual inhibitor that is able to target the tyrosine kinase (TK) domains of EGFR and HER2, has been approved for the treatment of breast cancer patients with HER2 overexpression. Lapatinib prevents the phosphorylation and subsequent signal transduction of the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K)/serine/threonine/kinase (Akt) pathways, resulting in inhibition of cell proliferation and cell death [9]. Lapatinib is approved in combination with other agents for the treatment of HER2 overexpressing metastatic breast cancer that progressed after combination treatment that included trastuzumab [10, 11]. Other EGFR inhibitors such as gefitinin and cetuximab have been developed and researched for the treatment of breast cancer, however they have not proven to be as effective as the EGFR inhibitors used in other cancers. This may be, in part, due to the tumor being constitutively resistant to these treatments or that the tumor cells have acquired resistance to these drugs. Here we review the mechanisms of EGFR resistance and the pivotal role they have in the resistance of breast cancer tumors to EGFR inhibitors.

Discovery of the EGFR

Ground-breaking work by Burnett and Kennedy first characterized the protein kinase enzyme activity responsible for regulating cellular metabolism in 1954 [12]. Receptor tyrosine kinases were found to be a subclass of cell-surface growth-factor receptors which regulate diverse functions in normal cells that demonstrated a crucial role in oncogenesis. Protein kinases were found to exert roles in almost every aspect of cellular function: metabolism, transcription, division, and apoptosis [12]. Protein phosphorylation was discovered to balance the action of protein kinases and phosphoprotein phosphatases making phosphorylation–dephosphorization an overall reversible process. A subsequent discovery led to the understanding that dysregulation of protein kinases occurs in many diseases including cancer and inflammatory disorders [12, 13]. Subsequent sequencing of the EGFR complementary deoxyribonucleic acid (cDNA) confirmed that the receptor contained a protein-kinase domain [13]. EGFR was the first receptor that provided evidence for a relationship between overexpression and cancer. It was also quickly realized that the tyrosine kinase receptors were potential cancer targets and rapid discovery of new targets ensued [14].

EGFR was then identified as a growth factor-regulated protein kinase, ushering in a new paradigm for hormonal signal transduction [12, 13]. Ullrich et al. presented

for the first time the complete amino acid sequence of a cell surface receptor for EGF and they hypothesized that the epidermal growth factor (EGF) induced a change in the external cell domain transmitted signals to the plasma domain. Most importantly, they found the EGF receptor levels in A431 vulvar cancer cell lines [15]. It was at this point that the investigation into breast as well as other cancer cell lines began. EGFR has subsequently become the most studied receptor TK owing to its overall role in signaling transduction and oncogenesis of certain tumors including breast cancer [12, 13, 15].

Epidermal growth factor receptors quickly became a therapeutic target in all cancer types. Overexpression of the "wild-type EGFR" found in breast cancers stimulated research into treatment strategies including anti-receptor antibodies, tyrosine kinase inhibitors, ligand-toxin conjugates and receptor antisense molecules [14]. Multiple breast cancer receptors were identified as potential targets, such as platelet-derived growth factor receptor (PDGFR), EGFR, HER2, HER3, HER4, and insulin-like growth factor 1 receptor (IGF-1R). The age of targeted molecular treatment modalities for breast cancer had begun.

Decades of research found the human EGFR receptor to be comprised of a family of four closely related cellular transmembrane glycoproteins (HER1, HER2, HER3, HER4), which contained extracellular binding sites and intracellular receptor tyrosine kinase domains [16]. At the cellular level, this ligands induced cell proliferation, altered adhesion and motility and prevented apoptosis while at the same time promoting cell invasion and angiogenesis [17]. Most importantly, EGFR gene overexpression was capable of increasing the metastatic potential of breast cancer cell lines especially in the breast cancer phenotype known as triple negative: an aggressive tumor type with absence of estrogen, progesterone and/or HER2 receptor overexpression [17].

The EGFR Pathway

The EGFR signaling pathway is a complex and tightly regulated network that is critical for the regulation of growth, survival, proliferation, and differentiation in cells. EGFRs are activated following ligand binding and receptor dimerization. Subsequently, several cytoplasmic proteins are recruited which increase EGFR function resulting in the activations of proteins that mediate cell survival, growth or differentiation. Details of the EGFR pathway can be seen in Fig. 1 [18].

Mechanisms of Resistance to EGFR Inhibitors

Breast cancer patients who initially benefit from EGFR-targeted therapies eventually develop resistance. Elucidating resistance mechanisms for anti-EGFR therapies is essential to developing strategies to prolong the efficacy of EGFR-targeted therapies in these patients. Currently, the mechanisms of resistance have been

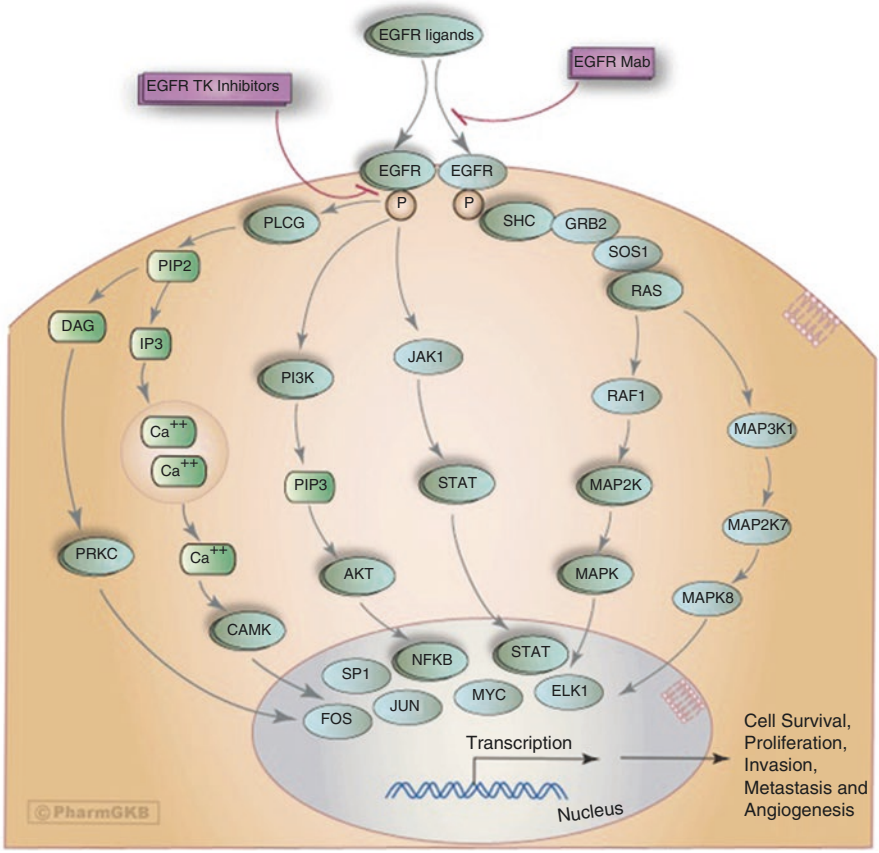

Fig. 1 EGFR is a transmembrane tyrosine kinase receptor that plays a central role in regulating cell division and death. EGFR belongs to the HER family of receptors which comprise four related proteins (EGFR(HER1/ErbB1), ERBB2(HER2), ERBB3(HER3) and ERBB4(HER4)). The HER receptors are known to be activated by binding to different ligands, including EGF, TGFA, heparin-binding EGF-like growth factor, amphiregulin, betacellulin, and epiregulin. After a ligand binds to the extracellular domain of the receptor, the receptor forms functionally active dimers (EGFR-EGFR (homodimer) or EGFR-HER2, EGFR-HER3, EGFR-HER4 (heterodimer)). Dimerization induces the activation of the tyrosine kinase domain, which leads to autophosphorylation of the receptor on multiple tyrosine residues. This leads to recruitment of a range of adaptor proteins (such as SHC, GRB2) and activates a series of intracellular signaling cascades to affect gene transcription, which in turn results in cancer cell proliferation, reduced apoptosis, invasion and metastasis and also stimulates tumor-induced angiogenesis. The pathways mediating downstream effects of EGFR have been well studied and three major signalling pathways have been identified. The first pathway involves RAS-RAF-MAPK pathway, where phosphorylated EGFR recruits the guanine-nucleotide exchange factor via the GRB2 and Shcadapter proteins, activating RAS and subsequently stimulating RAF and the MAP kinase pathway to affect cell proliferation, tumor invasion, and metastasis. The second pathway involves PI3K/AKT pathway, which activates the major cellular survival and anti-apoptosis signals via activating nuclear transcription factors such as NFKB. The third pathway involves JAK/STAT pathway which is also implicated in activating transcription of genes associated with cell survival. EGFR activation may also lead to phosphorylation of PLCG and subsequent hydrolysis of phosphatidylinositol 4,5 biphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), resulting in activation of protein kinase C (PRKC) and CAMK (Reprinted with permission from PharmGKB and Standford University [115])

predominantly determined from evaluating the tumors of patients with lung cancer, and they can be categorized as primary and acquired resistance. Acquired resistance has several mechanisms, including: (a) secondary mutation of EGFR, (b) independent or constitutive activation of downstream mediators, (c) activation of alternative TK receptors that bypass the EGFR pathway, and (d) activation of EGFR-independent, tumor-induced angiogenesis [19].

Primary Resistance

There are several tumor characteristics that contribute to primary resistance to EGFR-inhibitors. These include EGFR somatic mutations and germline polymorphism. EGFR somatic mutations, such as exon 20 insertions or duplications, G719X and L861X, can render the tumor resistant to tyrosine kinase inhibitors (TKI) [20–35]. Threonine (T)790methionine (M), V8431 variants have been identified in patients with germline polymorphism and familial cancer syndromes and these generally fail to respond to TKIs, either given alone or in combination with chemotherapy [36–40].

Acquired Resistance

Secondary Mutation of EGFR

Approximately 60% of EGFR-acquired resistance is due to T790M mutations, which substitute threonine (T) with methionine (M) at position 790 of exon 20 [41, 42]. This mutation results in acquired resistance by increasing the binding affinity of EGFR for adenosine triphosphate (ATP), relative to its affinity to TKIs, thus decreasing the sensitivity to EGFR inhibitors. In addition, the bulky methionine sterically prevents the binding of the inhibitor to the EGFR while preserving its catalytic activity, molecular alteration of related molecules, and genetic alteration in bypass signaling [43].

Independent or Constitutive Activation of Downstream Mediators

Alteration in signaling mediators leading to constitutive or EGFR independent activation of downstream mediators, thus bypassing the need for EGFR activation, can be seen in approximately 5% of patients who are resistant to EGFR inhibitors. This can occur due to the activation of mediators, such a PI3K as a result of direct gene amplification, activation mutations of the p85 subunit, overexpression of downstream effectors such as Akt, inactivation of mutations or loss of function of regulators such as the phosphatase and tensin homolog (PTEN) [44–58]. Less common dysregulation of downstream mediators, such as the mitogen-activated protein kinase (MAPK), Src TK family, and several members of the signal transduction and activator of transcription (STAT) family have also led to constitutive activation of multiple pathways that bypass the EGFR inhibition [36–40, 59–80].

Activation of Alternative TK Receptors that Bypass the EGFR Pathway

EGFR is able to control tumor growth through multiple downstream signaling pathways. However, cancer cells are able to switch to alternative survival mechanisms when the EGFR pathway is inhibited. This is accomplished by the activation of other TK receptor systems that are not related to EGFR, such as IGF-1R and PI3K/Akt signaling, *MET* gene, and *MAPK* amplification. IGF-1R activation can bypass inhibition of other TK receptors as evidenced by the correlation between the expression of IGF-1R and the cell growth inhibition capability of trastuzumab. Overexpression of IGF-1R is inversely correlated with the response of breast cancer cells to trastuzumab [81–93]. Overexpression of the hepatocyte growth factor (HGF), a ligand for c-MET, activates c-MET which restores phosphorylation of the downstream MAPK/extracellular signal-regulated kinases (ERK1/2) and the PI3K/AKT pathway, thus inducing resistance [94].

Activation of EGFR-Independent, Tumor-Induced Angiogenesis

Angiogenesis results from the tumor secretion of growth factors that act on host endothelial cells, such as the vascular endothelial growth factor (VEGF). VEGF binds to the TK receptors on the endothelial cells resulting in vasculogenesis and angiogenesis. Activation of EGFR leads to up-regulation of VEGF expression, consequently activating the VEGF-mediated angiogenesis [95–103]. Initially, most tumors treated with EGFR inhibitors respond to treatment, as evidenced by tumor shrinkage related to decreased angiogenesis; however, these eventually become resistant to treatment. This may be in part due to the inability of EGFR inhibitors to down-regulate VEGF production in cancer cells, rather than a change in the expression or a functional alteration of EGFR signaling, resulting in tumor angiogenesis that is independent of EGFR activation [104–108].

Resistance to EGFR Inhibitors in Breast Cancer

The mechanisms of resistance to EGFR inhibitors in breast cancer are not fully understood at this time. One proposed mechanism leading to EGFR resistance is increased estrogen (ER) and progesterone (PR) crosstalk and signaling, resulting in increased activity of anti-apoptotic proteins [34, 46]. These findings are based predominantly on the mechanism of resistance to lapatinib, an EGFR and HER2 TK dual inhibitor. Mutations in the kinase domain of EGFR, much like those seen in lung cancer EGFR domains, may confer EGFR inhibitor resistance [109]. Overexpression of HGF in breast cancer induces resistance, similarly to that in lung cancer, by activating alternative TK receptors that bypass the EGFR pathway [94]. Specific to resistance in breast cancer, two related oncogenes, family with sequence similarity 83 member (FAM83) A and B, have been implicated in making breast cancer tumor cells resistant to EGFR TKIs. An increase in the expression of these

genes increases proliferation and invasion of tumor cells [110]. Lapatinib resistance was found to be associated with the hyperactivation of the PI3K pathway, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutations, and phosphatase and tensin homolog (PTEN) tumor suppressor [111–113]. Lastly, overexpression of anexelekto (AXL), a membrane-bound tyrosine kinase receptor, is another mechanism of acquired resistance to lapatinib resistance, and it is believed that it does so via a crosstalk among the HER, AXL, and ER receptor pathways [114].

The Future of EGFR Inhibitors

The success of EGFR-directed therapies in the breast cancer setting requires new approaches to treat EGFR-driven breast cancer and to prevent or overcome acquired EGFR inhibitors resistance. Despite the potential benefits of EGFR-targeted therapy used either alone or in combination with other therapies in breast cancer, no consensus exists regarding the criteria for the use of these drugs. Nor do we have sufficient information regarding the EGFR overexpression in breast cancer types and its correlation with the therapeutic response. The current ongoing clinical trials with EGFR inhibitors, used alone or in combination with other therapies, in EGFR-overexpressing breast cancer will elucidate the benefit of these drugs in the treatment of all subtypes of breast cancer, and perhaps will lead to the development of good strategies for the assessment of molecular markers that can provide accurate and reliable evaluation of clinical trial results. In addition, therapies with multi-targeted drugs directed against EGFR receptors and downstream proteins that are relevant to this pathway are pivotal to overcoming the acquired EGFR resistance.

Conclusion

EGFR-targeted therapies are an important advance in breast cancer treatment. The clinical challenge is to determine which patients will benefit from EGFR inhibitors and how to overcome resistance. Validating the various mechanisms of resistance in clinical practice could lead to improvement of the effectiveness of EGFR-based therapies. The combination of targeted agents could prevent the onset of or prolong the development of resistance in breast cancer patients.

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Targeting FGFR for the Treatment of Breast Cancer

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Abstract Breast cancer has been detailed at the molecular level in very high definition. These molecular characterizations have allowed for the establishment of at least 5 distinct subtypes of the disease. Importantly, breast cancer subtyping can predict for tumor recurrence and drives the clinical application of endocrine and human epidermal growth factor receptor 2 (Her2)-targeted therapies. Recent studies have revealed that these subtypes of breast cancer are not static definitions and that through disease progression breast cancers have the ability to switch subtypes to acquire resistance to these therapies. In addition to therapeutic failure in the metastatic setting, other patients' primary tumors can only be defined as the poorly understood basal subtype, a classification that is synonymous with the description of triple negative breast cancer (TNBC). Unfortunately, these patients are not candidates for any currently approved molecular therapies and they are left with suboptimal, highly cytotoxic chemotherapies as treatment options. Therefore, recent research has focused on identifying the molecular drivers of TNBC and metastatic breast cancer that has undergone subtype switching and become resistant to endocrine and Her2-targeted therapies. One emerging target for the treatment of these advanced forms of breast cancer is the fibroblast growth factor receptor (FGFR). FGFR plays critical roles in the metastatic progression of TNBC and the acquisition of resistance to targeted therapies as well as chemotherapy. Herein, we review the current understanding of how FGFR is regulated in breast cancer and what approaches are currently being taken to pharmacologically target FGFR function as a therapeutic option for breast cancer patients. In addition to being amplified at the genomic level, FGFRs are highly inducible genes and their biology is made more complex by factors that include alternative splicing, differential subcellular localization and the presence of several different coreceptors and ligands. Finally, gatekeeper mutations in the receptor and activation of alternative growth factor pathways can give rise to acquired resistance to FGFR inhibitors. Recent clinical trials using

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FGFR kinase inhibitors emphasize that these biological factors need to be taken into diagnostic consideration when identifying the optimal patient population for FGFR-targeted therapies.

Abbreviations

ABC	ATP-binding cassette
BL1	Basal-like 1
BL2	Basal-like 2
BRCA1	Breast cancer 1
CDK	Cyclin-dependent kinase
CSFR1	G-CSF granulocyte-colony stimulating factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
ER-α	Estrogen receptor alpha
ESRP	Epithelial splicing regulatory proteins
FGFR	Fibroblast growth factor receptor
FIIN4	FGFR irreversible inhibitor 4
FLT3	fms-related tyrosine kinase 3
Her2	Human epidermal growth factor receptor 2
Her4	Human epidermal growth factor receptor 4
HSPGs	Hepran sulfate proteogyclans
Ig	Immunoglobulin
IL6	Interleukin 6
IM	Immunomodulatory
INFS	Integrative nuclear FGFR1 signaling
LAR	Luminal androgen receptor
M	Mesenchymal
mAb	Monocloncal antibody
MAP	Mitogen-activated protein
MDSCs	Myeloid derived suppressor cells
MSL	Mesenchymal stem-like
N-cad	N-cadherin
NCAM	Neural cell adhesion molecule
PAM50	Prediction analysis of microarray 50
PARP	Poly ADP-ribose polymerase
PDGFR	Platelet-derived growth factor receptor
PR	Progesterone receptor
Sp1 and 3	Specificity protein 1 and 3
TGF-β	Transforming growth factor-β
TNBC	Triple negative breast cancer
TNFα	Tumor necrosis factor-α
TRE	Thyroid hormone response element
VEGFR	Vascular endothelial growth factor receptor

Introduction

Targeted Therapies for Breast Cancer

The past three decades have witnessed the emergence of targeted therapeutics in clinical and translational breast cancer research. As the term implies, targeted therapies act by inhibiting very specific characteristics needed for tumor cell growth and survival, in contrast to traditional chemotherapies, which less specifically target hyperproliferating cells. By this mechanism, targeted therapies have a lower incidence of toxic side effects and a larger therapeutic index than chemotherapy. In recent years, the clinical application of targeted therapies has essentially tested the theory of oncogene addiction. Fundamentally, oncogene addiction states that despite their diverse array of genetic aberrations, tumor cells depend on one dominant oncogene for maintenance of the malignant potential, metastatic spread, and resistance to cytotoxic stress (reviewed in [1]). These observations fueled intense investigations to identify and target driver oncogenes in order to halt cancer progression and improve patient prognosis. Indeed, these efforts have resulted in the successful design and formulation of various targeted therapies for the treatment of breast cancer in the form of small-molecule inhibitors and monoclonal antibodies. Despite the initial success of many of these agents, breast cancer cells acquire resistance to molecularly targeted therapies by reactivating the inhibited oncogenic pathway or switching to alternative pathways for survival. Further, there are numerous reports of inherent resistance in breast cancer where targeting oncogenes identified from the primary tumor analysis does not yield clinical benefit for that patient in the metastatic setting. Overall, understanding the molecular plasticity that underlies both acquired and inherent resistance is of tremendous importance to reduce mortality due to metastatic breast cancer.

Clinical Classifications of Breast Cancer

Breast cancer is conventionally classified by pathological features such as tumor grade, size and node status, and by immunohistochemistry for estrogen receptor alpha (ER-α), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2) receptor. Since the late eighties, these tools have provided information regarding therapeutic decision making and patient prognosis. For example, breast cancer patients that express ER-α were reported by Fisher et al. to particularly benefit from anti-estrogens [2]. Moreover, Slamon et al. showed that Her2 overexpression correlates with aggressive behavior in breast and ovarian cancers [3]. Despite the clinical utility of these classification systems, accumulating evidence has suggested tumors with similar histological characteristics do not necessarily follow the same pathologic progression and display differential responses to similar treatments. Thus, ongoing research has aimed to understand the heterogeneity of

breast cancer subtypes and uncover druggable molecular targets for more accurate subtyping of breast cancers and effective therapeutic choices.

Molecular Subtypes of Breast Cancer

Molecular subtyping of breast cancer was described using cDNA microarrays that established the underlying diversity in gene expression patterns from various patient-derived breast tumors and cell-lines [4–7]. These distinctive molecular portraits of breast cancer subtypes were correlated with the traditional histological classifications to create the breast cancer subtypes: luminal A, luminal B, Her2-enriched, basal-like and caludin-low [6, 8]. The prognostic value of these subtypes was significantly improved by Parker et al. who introduced a 50-gene set that predicts patient outcome and responsiveness to chemotherapy, known as the Prediction Analysis of Microarray 50 (PAM50) [9]. Essentially, the PAM50 is a gene-list that faithfully differentiates breast cancer subtypes without the need for full genomic analyses [10]. Recently, the PAM50 is beginning to be applied clinically as NanoString and Prosigna have developed a clinical diagnostic based around analysis of the PAM50 leading to a Prosigna score that correlates to the tumor subtype and prognosis [11]. This and other molecular diagnostics such MammaPrint® and OncotypeDX® serve to better stratify patients and are beginning to strongly influence treatment decisions. Overall, the classification system of breast cancer continues to evolve to generate new subtypes and refine existing ones [12–15].

Targeted Therapies of Breast Cancer: Examples of Great Success Hindered by Resistance

Luminal Breast Cancer

The luminal A and B subtypes account for more than 60% of breast cancer cases, and while they differ in their gene expression profiles and prognosis, luminal A and B cells express ER-α and PR. Luminal A is the most common (~40% of all cases) and generally correlates with lower proliferative index and good overall prognosis. Thus current guidelines suggest luminal A patients receive endocrine therapy and be spared chemotherapy [16]. Indeed, multiple studies have demonstrated that the use of endocrine therapy in luminal A patients correlates with lower recurrence rates and is more beneficial than chemotherapy, thus anti-estrogens are likely to remain first-line treatment options for luminal A breast cancer [17–20].

The luminal B subtype accounts for ~20% of all breast cancer subtypes and is characterized by relatively lower ER-α expression, increased proliferation as measured by Ki67 staining, and poorer prognosis as compared to luminal A [21]. Unlike the luminal A subtype, luminal B breast cancer has been shown to be more sensitive

to chemotherapy than endocrine therapies [22]. This observation prompted investigations to identify molecular pathways for efficient drug development. Biomarker identification remains crucial to uncovering molecular targets for luminal B breast cancer as Ki67 staining and interpretation, the cornerstone of distinguishing luminal A and B subtypes, is known to be associated with significant variability that may impede the accurate classification of luminal A versus B and, thus, choice of endocrine versus chemotherapy [23]. Broader application of the PAM50 will help to alleviate much of this misdiagnosis, but additional targeted therapies for the luminal B subtype are still needed.

Acquired and Intrinsic Resistance in Luminal Breast Cancer

Extended adjuvant therapy with aromatase inhibitors after ER-α inhibitors prolongs disease-free progression in luminal breast cancer [24, 25]. However, both inherent and acquired resistance to endocrine therapy have been reported in metastatic luminal breast cancer. An established mechanism of acquired resistance to ER-α antagonists in initially-responsive patients is the downregulation of ER-α where these tumors become independent of estrogen-signaling for survival [26]. Inherent resistance to endocrine therapy involves loss of PR in metastatic tumors [27]. Despite these established mechanisms, molecular tools are needed to prospectively predict patient groups that will exhibit resistance. Further, the oncogenic drivers that allow for primary versus metastatic discordance in ER expression are yet to be identified for luminal breast cancers.

Her2-Enriched Breast Cancer

Her2-enriched breast cancer constitutes 15–20% of breast cancer subtypes, and as the name implies is characterized by high expression of Her2. Her2 is a member of the ErbB family of receptor tyrosine kinases and is a well-established proto-oncogene. The molecular mechanisms of Her2-mediated oncogenesis are complex and involve receptor oligomerization leading to constitutive receptor activity and activation of downstream signaling cascades to induce cell-proliferation, invasion and metastasis (reviewed in [28]). Given these findings, kinase inhibitors and monocloncal antibodies (mAbs) have been formulated to target Her2 expressing tumors. Trastuzumab was the first Her2-targeted mAb to be approved by the Food and Drug Administration (FDA) in combination with chemotherapy as an adjuvant therapy for Her2-overexpressing breast cancer patients with nodal involvement [29]. Trastuzumab binding to Her2 inhibits intracellular signaling and triggers antibody-dependent cellular cytotoxicity (reviewed in [30]). Similarly, pertuzumab is also a Her2-targeting mAb that binds a different domain of Her2 [31–33]. Recently, pertuzumab in combination with trastuzumab and chemotherapy was FDA-approved for the treatment of metastatic Her2-overexpressing breast cancer. Trastuzumab has also been chemically

linked to emtansine, a powerful chemotherapy, to produce an antibody-drug conjugate known as T-DM1 that effectively delivers emtansine specifically to Her2 overexpressing cells [34–36]. In addition to mAbs, several kinase inhibitors have also been developed for targeting Her2 and other ErbB members. Lapatinib competitively inhibits both Her2 and epidermal growth factor receptor (EGFR), and was the first FDA approved kinase inhibitor for Her2-amplified advanced breast cancer used in combination with chemotherapy [37, 38]. Other recently developed kinase inhibitors of Her2 and other ErbB family members include neratinib and afatinib, which covalently inhibit Her2 and EGFR, and another member of the ErbB family, human epidermal growth factor receptor 4 (Her4). Neratinib has been shown to significantly increase disease-free survival in Her2-overexpressing breast cancer patients that had previously received trastuzumab-chemotherapy combination or trastuzumab alone [39, 40]. These results have led to submission of a new drug application for neratinib in July of 2016. Overall, targeting the ErbB family in Her2-enriched breast cancer has revolutionized the treatment of patients of the Her2-subtype.

Acquired and Intrinsic Resistance in Her2-Enriched Breast Cancer

Despite the success of Her2-targeted therapies, clinical resistance remains a substantial problem. Studies have described mechanisms that alter the Her2 isoform expression or co-receptor expression that lead to inhibition of trastuzumab binding as a potential mechanism of resistance [30, 41]. Furthermore, resistance to trastuzumab has been demonstrated to result from activation of an interleukin 6 (IL6) signaling loop and essentially result in subtype switching to a TNBC phenotype [42, 43]. Similarly, resistance to lapatinib has recently been linked to general kinome reprogramming, leading to activation of several alternate growth pathways [44]. In attempts to overcome these mechanisms a recent clinical trial utilized neratinib as extended adjuvant therapy after completing trastuzumab standard therapy, which demonstrated a significant increase in disease-free survival (ExteNET Trial) [40]. These findings suggest that other ErbB family members that are not targeted by trastuzumab may be at play in facilitating resistance. Finally, as is the case with ER-α expression in luminal breast cancer, primary versus metastatic tumor discordance has also been described for Her2, and is an intuitive mechanism of resistance to Her2-targeted therapies [45]. Currently, the mechanism responsible for Her2 discordance and the emergence of new oncogenic drivers that accompany this phenomenon are yet to be established.

Basal-Like Breast Cancer

The basal-like subtype accounts for ~20% of breast cancer and is characterized by increased expression of basal/myoepithelial markers (cytokeratins 5/6, 14, & 17) and EGFR [10, 11]. While there is yet no unified positive definition of this subtype, the

basal-character correlates with the lack of ER-α, PR, and Her2 amplification, and thus the basal-like term is often used interchangeably with TNBC (characteristics of basal-like breast cancer extensively reviewed in [46]). Being a diagnosis of exclusion, TNBC has the worst prognosis of all breast cancer subtypes as it lacks targeted therapies [47]. Indeed, chemotherapy remains the mainstay of treatment for patients with the basal/TNBC subtype as it has been shown to be more sensitive to neoadjuvant chemotherapy compared to the luminal subtypes [46, 48]. However, TNBC is characterized by a higher incidence of breast cancer 1 (*BRCA1)* mutations [49]. BRCA1 along with Poly ADP-ribose polymerase (PARP) enzymes have critical roles in DNA-damage repair, thus TNBC patients with *BRCA1* mutations are particularly sensitive to PARP inhibitors. Indeed, PARP inhibitors have recently been approved for *BRCA1* mutant ovarian cancer and clinical trials are currently ongoing evaluating PARP inhibition in the context of BRCA1 mutant TNBC (NCT02032823). Another interesting finding is that TNBC is enriched for mutations in the tumor suppressor p53. Given the participation of p53 in cell-cycle arrest and induction of apoptosis in response to DNA damage, p53 mutant TNBC cells proceed in the cell-cycle in the presence of DNA damage resulting from chemotherapy. These observations prompted the initiation of trials assessing the efficacy of treating TNBC with cyclin-dependent kinase (CDK) inhibitors followed by a DNA-damaging chemotherapeutic. Indeed, this sequential combination was shown to induce synthetic lethality in TNBC cells resulting in a favorable patient response compared to either drug alone [50]. Finally, ongoing trials are also currently evaluating immune checkpoint inhibitors in the treatment of metastatic TNBC (NCT02555657). TNBC has been subclassified into five clinically-relevant subtypes: the basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and the luminal androgen receptor (LAR) subtype [51]. Further molecular characterizations such as these will continue to drive diagnostic criteria for appropriate stratification of patients into groups that will best respond to developing therapies.

Acquired and Intrinsic Resistance in Basal-like Cancer

While basal-like breast cancer is initially sensitive to chemotherapy, patients often relapse [52]. The mechanisms of this acquired resistance are likely to be several fold, but a major theme is the overexpression or activation of the ATP-binding cassette (ABC) transporters capable of efflux of chemotherapies from the cell [53]. EGFR overexpression is characteristic of basal-like/TNBC and has been intensely investigated as a potential candidate for targeted therapies, given its well-established oncogenic roles in other types of cancer. Yet, clinical trials assessing the effectiveness of EGFR kinase inhibitors and monoclonal antibodies have failed to improve outcomes of EGFR-positive patients with metastatic disease. The mechanism of this intrinsic resistance to EGFR inhibitors remains largely unknown; however, studies from our laboratory suggest a loss of EGFR expression and function in the metastatic setting in favor of fibroblast growth factor receptor (FGFR)-driven tumor growth [54, 55].

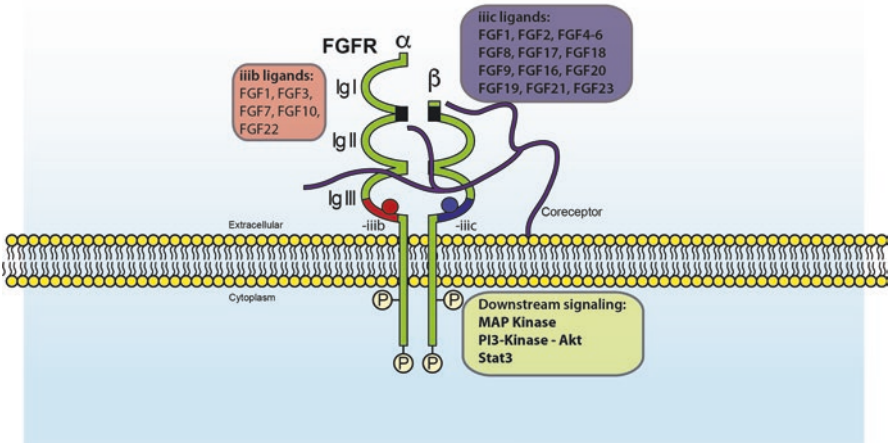

Fig. 1 The FGFR signaling system is highly diverse. FGFRs 1-4 are transmembrane receptors consisting of three extracellular immunoglobulin (Ig) domains numbered here as Ig I, Ig II, and Ig III. This full-length isoform (α) can be truncated via alternative splicing that excludes the third exon and encodes a receptor with just the two most membrane proximal Ig domains (β). Additional alternative splicing events in FGFR1-3 result in mRNA transcripts that encode for two different membrane bound ligand-binding domains in the Ig III domain (-iiib or -iiic). The -iiia isoform results in soluble receptor and therefore is not shown here. These domains bind the unique sets of ligands listed. Engagement of ligand with the FGFR and a coreceptor leads to activation of downstream signaling pathways that contribute to cellular migration, survival and proliferation

Targeting FGFR in Breast Cancer

Regulatory Mechanisms of FGFR Expression and Activity

Fibroblast growth factor receptors (FGFR1-4) are a four-member tyrosine kinase receptor protein family that binds 22 different fibroblast growth factor (FGF) ligands (Fig. 1) [56]. The aberrant activation of FGFRs leads to activation of several pro-migratory, pro-proliferative and pro-survival signaling pathways that support cancer development and progression (Fig. 1) [57–59]. At the molecular level, different transcription factors have been shown to regulate the transcription of FGFR genes. For example, expression of FGFR1 and 2 is induced by the E2F-1 transcription factor binding in response to proinflammatory cytokines such as tumor necrosis factor-α (TNFα) [60–62]. The FGFR1 promoter also contains a thyroid hormone response element (TRE) that can lead to hormonal stimulation of FGFR1 [63]. Specificity proteins 1 and 3 (Sp1 and 3) can enhance the transcription of FGFR3, while Sp1 was also shown to regulate FGFR4 expression in sarcomas [64, 65]. Upregulation of these transcription factors supports aberrant activation of FGFRs in various neoplasms. In breast cancer, the epithelial-mesenchymal transition (EMT) has been implicated in increased FGFR1 expression. Studies from our laboratory and others

demonstrated the upregulation of FGFR1 upon treatment with transforming growth factor-β (TGF-β) or overexpression of Twist, two "master regulators" of EMT [55, 66, 67]. However, the direct transcriptional regulatory mechanisms that drive FGFR1 expression during EMT remain to be fully elucidated.

In addition to transcriptional upregulation of FGFR1, EMT also greatly diversifies the biology of FGFR via the process of alternative splicing. The FGFR genes consist of up to 20 exons that encode for the three extracellular immunoglobulin (Ig) domains, the transmembrane domain and the intracellular kinase domains. FGFR1-3 undergo alternate inclusion of either exons 7, 8 or 9. Importantly, these exons encode for the ligand-binding portion within the most membrane proximal Ig domain. Therefore, the inclusion of one of these three exons produces FGFR receptors (iiia, iiib, iiic) that have differential specificities for the 23 different FGF ligands (Fig. 1). The iiia isoform produces a soluble receptor that can bind ligands but does not signal. The –iiia receptors are capable of binding and sequestering FGF ligands, but the impact of this event in the tumor microenvironment remains poorly understood [68]. Both the iiib and iiic isoforms bind FGF1, also known as acidic FGF. In contrast, FGF2 also known as basic FGF is specific for the iiic isoform, while FGF7 demonstrates specificity for the iiib isoform (Fig. 1).

Epithelial cells express high levels of the epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) [69]. These factors drive inclusion of the eighth exon leading to the production of the iiib isoform of FGFR1 and FGFR2. Importantly, the ESRPs are downregulated by the EMT transcription factors Zeb1 and Zeb2 resulting in inclusion of the ninth exon and production of the iiic isoform in breast cancer cells undergoing EMT [70]. These changes in the FGFR isoforms have important implications in paracrine signaling as stromal-epithelial signaling is mediated through reciprocal ligand production and iiib (epithelial) vs iiic (stromal) receptor expression. For a thorough review of FGF ligand receptor specificities and stromal-epithelial paracrine signaling, the reader is directed to [71]. Additionally, the exclusion of the third or α exon in FGFR1 or 2 leads to the production of a receptor lacking the outermost Ig domain. This truncated receptor, or β receptor, has been reported to have greater binding affinity for its cognate ligand and is preferentially expressed in several cancers [72]. Interestingly, only the extracellular domains of the receptor are affected by alternative splicing, but distinct downstream signaling events have been reported from α versus β and iiib versus iiic isoforms [73]. In fact, overexpression of the full length FGFR1-α-iiic isoform can actually inhibit tumor growth in some breast cancer systems [55]. Clearly, factors that contribute to the pro- versus anti-tumorigenic effects of FGFR need to be better understood to prospectively identify patients for treatment with FGFR inhibitors.

FGFR1 is also frequently amplified at the genomic level in breast cancer. Analysis of the 2015 TCGA dataset for invasive breast cancer indicates *FGFR1* amplification in 12.8% of samples [12]. Another study analyzing 522 cases of breast cancer recently reported 14% of patients display *FGFR1* amplification, whereas *FGFR2-4* amplifications were less frequent [74]. Interestingly, this study also concluded that while co-amplification of *FGFR1* and *ERBB2* was a very rare event, co-amplification with *MYC* was very common (22 of 73 cases). This is somewhat

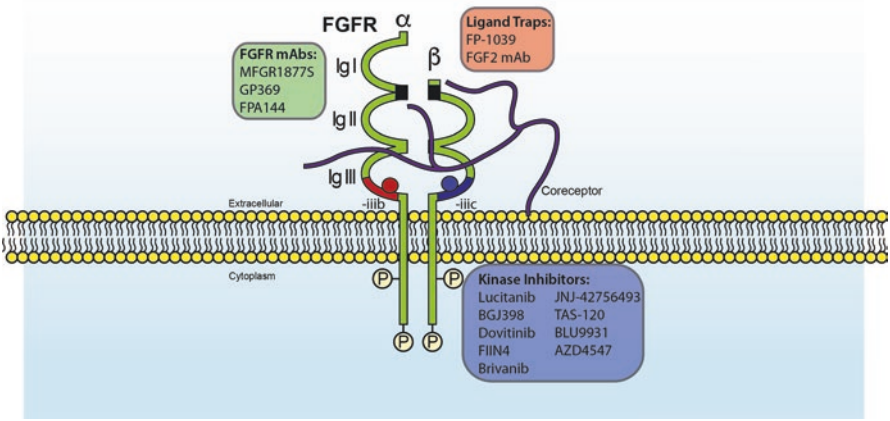

Fig. 2 Targeting FGFR for the treatment of breast cancer. Inhibition of FGFR signaling is being therapeutically targeted using the indicated kinase inhibitors. As detailed in the text, these compounds vary in specificity for particular FGFRs and other growth factor receptors. These compounds block the activity of all differential receptor isoforms created by alternative splicing. More specific blockade of particular FGFRs is also being explored using monoclonal antibodies and ligand binding traps

expected as *MYC* and *FGFR1* are located in close proximity on chromosome 8. As discussed below, these coamplification events raise concerns about passenger versus driver effects in patients with amplification of chromosome 8p11-12. Overall, these events likely impede our ability to use *FGFR1* genomic amplification as a potential diagnostic for anti-FGFR1 therapies. FGFRs can also undergo mutational activation and various translocation events leading to production of constitutively active molecules, but these events are rarely observed in breast cancer [74, 75].

The FGFR signaling system relies very heavily on the expression and interaction of co-receptors (Fig. 2). Indeed, various heparan sulfate proteogyclans (HSPGs) have been found to both enhance and inhibit FGFR signaling [76, 77]. If a particular HSPG only binds ligand and not receptor it can act to inhibit FGFR signaling. However, if HSPGs form a ternary complex with the FGF ligand and the receptor signaling will be enhanced. In particular, the syndecans are a group of HSPGs known to interact with FGFRs and drive signaling in breast cancer [78]. Syndecans do seem to do more than just stabilize a receptor-ligand complex as the cytoplasmic portion of these proteins is also critical for FGFR signaling [79]. While some FGF ligands have high affinity for HSPGs, the endocrine FGFs (FGF19, FGF21, and FGF23) utilize the Klotho family of co-receptors for more efficient interaction with their cognate FGFR [80]. Other membrane receptors have also been demonstrated to interact with FGFRs in breast cancer. For instance, N-cadherin (N-cad) interacts with FGFR during neuronal outgrowth and expression of N-cad in MCF-7 breast cancer cells greatly enhances their responsiveness to FGF ligand stimulation [81, 82]. Along these lines, β3-integrin has been identified as being capable of binding FGF and mediating its activity of FGF2 in endothelial cells [83, 84]. Similar to

N-cad, β3-integrin is potently upregulated along with FGFR1 during EMT and we and others have recently established that β3-integrin is required for FGF signaling in breast cancer cells following EMT induction [67, 85]. Neural cell adhesion molecule (NCAM) also interacts with FGFR and can stimulate its signaling in a ligand independent manner [86]. Finally, the HSPG binding FGF ligands can also interact with Neuropilin-1, which similarly acts as a co-receptor during ligand-induced FGFR signaling [87]. In contrast to these co-receptors that facilitate FGFR signaling, interaction with E-cadherin prevents the internalization and FGFR1, thus reducing ligand induced signaling [88]. Overall, several of these interactions are significant in breast cancer as loss of E-cadherin and upregulation of N-cad, NCAM, β3-integrin, Neuropilin-1 and FGFR1 itself are all prototypical markers of EMT [89]. Taken together, these studies suggest a fundamental change in cell signaling during EMT that leads to several factors supporting enhanced FGFR signaling in breast cancer.

In addition to signaling emanating from the plasma membrane, several studies have observed the presence of FGFR in the nucleus where it has been linked to enhanced cell proliferation [88, 90, 91]. The transit of FGFR1 from the plasma membrane to the nucleus can be influenced by external stimuli resulting in the interaction of FGFR1 with transcriptional regulators and a process termed integrative nuclear FGFR1 signaling (INFS) [92–94]. However, the mechanisms capable of driving FGFR1 into the nucleus and its functional role in this subcellular compartment with relation to breast cancer progression remain to be defined. Because of this, it is unclear how nuclear localization might be used for diagnosis of breast cancer patients with FGFR kinase inhibitors. These points are further discussed below.

EMT and FGFR Signaling in Resistance to Anticancer Drugs

Estrogen has been found to expand the pool of functional breast cancer stem cells through a paracrine FGF signaling axis [95]. Along these lines FGFR1 amplification has been linked to resistance to endocrine therapy in the luminal B subtype of breast cancer [96]. FGFR signaling has also been identified as a bypass mechanism utilized by breast cancer cells during acquisition of resistance to Her2 therapies [97]. Finally, kinase inhibition of FGFR is capable of sensitizing breast cancer cells to chemotherapy via blockade of the ABC transporter-mediated multidrug resistance [98]. We have recently connected the concepts of EMT and drug resistance with upregulation of FGFR by showing that breast cancer cells with acquired resistance to lapatinib undergo a dramatic EMT that includes upregulation of FGFR. Importantly, these lapatinib resistant cells could be readily eliminated using covalent kinase inhibitors of FGFR [99]. In the basal-like subtype of breast cancer, drug resistance and metastasis are strongly linked to induction of a cancer stem cell phenotype. Recent studies from our laboratory and others indicate that FGFR functions upstream of mitogen-activated protein kinase (MAP kinase) and Notch signaling to functionally participate in a cancer stem cell phenotype, contributing to drug resistance and metastasis [99, 100].

Therapeutic Targeting of the FGFR Pathway (Table 1)

Preclinical genetic studies have underscored the involvement of FGFR signaling in breast cancer progression [55, 101, 102]. Furthermore, two laboratory compounds PD173074 and SU5402 have demonstrated potent efficacy against several cell lines and *in vivo* mouse models [58, 98, 103, 104]. In addition to directly targeting tumor cells, systemic inhibition of FGFR and the shared off targets of these compounds such as VEGFRs likely contribute to antiangiogenic affects as well. These findings have attracted vast research interests among academic and pharmaceutical research groups to develop various therapeutic approaches to target FGF signaling. Current approaches are primarily using selective and non-selective tyrosine kinase inhibitors, but monoclonal antibodies to block receptor function and sequester ligand are also being pursued. Below is a non-comprehensive review of the current status of several FGFR targeting therapeutics (Fig. 2).

Brivanib Alaninate It is a compound that primarily inhibits vascular endothelial growth factor receptor 2 (VEGFR2) but also has inhibitory activity against FGFR. *In vitro* studies have demonstrated the ability of brivanib to inhibit FGFR signaling,

Table 1 Therapeutic targeting of FGFRs: the following table summarizes the specificity and current developmental status of the listed anti-FGFR agents

	Name	Specificity	Status
Small molecule tyrosine kinase inhibitors	Brivanib	Inhibitor of VEGFR2 and FGFR	Preclinical
	Dovitinib	Inhibitor of FGFR1-3, VEGFR1-3, c-KIT, FLT3, PDGFRβ, CSFR1	Improved responses were observed in phase II trial
	Lucitanib	Inhibitor of FGFR1-2, VEGFR1-3, PDGFR α/β,	Ongoing multiple phase II trials
	BGJ398	Inhibitor of FGFR1-4	Ongoing multiple phase II trials
	AZD4547	Inhibitor of FGFR1-3	Completed phase II trials
	FIIN4	Inhibitor of FGFR1-4, PDGFRβ, CSFR1, RET, VEGFR2, FLT4 and cKIT	Preclinical
	BLU9931	Inhibitor of FGFR4	Preclinical
	TAS-120	Inhibitor of FGFR1-4	Ongoing phase I trial
	JNJ-42756493	Inhibitor of FGFR1-4	Ongoing phase I trial
Monoclonal antibodies	GP369	Anti-FGFR2-iiib	Preclinical
	FPA144	Anti-FGFR2-iiib	Phase I trial
	MFGR1877S	Anti-FGFR3	Phase I trial
	IMC-A1	FGFR1-iiic	Preclinical
Ligand traps	FP-1039	Sequester FGF1, FGF2 and FGF4	Phase I trial
	GAL-F2	Neutralize FGF2	Preclinical

and cell lines with *FGFR1* amplification are more sensitive to brivanib as compared to those without [105].

Dovitinib It is a multi-kinase inhibitor of FGFR1-3, VEGFR1-3, c-KIT, fms-related tyrosine kinase 3 (FLT3), platelet-derived growth factor receptor (PDGFR) β, and colony-stimulating factor receptor 1 (CSFR1). Dovitinib is very effective in the 4T1 mouse model of metastatic breast cancer [101, 106]. A phase II trial using dovitinib in breast cancer demonstrated an improved overall response in patients with FGFR1 amplification [107]. Furthermore, qPCR analyses of these patients demonstrated a 21.1% decrease in target lesion size in patients that were amplified for *FGFR1* and/or *FGFR2* and/or *FGF3*.

Lucitanib It is an inhibitor of FGFR1-2 as well as VEGFR1-3 and PDGFR-α/β. Lucitanib was recently evaluated in a phase I/IIa trial where 6 out of 12 patients demonstrated a partial response [108]. In this trial patients were selected based on *FGFR1* or *FGF3/4/19* amplification (these three ligands are encoded on the same amplicon), if their tumor was newly progressing following response to a previous antiangiogenic therapy, or if their tumor was histologically determined to be potentially sensitive to antiangiogenic therapy. These results have led to the international FINESSE phase II trial that is currently underway. This trial is similarly using *FGFR1* and/or *FGF3/4/19* amplification to establish three cohorts to measure safety and efficacy of 15 mg Lucitanib daily (NCT02053636). Importantly, in this trial biopsy material for FISH analysis is being collected from metastatic sites. Clovis oncology is also initiating a multicenter phase II trial to evaluate similar patient cohorts for the safety and efficacy of 10 mg daily Lucitanib (NCT02202746).

BGJ398 It is a more specific inhibitor of FGFR [109]. Studies from our laboratory have shown reduction in *in vivo* pulmonary tumor growth of the D2.A1 model of metastatic breast cancer [55]. Currently, several clinical trials are underway to characterize the dose schedule and safety of BGJ398 including a phase II dose escalation trial in solid tumor patients with FGFR1/2 amplification or FGFR3 mutation (NCT01004224).

AZD4547 It is an orally available tyrosine kinase inhibitor selective for FGFR1-3 [110]. Similar to dovitinib, AZD4547 effectively inhibits *in vivo* tumor growth and metastasis of the 4T1 cells [111]. A recently reported translational clinical trial utilized FISH to identify FGFR1 amplification in 18% of advanced HER2-negative breast cancers [112]. Eight patients with FGFR1 amplification were treated with AZD4547 and one patient had a confirmed response. This study concluded that high levels of FGFR2 amplification in gastric cancers may be predictive for response to AZD4547, the focus of which was further evaluated in a recently completed larger phase II study (NCT01457846).

FIIN4 In collaboration with the laboratory of Nathanael Gray, we recently characterized the FGFR irreversible inhibitor 4 (FIIN4) [67]. FIIN4 is the final of a series of compounds developed in the Gray laboratory using a structure guided approach [113, 114]. It is a covalent inhibitor of FGFR1-4, but kinome scan

analyses demonstrated additional inhibition of PDGFRβ, CSFR1, RET, VEGFR2, FLT4 and cKIT. The enzymatic IC50 values for FIIN4 were in the low nanomolar range for FGFR1-3, and in a direct comparison of IC50 values for FIIN4 they were nearly tenfold lower than AZD4547 for FGFR1-3 and more than 100 fold lower for FGFR4. FIIN4 forms a covalent bond with a conserved cysteine in the P-loop of ATP binding pocket of FGFR1-4s and stabilizes the inactive state of the receptors. These compounds are able to overcome mutations in FGFR that render BGJ398 and AZD4547 inactive [113]. Additionally, we have demonstrated prolonged inhibition time and more effective cell targeting using FIIN4 as compared to BGJ398 [67, 99]. Using the 4T1 cells and patient-derived xenograft models we have also demonstrated the *in vivo* ability of orally administered FIIN4 to effectively inhibit pulmonary metastasis [67]. This inhibitor is yet to be evaluated in the clinical setting.

BLU9931 It is a covalent inhibitor that is specific for FGFR4 [115]. In contrast to FIIN4 that targets a conserved cysteine among FGFR1-4, BLU9931 forms a covalent bond with cysteine 552 that is unique to FGFR4. This interaction yields nearly a 100-fold selectivity to FGFR4 as opposed to FGFR1-3.

TAS-120 Similar to FIIN4, TAS-120 is described as a covalent inhibitor of FGFR, but these data have not been published. Currently, a dose finding phase 1 trial is ongoing with this compound in solid tumors and multiple myelomas with *FGFR* amplification and mutation events (NCT02052778).

JNJ-42756493 It is an inhibitor with low nanomolar IC50 values for FGFR. A phase 1 dose escalation study, using intermittent dosing (7-days-on/7-days-off), noted several responses in patients that harbored FGFR translocation events [116].

Anti-FGFR Therapeutic Antibodies FGFR signaling can also be more specifically blocked using monoclonal antibodies targeting individual FGFRs. Such antibodies either block ligand binding or block receptor dimerization. Currently, there are multiple therapeutics under preclinical development. For instance, treatment of breast cancer models with GP369, an antibody targeting the FGFR2-iiib isoform, showed efficacy [117]. Similarly, FPA144 is a mAb currently being evaluated in FGFR2 amplified gastric cancer (NCT02318329). Preclinical success has also been demonstrated with mAbs targeting FGFR3 in models of bladder cancer, and a dose escalation trial of the FGFR3 mAb, MFGR1877S, has recently been completed (NCT01363024) [118]. In contrast, monoclonal antibodies targeting FGFR1-iiic have been toxic in preclinical studies [119].

Ligand Traps FP-1039 has been developed by fusing the extracellular domain of FGFR1-iiic to the Fc region of IgG1 [120]. This ligand trap can sequester FGF1, FGF2 and FGF4. Also, Galaxy pharmaceutical has recently developed a novel mAb that neutralizes FGF2. Whether or not such ligand traps will be successful in *FGF* or *FGFR1* amplified breast cancer models remains to be determined.

Unique Benefits and Challenges to Targeting FGFR in Breast Cancer

As mentioned above several of the FGFR kinase inhibitors currently being pursued clinically also inhibit VEGFR yielding a potential benefit of dual targeting of tumor cells and angiogenesis. In addition, several recent studies point the ability of systemic FGFR inhibition to modulate the immune make-up of tumors [103, 121]. Indeed, recent studies utilized BGJ398 to delineate a mechanism by which FGFR:Akt signaling drives the expression of granulocyte-colony stimulating factor (G-CSF). This cytokine in turns enhances recruitment of myeloid derived suppressor cells (MDSCs) that potentiate an immune suppressive tumor microenvironment [121]. These studies have added to the biological complexity of FGFR signaling in driving tumor growth, but they also strongly suggest an exciting potential for the combination of the FGFR-targeted therapies with evolving immunotherapies. However, FGFR is also expressed on T-cells and contributes to their activation [122]. This presents the possibility that systemic targeting of FGFR may actually be blocking antitumor immunity. FGFR targeted therapies face other unique challenges associated with off-target effects. Compounds such as Dovatinib and Lucitanib are non-specific inhibitors of FGFR and several other tyrosine kinases. The development of BGJ398 and AZD4547 have allowed for specific targeting of the FGFR kinases. However, as mentioned above the diversity in FGFR expression is increased by alternative splicing events that generate unique extracellular domains. Indeed, all isoforms of FGFR encode similar kinase domains and are similarly subject to kinase inhibition. Surprisingly, we and others have clearly identified anti-tumorigenic functions for particular FGFR isoforms, and therefore pan-kinase inhibition of these particular isoforms may contribute to undesired affects [55, 72]. Finally, as mentioned above *FGFR1* is commonly co-amplified with the powerful oncogene *MYC*, and it is not clear if *MYC* co-amplification nullifies the tumor driver effect from upstream FGFR signalling. Lack of understanding in splicing and co-amplification events could be contributing to the failure of *FGFR1* amplification to predict for the patient response to the AZD4547 [112].

Conclusions

As our molecular understanding of breast cancer increases, targeted therapies will continue to be developed and more accurately applied to patients most likely to benefit from them. Overall, FGFR kinase inhibitors are quickly evolving and becoming an exciting new area of therapeutic development in breast cancer. However, clinical success of these compounds will be limited by our current inability to properly identify the proper patient population. Unlike Her2, gene amplification of FGFR1 does not seem to effectively identify patients, and while FGFR2 amplification does seem to have better success, this molecule is rarely amplified in

breast cancer. To properly stratify breast cancer patients, we must take into account several aspects including co-amplification of other potential driver genes, identification of receptor isoforms, subcellular localization and identification of potential receptor cofactors. Such comprehensive diagnostic profiling is not currently feasible at most medical centers, but as has been the case with several other kinase inhibitors, the full potential of FGFR as a therapeutic target in breast cancer will not be realized until the optimal small molecule is matched with the proper companion diagnostic.

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Targeted Therapies in Breast Cancer

Anna T. Lyons and Jenifer R. Prosperi

Abstract As the most prevalent form of cancer found in women, breast cancer is an active area of research and clinical study. Treatments for cancer patients have traditionally relied upon chemotherapy, but an increasing emphasis is placed on targeted therapies as a safer and more effective way to treat cancer. Therapies that target specific aspects of cancerous cells may be especially significant for triple negative breast cancer (TNBC), which is often associated with poor patient prognosis and currently lacks reliable targeted treatment options. Many therapies for breast cancer have been extensively studied and examined in clinical settings, while others show future potential and require further investigation. This chapter will focus on six targeted therapies, describing mechanisms of action and current methods of inhibition. Inducible nitric oxide synthase (iNOS) overexpression is correlated with increased cell proliferation and mammosphere production, while inhibition has been shown to minimize these effects in cancerous cells. The PI3K pathway is an essential component of intracellular signaling that is frequently dysregulated in tumors. Poly(ADP-ribose) polymerases (PARP) are implicated in DNA damage repair, and are the focus of numerous ongoing clinical trials. Protein tyrosine kinase 6 (PTK6) has been associated with cell proliferation and tumor growth in breast cancer cells, while the serine/threonine cyclin-dependent kinase (CDK) protein family is a crucial component of cell cycle regulation and an important area of research for targeted therapies. Finally, aberrant activation of the Wnt/β-catenin signaling pathway has been implicated in multiple breast cancer subtypes, particularly with respect to the prevalence β-catenin mutations in TNBC. These pathways represent promising topics in the

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field of targeted therapies, and with increased research may contribute to the development of superior and precise treatments for breast cancer.

Abbreviations

ABC	ATP-binding cassette
APC	Adenomatous polyposis coli
CDK	Cyclin-dependent kinase
CK1α	Casein kinase-1α
CSC	Cancer stem cell
DSB	Double-stranded break
DVL	Dishevelled
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FAK	Focal adhesion kinase
FOXM1	Forkhead box M1
FZD	Frizzled
GSK3β	Glycogen synthase kinase 3β
HER2	Human epidermal growth factor 2
HR	Homologous recombination
HR+	Hormone receptor-positive
iNOS	Inducible nitric oxide synthase
NOS	Nitric oxide synthase
NSAID	Nonsteroidal anti-inflammatory drug
PARP	Poly(ADP-Ribose) polymerase
PARPi	PARP inhibitor
PI3K	Phosphoinositide 3-kinase
Prcn	Porcupine
PTEN	Phosphatase and tensin homolog
PTK6	Protein tyrosine kinase 6
Rb	Retinoblastoma
RTK	Receptor tyrosine kinase
SSD	Single-stranded break
STAT	Signal transducer and activator of transcription
TCF/LEF	T-cell factor/lymphoid enhancer factor
TNBC	Triple-negative breast cancer

Introduction

Treatment of breast cancer has increasingly focused on targeted therapies as a form of specific and effective treatment for patients. These therapies employ a variety of mechanisms with the ultimate goal of halting tumor cell survival and proliferation without damaging noncancerous cells. There are multiple pathways that have shown promise as targeted therapies in breast cancer. While the most common ones have been discussed in significant detail throughout the book, this chapter will briefly discuss the following potential targets: inducible nitric oxide synthase (iNOS), phosphoinositide 3-kinase (PI3K), poly(ADP-ribose) polymerases (PARP), protein tyrosine kinase 6 (PTK6), cyclin-dependent kinase 4/6 (CDK4/6), and the Wnt/β-catenin pathway. Within each pathway, we will discuss background information and basic science research. We will then provide an overview of methods of inhibition and clinical studies for each target. A better understanding of these targets, as well as mechanisms of inhibition, may be beneficial to the advancement of breast cancer treatment.

Inducible Nitric Oxide Synthase (iNOS)

Introduction and Basic Research

Nitric oxide is a free radical produced by three nitric oxide synthase (NOS) isoforms: neuronal, endothelial, and inducible synthases. The endothelial and neuronal isoforms generate smaller amounts of NO, and the effects of NO production have shorter durations using these isoforms. However, inducible nitric oxide synthase (iNOS) has been identified as a target for breast cancer. High levels of iNOS have been implicated in increased cell proliferation and self-renewal of cancer stem cells (CSC) [1]. iNOS has been examined for potential correlations with increased p53 mutations and increased cell motility [2]. Inhibition of iNOS has been show to decrease migration of cells and reduce levels of the epithelial-mesenchymal transition (EMT) transcription factors Snail, Slug, and Twist1 [1]. Although many of the mechanisms by which iNOS influences cancer cell progression are unknown, more attention is being paid to the clinical applications of iNOS inhibition and its status as a therapeutic target, especially in triple-negative breast cancer (TNBC).

iNOS is moderately to strongly expressed in 70% of breast tumors, and high expression of iNOS is correlated with increased p53 mutations [2]. High levels of iNOS are especially seen in ER-negative cells, and NO production has the ability to induce cell motility in these cells. The relationships between iNOS expression and the gene expression profile in ER-negative breast tumors has also been examined, with high levels of iNOS correlating to increased levels of IL-8, which is associated with an invasive phenotype of cancer cells [2]. Additionally, increased NO production leads to increased phosphorylation of epidermal growth factor receptor (EGFR) at residues Tyr1045 and Tyr1173 [2]. Activation of EGFR is a marker of poor prognosis

and promoter of angiogenesis, and EGFR signaling has been linked to upregulation of iNOS in breast cancer. Studies demonstrating the role of NO in increased breast cancer cell motility, invasion, and proliferation make iNOS an important subject for research and targeted therapies.

Inhibition of iNOS and Clinical Studies

Possible inhibitors for clinical testing include the NOS inhibitors, L-NMMA and L-NAME, and the iNOS specific inhibitor 1400 W. 1400 W decreases cell proliferation and migration in TNBC cells. Additionally, inhibition of iNOS with 1400 W reduced levels of EMT transcription factors normally expressed during tumor invasion, thereby decreasing migration of TNBC cell lines [1]. The NOS inhibitor L-NAME reduces tumor growth in mouse models, and administration of L-NMMA either alone or with docetaxel reduced tumor growth and tumor initiating abilities. In addition, recent studies have demonstrated that platinum drugs may be more effective in treating TNBC when iNOS is inhibited [3].

Increased expression of iNOS in TNBC is a predictor of poor patient survival due to the correlation of iNOS with increased cell proliferation, efficiency of mammosphere production, and heightened levels of EMT transcription factors [1]. This was verified by analyzing the relationship between iNOS levels and patient outcomes in multiple databases of TNBC. Inhibitors of iNOS have been shown to decrease tumor cell proliferation as well as cancer stem cell renewal, making iNOS a promising area of research regarding targeted therapies for TNBC.

Phosphoinositide 3-Kinase (PI3K) Pathway

Introduction and Basic Research

The phosphoinositide 3-kinase (PI3K) pathway is a cell-signaling pathway that regulates cell growth, proliferation, motility, and survival. PI3Ks constitute a family of kinases that are responsible for phosphorylating phosphoinositides, a group of lipids heavily involved in intracellular communication [4]. The pathway begins with activation by receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCRs), which allows for phosphorylation of PIP_2 to form PIP_3. Binding of the AKT nodal kinase to PIP_3 initiates a cascade of signaling events influencing cell survival and proliferation. The pathway is opposed by the lipid phosphatase and tensin homolog (PTEN), which dephosphorylates PIP_3 to halt AKT activity [5]. Importantly, mutations or amplifications in every major component of this pathway are seen in cancer, making the PI3K pathway an attractive subject for targeted therapies. PI3Ks can be divided into three classes (I, II, and III), with current research in targeted therapies focused on Class I PI3Ks. This class can be further subdivided into Class IA and IB [6]. Class IA of the PI3K family is comprised of PI3Kα, PI3Kβ, and PI3Kδ, which is

a heterodimer of a regulatory subunit (p85α, p55α, p50, p85β, or p55γ) and a catalytic subunit (p110α, p110β, or p110δ) [4–6]. Class IB consists of PI3Kγ, also a heterodimer, which is distinguished by its inability to bind to the p85 regulatory subunit type [6]. PI3Ks of Class IA are most commonly implicated in human cancers; however, current studies focus on developing both pan-specific and isoform-specific PI3K inhibitors as forms of targeted therapy. Isoform-specific small-molecule inhibitors are of special interest due to the distinct genes, structures, and substrate preferences amongst different classes of PI3Ks [7].

Inhibition of PI3K and Clinical Studies

The PI3K pathway is an active area of research as nearly every major component is altered in tumors, including upstream epidermal growth factor receptor (EGFR) responsible for activating PI3K, the downstream Protein Kinase B (PKB/AKT), and the negative pathway regulator PTEN [5]. Clinical research has focused on both pan and class-specific inhibitors as potential therapies. Two relevant pan inhibitors are the competitive ATP-binding proteins, wortmannin and LY294002. Wortmannin is a fungal product that reacts with the p110 catalytic subunit of PI3K and has been shown to be a powerful pan inhibitor. However, the study of wortmannin is hindered by its short half-life and instability in culture media [8]. The synthetic compound LY294002 is another significant pan PI3K inhibitor that has been shown to be an effective in combination with radiation; however, clinical trials have yet to be undertaken [9].

The significance of this pathway in tumor initiation, cell growth, and proliferation has made it an active area of clinical research, especially with regards to isoform-specific inhibitors. Clinical studies examining isoform-specific inhibitors have focused on Class IA and IB PI3K isoforms. PI3Kα of Class IA is highly mutated in solid tumors, and the PI3Kα-specific inhibitor A66 has shown promise in preclinical trials for its ability to inhibit proliferation. The compound Alpelisib (BYL-719) is a PI3Kα inhibitor that has undergone phase I and II clinical trials and induces apoptosis in certain cell types. Additionally, this compound may impede the role of PI3K in angiogenesis, as indicated by decreased glucose consumption of the cell [7]. Research regarding the mechanisms of this pathway has revealed the context-specific and physiologically significant role of individual PI3K isoforms. For instance, the PI3Kδ isoform is important in mediating the immune modulation and function of T cells, B cells, mast cells and neutrophils. Examples of PI3Kδ-specific inhibitors in clinical studies include IC87114 and CAL101, a potential therapy that has undergone phase I, II and III trials and has shown promising responses in patients with leukemia. Other potential targeted therapies for breast cancer include AZD8186, a PI3Kβ inhibitor that has undergone phase I clinical trials, and GDC0032, which targets PI3Kα, PI3Kδ and PI3Kγ members of the Class I family [7]. The multiple isoforms of PI3K and its important role in cell signaling make it an important and ongoing area of research for targeted therapies, and current studies prove the large potential of PI3K isoform inhibitors in clinical settings.

The complete oncogenic mechanisms of the PI3K pathway are not yet understood, but a more enhanced understanding of this pathway would aid in differentiating between patients who would benefit from PI3K inhibitors alone versus those requiring a combination therapy [5]. Recent studies have focused on human epidermal growth factor receptor 2 (HER2), as it is overexpressed in 25–30% of breast and ovarian cancers and is associated with poor prognosis and decreased survival [10]. Trastuzumab (Herceptin) is a monoclonal antibody that targets HER2, and activation of the PI3K pathway has been shown to mediate trastuzumab resistance in breast cancer cells [11]. Preclinical studies demonstrated that the inhibitor BAY 80-6946, which selectively inhibits the Class IA isoforms PI3Kα and PI3Kδ, decreases proliferation in HER2-positive breast cancer cells [12]. It was also shown that the combination of BAY 80-6946 with traditional HER2-targeted therapies including trastuzumab may be a potential clinical treatment for patients whose cancers show resistance to HER2-targeted therapies, since a combination of the two may restore sensitivity of HER2-positive cells to traditional therapies [12].

Poly(ADP-Ribose) Polymerase (PARP)

Introduction and Basic Research

Poly(ADP-ribose) polymerases (PARP) are a group of enzymes involved in DNA-damage repair. Eighteen enzymes have been identified in this category; however, DNA damage drives only the activation of PARP-1, -2, and -3. PARP synthesizes ADP-ribose polymers that mark locations of DNA damage, which then signal for the formation of DNA-repair complex sites [13]. PARP inhibition is an important subject for targeted therapy research in breast cancer because of its effects in TNBC cells exhibiting BRCA1 and BRCA2 mutations. Mutations in the BRCA tumor suppressor genes cause damage to the DNA homologous recombination (HR) pathway, which is crucial in repairing double-stranded breaks (DSB) that occur at the replication fork. In BRCA-proficient cells, the HR pathway can repair DSBs, resulting in cell proliferation and survival. In BRCA-mutant cells, the HR pathway is unable to repair DNA at the replication fork, leading to increased DNA damage and cell death. PARP is primarily implicated in single-strand break (SSB) sites, where it repairs DNA damage through poly(ADP-ribosyl)ation of enzymes responsible for histone and chromatin modification. Additionally, PARP recruits DNA damage repair proteins, which lead to the correction of SSB errors and continued survival and proliferation of the cell. PARP inhibition may cause an increase in SSBs, which are converted to irreparable DSBs [14]. Because of the important role of PARP in DNA damage repair and its relationship with BRCA1 and 2 proteins, inhibition of PARP is a well-researched and clinically tested targeted therapy.

Inhibition of PARP and Clinical Studies

The mechanism through which PARP inhibitors lead to cell death in BRCA1 and 2 deficient tumors has been labeled "synthetic lethality" [14]. There are currently two relevant models explaining synthetic lethality. In the first model, inhibiting PARP effectively "traps" PARP at the DNA repair site, which blocks the replication fork and relies on the HR pathway to fix the damaged site. However, in the second model PARP itself is involved in restarting the stalled replication fork in a pathway independent of HR. In both models, PARP is essential to protecting the stalled replication fork, and inhibition of PARP leads to the accumulation of DNA damage and the inability of the cell to repair single- and double-strand errors. PARP inhibitors (PARPi) including veliparib prevent the DNA damage repair complex from forming, leading to an accumulation of SSBs and cell death in the case of an inefficient HR pathway. Other PARPi (olaparib, talazoparib, rucaparib, and niraparib) work later in the overall pathway by trapping PARP at the replication fork, which prevents dissociation of the complex from the site of DNA damage and leads to DSBs [13]. While PARP inhibition is promising, resistance to PARPi has been observed. The mechanisms of PARPi resistance include restoration of BRCA functionality through secondary mutations in BRCA1 and 2, rewiring of DNA damage repair through mutations in p53 binding protein 1, and increased drug efflux resulting in a decreased amount of intracellular PARPi [15]. However, breast cancer cell sensitivity to PARP inhibition has been demonstrated in various cases. Increased sensitivity to PARPi has been found in breast cancer cells low in ataxia telangiectasia mutated (ATM) kinase [16], as well as in cells that overexpress lysine-specific histone demethylase (LSD1) [17]. Despite the observation of PARPi resistance, clinical trials have proven that in general, PARP has been an effective target for therapy in TNBC.

Clinical trials have focused on using PARPi either by themselves as targets for cancer cells with specific features (with a focus on BRCA1 and 2 mutations), or in combination with other cytotoxic drugs including cisplatin, carboplatin, and topotecan [13]. The six PARPi compounds that have been the most extensively studied in clinical trials are olaparib, veliparib, niraparib, talazoparib, rucaparib, and CEP-9722. Phase I testing of olaparib showed a partial response in 47% of patients with BRCA-associated breast, ovarian, and prostate cancers, with 63% of patients reporting clinical benefit as defined by tumor marker decrease or disease stability for at least 4 months. Current phase III trials are examining the effectiveness of olaparib as a monotherapy [18]. Veliparib has been examined primarily for its efficacy as part of combination therapy. This PARPi has been shown to increase the cytotoxic effect of temozolomide, an oral chemotherapy drug. A recent phase II trial showed that combination therapy of temozolomide and veliparib resulted in a response rate of 22%, with 50% of participants in the 41 person-large study reporting clinical benefit. Niraparib and talazoparib, while less extensively studied than other inhibitors, demonstrated a 50% and 33% objective response rate, respectively, in phase I trials. Both are being examined in phase III testing as monotherapeutic inhibitors [19]. Rucaparib is being examined in two phase II trials, in one case as a monotherapeutic inhibitor and in the other as a treatment in combination with cisplatin. Effects of the

inhibitor CEP-9722 are being studied in phase II trials in solid tumors. The toxicity of these inhibitors is thought to be similar to the level of toxicity found in other chemotherapeutic agents; however, it is unknown if the use of DNA damage repair inhibitors may lead to a higher chance of developing new malignancies [13]. This has been observed in a very small number of cases in which patients were previously treated with other chemotherapeutic drugs known to cause DNA damage and is a subject of current research. The effectiveness and variety of targeted therapies, which work to inhibit PARP at various stages of DNA repair, make them especially relevant as therapies for BRCA-mutated breast cancers.

Protein Tyrosine Kinase 6 (PTK6)

Introduction and Basic Research

Protein tyrosine kinase 6 (PTK6) is a member of a distinct group of kinases found in both normal and cancer cells. PTK6 is an auto-phosphorylating protein comprised of 451 amino acids and has three regions responsible for protein and intracellular reactions: the tyrosine kinase domain, SH2, and SH3. PTK6 lacks amino-terminal myristolation/palmitoylation signals, resulting in flexibility in intracellular location. Studies suggest that the specific localization of PTK6 may influence its function; however, the mechanisms leading to PTK6 localization are unknown [20]. PTK6 is common in epithelial lining cells, particularly in the gut, and in normal cells has been shown to play a role in cell differentiation and survival [21]. However, PTK6 in breast cancer cells has been shown to play a role in cell proliferation and migration as well as tumor growth [20]. Because of its presence in over 60% of breast cancer cells, PTK6 is a promising area of study in terms of targeted therapies for breast cancer.

PTK6 activates signaling pathways that promote growth in breast cancer cells. The ErbB receptor tyrosine kinase is activated by PTK6, and PTK6 is overexpressed with both ErbB3 and ErbB4 in many breast cancer cells. ErbB3 stimulation is related to an increase in epidermal growth factor (EGF) signaling, which in turn promotes cell proliferation [22]. PTK6 can regulate the phosphorylation of paxillin by EGF and increase the sensitization of cancer cells to EGF. Phosphorylation of paxillin by EGF activates the GTPase Rac1 and leads to increased cell motility and migration [23]. Additionally, PTK6 activates members of the signal transducer and activator of transcription (STAT) family, including STAT3 and STAT5b, which promote cell transformation, differentiation, and inflammation [24, 25]. PTK6 interacts with focal adhesion kinase (FAK), whose activity is related to increased cell survival and proliferation. FAK is subsequently activated by the AKT pathway, which can inhibit apoptosis-promoting proteins and is commonly activated in cancer cells as described above. Through interactions with FAK and AKT, PTK6 expression results in resistance to apoptosis and a survival advantage in an anchorage-independent manner [26]. The critical roles of PTK6 in cell survival and proliferation has

made it the focus of an increasing number of clinical studies on targeted therapies with the intent to inhibit PTK6 and decrease its activity in breast cancer.

Inhibition of PTK6 and Clinical Studies

Overexpression of PTK6 in breast cancer and its role in promoting apoptosis resistance make it a significant therapeutic target. PTK6 complexes with IGF-1 receptor (IGF-1R), and regulates its phosphorylation and expression to mediate anchorage-independent survival of cells [27]. PTK6 is highly expressed in human epidermal growth factor 2^+ ($HER2^+$) breast cancers, some of which are resistant to the targeted therapy Lapatinib. In an *in vitro* model, PTK6 overexpression resulted in resistance to Lapatinib treatment [28], suggesting that PTK6 expression may be a marker for Lapatinib resistant $HER2^+$ breast cancers. Inhibition and down-regulation of PTK6 using shRNA leads to increased expression of Bim, a protein required for apoptosis. Bim is not expressed in Lapatinib-resistant breast cancer cells; however, inhibition of PTK6 leads to the activation of the p38 mitogen-activated protein kinase (MAPK), which in turn increases expression of the pro-apoptotic protein Bim [28]. Combined, the impact of PTK6 overexpression is correlated with resistance to $HER2^+$ targeted therapies. Additional studies have examined the prevalence of PTK6 overexpression in TNBC. PTK6 overexpression has been observed in 70% of TNBCs, where it promotes cell migration and survival as well as contributes to EMT [29]. In one study, inhibition of PTK6 via siRNA or shRNA vectors was shown to reduce migration, induce anoikis, and halt metastases of TNBC cells, further demonstrating the potential in targeting this pathway [29].

The interaction of PTK6 with HER2 provides an option for therapeutic targets, as HER2 and PTK6 interaction promotes growth of breast cancer tumors. Trastuzumab (Herceptin), a monoclonal antibody targeting HER2, is correlated with improved survival rates in patients overexpressing the HER2 protein [30]. However, resistance to trastuzumab commonly develops and additional therapies targeting PTK6 and the HER2 receptor tyrosine kinase should be examined [20]. The ability of PTK6 to promote anchorage-independent growth of tumor cells as well as increase cell proliferation, migration, and tumor growth make it an important subject for future targeted therapy studies.

Cyclin-Dependent Kinases CDK4/6

Introduction and Basic Research

The serine/threonine cyclin-dependent kinase (CDK) family of proteins plays an essential role in regulating the cell cycle. A dividing cell must progress through the G1 (pre-DNA synthesis), S (DNA synthesis), G2 (pre-division), and M (mitosis)

phases, and its ability to do so is monitored by CDKs at each step [31]. Dysfunctional cell cycle regulation results in uncontrolled proliferation, which is frequently seen in cancerous cells [32]. The importance of these kinases in functional cells as well as their implication in tumorigenic cells has made them a promising target for inhibition and potential therapeutic treatments. Two members of this family, CDK4 and CDK6, are of particular interest due to their regulation of the G1-S transition [31]. The two kinases share 71% amino acid identity and function primarily by regulating phosphorylation of retinoblastoma (Rb) at the end of G1 phase via association with D-type cyclin proteins [31]. Rb is crucial in regulating cell cycle progression. Phosphorylation and inactivation of Rb allows dissociation of the Rb-EF2 complex, which permits EF2 transcription factors to activate genes necessary for progression into S phase. When Rb is unphosphorylated in its active form, EF2 transcription factors are suppressed and cells are unable to enter S phase, halting the cell cycle [31]. Many types of cancer cells display overexpression of CDK4/6 or loss of CDK4/6 negative regulators, both of which result in uncontrolled cell proliferation. Dysfunction of these kinases is especially significant in breast cancer, where studies have shown that the CDK4-encoding gene is amplified in 16% of cases and CDK6 levels are increased in 17%. Additionally, the cyclin D protein has been shown to be overexpressed in 50% of breast cancer cases [33]. The importance of CDK4/6 in regulating cell proliferation and its direct association with numerous cancer types has led to increased study of CDK4/6 inhibition and its potential in a targeted therapy approach.

Inhibition of CDK4/6 and Clinical Studies

Natural CDK4/6 inhibitors include proteins from the INK4 family ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$), as well as Cip and Kip family proteins ($p21^{CIP1}$ and $p27^{KIP1}$) [34]. The inhibitor $p16^{INK4A}$ facilitates formation of the cyclin D-CDK complex, and has proven to be an important tumor suppressor [35]. Initial efforts to inhibit CDK4/6 focused on pan-CDK inhibitors. The drug flavopiridol was initially implicated as a potential therapy, but was discontinued due to low CDK specificity and adverse results [36]. Recent efforts have focused on monoclonal approaches using specific CDK4/6 inhibitors, as well as using these treatments in combination with other types of therapy.

Three selective CDK4/6 inhibitors have emerged as the most relevant forms of monotherapeutic treatment. The first is palbociclib, which prevents cell growth by preventing phosphorylation of Rb, which down-regulates E2F transcription factor activity and stalls cell growth [37, 38]. Additionally, it inactivates the FOXM1 transcription factor to reduce cell proliferation [39]. This drug performed well in Phase II trials, preventing disease progression in 165 breast cancer patients for over 2 years. Palbociclib showed additional potential when used in combination with letrozole, an aromatase inhibitor [38]. The second specific CDK4/6 inhibitor is ribociclib, which functions by arresting cells in the G1 phase, preventing growth and

division. Like palbociclib, this drug works especially well when used in combination with other therapies, including letrozole [40]. The PI3K/AKT/mTOR pathway is an upstream regulator of CDK4/6 activity, and combined treatment of ribociclib and PI3K pathway inhibitor alpelisib showed promising results in breast cancer tumor growth in mouse models [41]. The third specific CDK4/6 inhibitor, abemaciclib, is an effective treatment for cancer cells over-expressing ATP-binding cassette (ABC) transporters, which contribute to multi-drug resistance [36]. A Phase I study of treatment with abemaciclib showed a 30% or more decrease in tumor size from initial levels, and another Phase I trial showed partial responses in 50% of hormone receptor-positive (HR+) cases and 33% of HR- cases [42, 43].

These three CDK4/6 specific inhibitors have been examined in combination with endocrine therapy with promising results. Palbociclib, in particular, was shown to inhibit proliferation of endocrine-resistant cells, which present a major problem in the treatment of HR+ breast cancer [44]. Studies of CDK4/6 inhibitors in combination with chemotherapy have shown conflicting results. Inhibition was shown to sensitize neuroblastoma cells to doxorubicin-induced apoptosis; however, other studies have shown that palbociclib reduces the toxicity and efficiency of platinum agents and anthracyclines [45, 46]. Due to the importance of CDK4/6 in normal cell functioning and its activity in a wide variety of cancer types, inhibition is an active area of research. CDK4/6 inhibitors show great potential when used alone or in combination with other forms of treatment, and more studies are in progress to examine the mechanisms of inhibition and the way to most effectively target this important component of the cell cycle and proliferation.

Wnt/β-Catenin Pathway

Introduction and Basic Research

The Wnt/β-catenin signaling pathway has been appreciated for its essential role in tissue development, cell proliferation, polarity, and stem cell stability. The Wnt family of glycoproteins is a highly conserved component of embryonic and mammary gland development, with pathway dysregulation occurring in various breast cancer subtypes including TNBC [47]. Wnt signaling is divided into canonical and non-canonical pathways; however this piece will focus on mechanisms and possible modulators of canonical signaling. In the inactive state, the intracellular signal transducer β-catenin remains localized in the cytosol and is targeted for proteasomal degradation by the "destruction complex" (Axin, glycogen synthase kinase 3β (GSK3β), Adenomatous Polyposis Coli (APC), and casein kinase-1α (CK1α)). Aberrant activation of the pathway has been shown to contribute to tumorigenesis in various cancer types [48]. Activation of the signaling pathway occurs when Wnt ligands, acetylated by the membrane-bound Porcupine (Prcn), bind to the transmembrane receptor frizzled (FZD) and co-receptors LRP5/6. This permits interaction with the scaffolding proteins Dishevelled (DVL) and Axin, rendering the

previously described “destruction complex” unable to function. The pathway can also be activated by any disruption to a protein in the destruction complex or a stabilizing mutation to β-catenin itself. β-catenin mutations occur at a high frequency in TNBCs and have been shown to drive tumorigenesis [49]. Any method of pathway activation results in β-catenin accumulation and translocation to the nucleus, where it binds to T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors [48]. Pathway activation ultimately results in transcriptional regulation of Wnt target genes commonly implicated in cell growth and tumorigenesis, cMyc and cyclin D1 [50]. Notably, activation of the Wnt pathway has been implicated in survival and maintenance of CSCs, further highlighting the necessity of targeted therapies [51]. The association of Wnt signaling with poor patient outcome has made it an attractive target for potential therapies; however, the complex role of Wnt signaling in normal and cancerous cells must be further examined.

Inhibition of the Wnt/β-Catenin Pathway and Clinical Studies

Despite the demonstrated need for therapies targeting the Wnt pathway, development of inhibitors has been complicated by the importance of Wnt signaling in mammary gland development and tissue regeneration [47], in addition to the importance of the absolute level of Wnt signaling. Our laboratory has previously described the use of Wnt pathway inhibitors in breast cancer [47], and will herein provide an update and discuss innovative approaches to Wnt pathway inhibition that are currently under investigation. Multiple small molecule inhibitors have been identified in preclinical trials due to their ability to antagonize or modulate various components of the pathway. The most notable of small molecules is the Prcn inhibitor LGK974, which prevents acetylation of Wnt ligands and subsequent activation of signaling. LGK974 is currently undergoing Phase I clinical trials for a variety of cancers including TNBC and has been shown to decrease Wnt signaling *in vivo* and *in vitro* [52]. However, the anti-FZD receptor monoclonal antibody vantictumab, has been shown to effectively target the canonical pathway while hindering osteogenesis [53]. One possible approach may be the combination of Wnt inhibitors with therapies targeting other pathways. For example, a 2017 study demonstrated the potential of combining LGK974 with the pan-PI3K inhibitor buparlisib in treating TNBCs [54]. Recent efforts have focused on the utilization of previously approved drugs to target components of the Wnt/β-catenin pathway. For instance, the antihelminthic drug niclosamide has been shown to inhibit Wnt signaling through suppression of the co-receptor LRP6, preventing the accumulation of nuclear β-catenin and subsequent transcription of Wnt target genes [55]. The antibiotic salinomycin is being investigated as a potential therapy for TNBC due to its ability to damage CSCs by way of Wnt pathway interference [56]. Other studies have explored the possibility of repurposing nonsteroidal anti-inflammatory drugs (NSAIDs) in order to target the pathway. Extended intake of these drugs, including sulindac, aspirin, and celecoxib, has been correlated with tumor reduction in breast cancer and interference of the Wnt-associated COX enzymes [48]. Despite the challenges faced in

targeting Wnt/β-catenin signaling, studies to determine mechanisms of action and inhibition may benefit many patients faced with poor prognosis and limited treatment options. In addition, we have just published a detailed overview on the Wnt pathway in epithelial cancers, with a discussion on the benefits and challenges in targeting this diverse and dynamic pathway [57].

Summary

As the mechanisms and significance of cellular pathways in breast cancer are elucidated, the need for targeted therapies becomes increasingly clear. While previous chapters have focused on the most common targeted therapies in breast cancer, this chapter serves as an overview of selected new and upcoming pathways in the treatment of breast cancer. The targets described in this chapter do not comprise an exhaustive list, and various methods of targeting are beyond the scope of this piece. For example, we have focused on the targeting of oncogenic pathways; however, targeting tumor suppressors and their associated signaling pathways also holds great potential in terms of therapeutic treatment. The pathways and inhibitors described in this chapter highlight significant advances made in the area of targeted therapies, but further research must be done to maximize the potential of these treatments. This book concludes with a discussion of the challenges and future directions of targeted therapies, and it is probable that other treatments may be on the horizon. Targeted therapies are a precise and effective form of treatment for breast cancer and, with increased understanding and experimentation, may contribute to improved patient prognosis and a more efficient, powerful way to manage this disease.

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Future Paradigm of Breast Cancer Resistance and Treatment

Ravi Velaga and Masahiro Sugimoto

Abstract Despite advances in early detection and the understanding of the molecular bases of breast cancer biology, the real challenges in therapeutics lie in detecting the disease progression and relapse. Resistance to therapy is not only common but expected. Multidisciplinary joint efforts are required in making necessary progress with breast cancer treatment. With the recent advances in multiplex genotyping and high-throughput genomic sequencing technologies, breast cancer is now considered as a group of diseases characterised by varied clonal evolution with different molecular and cellular mechanisms which drive tumour initiation, proliferation and progression with underlying resistance. Using the liquid biopsy, attempt to discover clinical molecular biomarkers are progressing rapidly as we begin to understand the complex mechanisms that transform a normal cell to a cancer cell and leading to a resistant cell. One of the examples of these molecularly targeted biomarker therapies in HER2/neu-positive breast cancer is HER2/neu blockage. Following endocrine therapy, the occurrence of secondary resistance, such as ESR1 mutations, poses a significant challenge. Drugs like lapatinib may be effective to overcome EGFR therapy resistance but it needs to be established yet. FGFR target therapy may also be interesting, but still little is known about its clinical significance. Analysis of liquid biopsy has the potential to change the clinical practice by exploiting the blood rather than the tissue as a source of underlying mechanisms. Multiple clinical studies on liquid biopsies have already been used to monitor disease response and track the emergence of drug resistance. With the computing power, the sheer amounts of data generated through sequencing and other technologies are exponentially increasing with each day. This also creates a gap between the possibilities and which can be practiced clinically. Artificial intelligence algorithms could help to determine what type of resistance a patient attains with the disease relapse and whether a tumor is a new cancer or a recurrence of previous disease, all

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of which has implications for treatment. Targeted therapeutics including EGFR and FGFR amplifications have been detected and associated with endocrine resistance in hormone receptor-positive breast cancers have been discussed in the previous chapters of this book. In this chapter, we present the potential of circulating tumour DNA in improving and understanding the possible resistance mechanisms through cancer genomics and integrating with artificial intelligence.

Abbreviations

ADTree	Alternative decision tree
AI	Artificial intelligence
AIs	Aromatase inhibitors
ARMS	Amplification refractory mutation system
ASCO	American Society of Clinical Oncology
BEAM	beads, emulsion, amplification and magnetics
CDK	Cyclin dependent kinase
cfDNA	Cell free DNA
CIN	Chromosomal instability
CTC	Circulating tumour cells
ctDNA	Circulating tumour DNA
CTLs	Cytotoxic T cells
ddPCR	droplet digital PCR
ER	Estrogen receptor
FFPE	Formalin-fixed paraffin-embedded
Her2	Human epidermal growth factor receptor 2
HR	hormone receptor
HRD	Homologous recombinant deficiency
ICGC	International Cancer Genome Consortium
JBCRG	Japan Breast Cancer Research Group
LOH	Loss of heterozygosity
LST	Large scale transitions
MATCH	Molecular analysis for therapy choice
MBC	Metastatic breast cancer
MFA	Multi-factorial, principal component analysis
ML	Machine learning
MLR	Multiple logistic regression
MPACT	Molecular profiling-based assignment of cancer therapy
NCI	National Cancer Institute
NGS	Next generation sequencing
NLP	Natural language processing
PAP	Pyrophophorolysis-activated polymerization
pCR	Pathologic complete response
PD1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1

PDL1/2	Programmed cell death ligands 1 or 2
PFS	Progression-free survival
PR	Progesterone receptor
SVM	Support vector machine
TAI	Telomeric allelic imbalance
TAM Seq	Tagged-amplicon deep sequencing
TAMs	Tumour associated macrophages
TAPUR	Targeted agent and profiling utilization registry
TCGA	The Cancer Genome Atlas
TEPs	Tumour associated platelets
TILs	Tumour infiltrating lymphocytes
TNBC	Triple negative breast cancer

Introduction

Owing to the clonal evolution and selection [1, 2], the tumour develops resistance to treatment over a period of time. This is when the cancer cells figure out how to survive against the standard treatments. The cells that escape and survive from preoperative (neoadjuvant) and/or postoperative (adjuvant) systemic therapy become resistant cells. The resistant cells in metastatic distant organs eventually grow and result in the recurrence of the disease. Though the breast cancer survival outcome has largely improved in the past two decades, unfortunately, many breast cancer patients still develop relapse of the disease showing resistance to the conventional treatments. The advent of next generation sequencing (NGS) has enabled more powerful and near accurate analysis of tumor evolution and has improved our understanding of tumor initiation and development. Despite these advances, knowledge of the intra tumour heterogeneity, clonal evolution and the potential for competitive release of resistant subclones is infrequently considered in the therapeutic setting. Therefore, understanding the mechanisms that lead to treatment resistance through clonal evolution is crucial in developing novel diagnostics and therapeutics, and in improving the overall breast cancer survival. The NGS application to matched primary and metastatic samples helps in identifying sets of shared and private mutations, sample relatedness and in determining an approximate evolutionary relationship. Currently, based on the primary tumour biology, the breast cancer clinical management primarily relies on relatively three prognostic/predictive clinical markers (estrogen receptor - ER, progesterone receptor -PR and human epidermal growth factor receptor 2 - HER2). Intra tumour heterogeneity which is known to foster cancer clonal evolution and given the dynamic nature of cancer evolution, tumour biopsies are known to be limited in offering the knowledge. In the past decade and most especially in the past 5 years, a new source of tumour DNA and RNA was referred to as “liquid biopsy”. Blood which essentially acts as a hub for storing circulating biomarkers such as circulating tumour DNA (ctDNA), circulating tumour cells (CTCs), tumour educated platelets (TEPs), tumour associated macrophages (TAMs) and circulating

RNA offers the potential to learn and shift the current clinical paradigm in assessing tumour biology in real time. In the past decade, many clinical studies have focused on the use of CTCs as prognosis and a response prediction marker in breast cancer. CTCs, though are detected in extremely limited numbers, studying them yields an advantage in telling a near full story that includes events at DNA, RNA and protein levels. Molecular alterations, which can be detected in ctDNA by applying ever advancing NGS technologies, span the types of genomic alterations identified in tumors and include point mutations, rearrangements, amplifications, and gene copy variations. Few cancer genomic centers across the globe have already started using ctDNA and CTCs to monitor disease response and molecular events that emerge and influence drug resistance. Below, we focus on how understanding cancer clonal evolution, through the use of knowledge obtained from carrying out cancer genomics of ctDNA and integrating artificial intelligence, might help in discovering new therapeutic resistance drug targets beyond the usual EGFR, FGFR, HER2 suspects.

Cancer Genomics

The advance in cancer genomics research has helped to reveal the underlying tumour heterogeneity for each breast tumour that consists of several molecular subsets. Each molecular subtype is driven by distinct molecular alterations, indicating that tumours could be treated according to not only these tumour subtypes but also their individual molecular landscape. Despite the exciting potential for personalized medicine, ER and HER2 are currently the established major molecular alterations with confirmed predictive and prognostic values [3, 4]. Genomics can be applied to improve and enhance the patient outcomes. One of the applications is that it helps in the identification of oncogenic driver genes. A genomic driver could be defined as the molecular alteration responsible for cancer progression and appears at high frequency in the disease population. Hence, targeting the oncogenic driver gene is expected to show some therapeutic effect. Genomics can also be used in identifying the resistant clones which can be the result of the patient developing the resistance to a particular treatment. The other applications of genomics include identifying the DNA-repair defects, mutational processes, defects in the DNA duplication and the immune escape mechanism. Even with every day advances in genomic technology, the identification of very low amounts of ctDNA in blood samples with variable amounts of cell free DNA (cfDNA) remains challenging. cfDNA is a blend of DNA that originates from normal cells and by a relatively small fraction derived from tumor cells. Sanger sequencing, which is a gold standard for DNA sequencing does not hold enough sensitivity and specificity while detecting the genomic changes in the ctDNA. The accumulation of mutations creates varied distinct populations of cells, which are also called clones, that differ in their treatment response and resistance. Molecular profiling of ctDNA at different time points (time course events) before and during the treatment could help perceive the clonal evolution to reveal any complex clonal relationships between the primary and metastasis those which can be conferred using the tumour tissue. Cancer clonal evolution can help capture temporal, phylogenetic and spatial aspects

of a tumour which can be reflected by the state of ctDNA. Hence, understanding the cancer evolution can help unlock the underlying mysteries, otherwise can act as limitations in the discovery of underlying resistance mechanisms. The following content sheds light on different but accepted and evolving cancer evolutionary paths.

Cancer Evolution

Three decades ago the existence of multiple phenotypes within a single tumour has been proposed [5]. With the advance of NGS it has enabled the more detailed understanding of cancer evolution and intra-tumour heterogeneity. Genome instability which refers to genetic aberrations ranging from point mutations to chromosomal rearrangements, gains and losses [6, 7] facilitates the cancer cell with the potential to generate new genetic aberrations in daughter cells. Once the daughter cell acquires a selection advantage, it results in distinct sub-clones within a single tumour. Many cancers show hallmarks of genome instability that support the development of intra-tumour heterogeneity [27], evidenced by elevated rates of point mutations [8–10], chromosomal rearrangements [11], and somatic copy-number aberrations [11, 12]. The influence of genome instability and intra tumour heterogeneity upon the clinical outcome is becoming clearer. Chromosomal instability (CIN) is a specific form of genome instability that may include loss of heterozygosity (LOH), telomeric allelic imbalance (TAI) and large scale transitions (LST) and collectively termed as homologous recombinant deficiency (HRD). In a Phase II neoadjuvant clinical trial in women with Triple negative breast cancer, the HRD score and status significantly predicted the pathologic complete response (pCR) [13]. Different genomic events in cancer are of clinical relevance and help to elucidate how cancers tolerate the somatic events while increasing the clonal diversity and branched evolution, leading to cancer progression and therapeutic resistance. A study carried out by Swanton et al. to decipher whether any actionable driver mutations are found in all or a subset of tumour cells in nine different cancer types reported that the known driver gene mutations typically occurred early in cancer evolution. They also identified later sub-clonal "actionable" mutations including BRAF (V600E), IDH1 (R132H), PIK3CA (E545K), EGFR (L858R), and KRAS (G12D), which may compromise the efficacy of targeted therapy approaches [14]. A key limitation while understanding the clonal composition of each tumour tissue biopsy is that it is determined by the presence or absence of private or shared mutations. This may not allow the exact estimation of clonal frequencies, which is vital for the accurate evolutionary reconstruction and the identification of clones. Time course of ctDNA molecular changes, if matched with the constituent subclonal mutations between pairs of primary and metastatic tissue biopsy samples, provide an opportunity to derive the ancestral relationships among tumor clones rather than between tumor samples. The general belief is that cancer progresses via the multistep process of oncogene activation, tumour suppressor gene loss, and subsequent clonal sweeps by the fittest clone [15, 16]. Starting from Darwin to modern day researchers, few cancer evolutionary postulates have been put forth and which are discussed below.

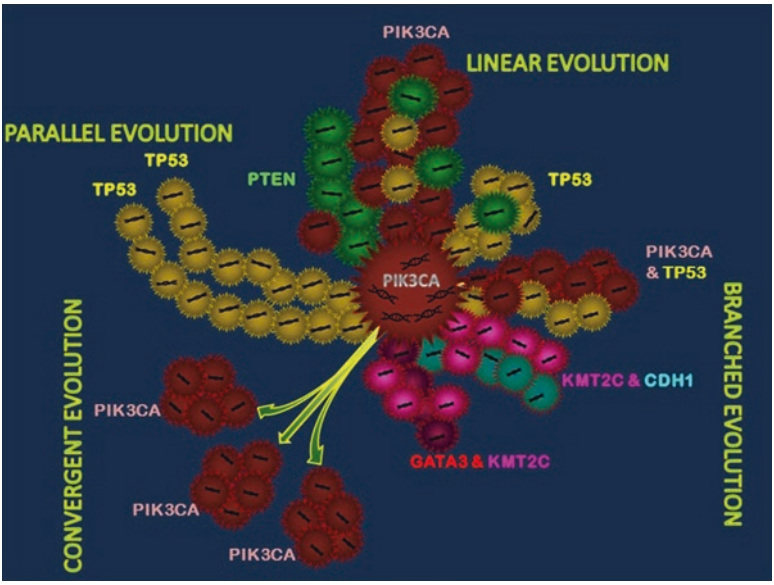

Fig. 1 Modes of cancer evolution (Figure adapted from Venkatesan S and Swanton C, ASCO educational book, 2016 with ASCO's permission)

Contemporary Postulates in Cancer Evolution

Due to the advances in NGS in the past decade, different thoughts of cancer evolution have been put forth, which include models of macroevolution [17–19] and neutral evolution [20–22]. Recently, Venkatesan and Swanton [27] have summed up very brilliantly about the Darwinian cancer evolution and the Neutral evolution [21]. They suggested that the trunk of the phylogenetic tree represents the clonal events where the founder driver events are present. As the trunk forms the main part of a growing tree, the trunk driver events are also present in all cancer cells derived from the cell of origin. As the cancer genome evolves during cancer progression, sub-clonal events are introduced into a subset of the progeny, termed branched events (Fig. 1). It was suggested that a clonal sweep occurs if a branch driver event increases the fitness of a sub-clone to the extent that it out-competes all other sub-clones in the tumour. This mode of cancer evolution has been termed linear evolution (Fig. 1). Convergent and parallel cancer evolutionary concepts were proposed, where the process of independent tumours within the same patient acquire (epi)genetic alterations in similar genes, protein complexes, or signaling pathways in the former, while in the later the sub-clones derived from the same parental clone acquire (epi)genetic alterations in similar genes, protein complexes, or signaling pathways (Fig. 1) [23–25]. In the wake of developments in the use of multi-region and single cell sequencing the concepts of convergent and parallel evolution are increasingly finding their place in the cancer evolution and are emerging as potential exploratory ideas to understand the breast cancer evolution. Different data sets from tissue

sections, small biopsies and the more recent single cell analyses [26] reflect the fact that the different evolutionary paths that are seeing the light of the day are complex and branching (Fig. 1), providing a parallel with the Charles Darwin's iconic evolutionary speciation tree. Sottoriva et al. [21] proposed a "big bang model" or neutral cancer evolution in which they found that a proportion of the aberrations to be clonal, but unexpectedly, the same set of sub-clonal (or private) mutations could be found in different tumour glands on opposite sides of the resection. The neutral cancer evolution model states that after malignant transformation, despite showing distinct mutational patterns, individual sub-clones grow at similar rates. Once treatment is initiated, it is likely that other forms of the Darwinian cancer evolution take over and eventually force the selection of treatment resistant sub-clones [27].

With each day, as our understanding of the genomic landscape is improving, elucidating breast cancer has advanced significantly. Along with other cancer types, the genetic alterations of breast cancer have also been outlined through initiatives by The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC). The most common genes with genetic alterations across all genomic subtypes of breast cancer include *TP53* and *PIK3CA* mutations, which account for 28% of all cases [28]. A proteomic analysis of the TCGA dataset has demonstrated that the PI3K/AKT/mTOR, p53, and CCND1/CDK4/Rb are the three pathways that are activated across all subtypes in early-stage breast cancer [28]. PIK3CA forms the most promising oncogenic target in metastatic breast cancer (MBC) including hormone receptor-positive (34.5%), human epidermal growth factor receptor 2 (HER2)-positive (22.7%), and triple-negative (8.3%) breast cancers [29]. Pre-clinical studies showed that a selective inhibitory effect of the PI3Kb subunit-specific agent in PTEN-negative cancer cells [30]. FGFR1 amplification has been detected in 10% of breast cancer cases, and has been associated with endocrine resistance in highly proliferative hormone receptor-positive breast cancer [31]. Other clinical trials are being carried out to investigate rarer gene alterations, such as AKT1 and ERBB2 mutations and EGFR amplifications as therapeutic targets in combination with tamoxifen, inhibitors of cyclin dependent kinase (CDK) 4 and CDK 6, 2 components of the cell cycle regulatory machinery that have shown promising results in the treatment of breast cancer [32]. A phase II trial in postmenopausal women with locally advanced or metastatic hormone receptor-positive breast cancer, PALOMA-1/TRIO-18, showed significant improvement in progression-free survival (PFS) in those treated with palbociclib, an inhibitor of CDK 4/6, versus letrozole alone as first-line treatment. Encouraged by the phase II results, palbociclib was recently approved by the FDA for use in combination with letrozole in women with hormone receptor-positive MBC [33]. The results of the PALOMA-3 clinical trial demonstrated longer PFS with palbociclib combined with fulvestrant in comparison to fulvestrant alone in patients with hormone receptor-positive MBC who had progression of disease during endocrine therapy [34].

With the advances in genomic technologies and access to different sequencing platforms many laboratories are detecting gene alterations, including mutations and amplifications using both tumour biopsies and liquid biopsies that are recognized to be important in the biology of invasive and non-invasive mammary tumours. In the recent years, along with the application of artificial intelligence, machine learning

techniques and the genomic panels included in the testing platforms, an enormous amount of data were generated for testing, have evolved significantly. Nascent as it is, BOLERO-2 and PALOMA-3 clinical trials [33, 35] have shown that there is no proven genomic marker identified by molecular testing to select patients for targeted therapy with currently approved agents, including the mTOR inhibitor everolimus and the CDK4/6 inhibitor palbociclib for the treatment of advanced HER2-positive and HER2-negative metastatic breast cancer. Precision medicine based on targeted therapy is a promising strategy in treating the breast cancer patients. A meta-analysis of Phase II clinical trials with single-agent arms across the malignancies revealed that a personalized strategy was an independent predictor of better outcomes and fewer toxic deaths [36]. The authors also reported that non-personalized targeted therapies were associated with significantly poorer outcomes than cytotoxic agents, which in turn were worse than personalized targeted therapy [36].

A multi-center (SAFIR01/UNICANCER) molecular screening study based on the biomarker approach in metastatic breast cancer has been shown to be associated with improved efficacy outcomes in FDA-approved anticancer agents, indicating the need to establish robust genome-driven biomarkers for the success of personalized therapy [37]. Since ER, HER2, and PIK3CA genomic alterations are more frequent and others being rare and recruiting the study participants in randomized trials of drugs matched to given genomic alterations becomes a very lengthy process it is important to address the issue before the study design. To overcome the shortcomings, large molecular screening programs are currently in place to select patients with candidate genomic alterations under two different clinical trial designs; (1) basket trials, to test the effect of a single drug on a molecular alteration in a variety of cancers and (2) umbrella trials, to assess the effect of different drugs in different molecular alterations in either one or several tumours.

Other than the SAFIR01 and AURORA trials which are the two umbrella trials, many molecular screening programs that include breast cancers are the National Cancer Institute (NCI) molecular profiling-based assignment of cancer therapy (MPACT), targeted agent and profiling utilization registry (TAPUR), and the NCI-molecular analysis for therapy choice (MATCH) trial [38–40]. The use of ctDNA and CTCs obtained from the peripheral blood of patients with MBC holds promise as a substitute for tissue biopsy to perform baseline and serial longitudinal genomic testing. The promising potential of liquid biopsies for monitoring drug efficacy and detecting the genomic alterations involved in resistance needs to be established by clinical trials. The liquid biopsy aspect involving ctDNA will be discussed in more detailed in the following sections.

Circulating Tumour DNA

In order to examine and identify the genomic alterations and clonal evolution before and after the treatment, repeated biopsies are difficult, invasive and may be faced with a challenge of intra-tumour heterogeneity [41, 42]. For clinical

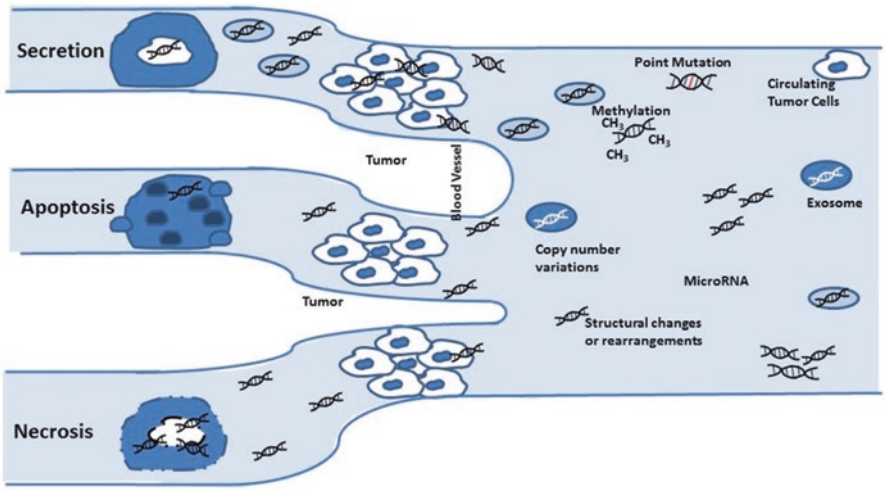

Fig. 2 Mechanisms depicting the release of small fragments of cell-free DNA into circulation by the tumour cells. Cancer-associated genetic and epigenetic alterations can be detected in circulating cell-free DNA using different NGS methods (Figure adapted from Diaz & Bardelli. J Clin Oncol. 2014 with ASCO's permission)

investigation and translational research, though tumour biopsy remains the gold standard in understanding and establishing the primary and secondary mechanisms of resistance, along with the difficulty in accessing the tumour tissue other technical difficulties also remain as hurdles. For instance, tumour tissue is preserved in formalin-fixed paraffin-embedded (FFPE) blocks, which crosslink DNA and in some cases can result in FFPE samples being inadequate for molecular analysis. Other aspects like tumour cellularity also limits the efficiency of tumour biopsy.

Within the past decade, studies have shown that along with the genomic alterations, resistant mutations in solid cancers can be identified and tracked by next generation sequencing of ctDNA, which is released from the cancer cells into the plasma [43, 44]. In general, patients with cancer have been reported with much higher levels of ctDNA than healthy individuals [45–47]. With the increase of tumour volume, there are also increases in the cellular turnover and, hence, the number of apoptotic and necrotic cells [48, 49]. Under normal physiological conditions, apoptotic and necrotic cell remains are cleared by infiltrating phagocytes. However, this does not happen efficiently within the tumoural mass, leading to the accumulation of cellular debris and its inevitable release into the circulation (Fig. 2). When the length of ctDNA strands are measured, they often assume the classic ladder pattern in integer multiples of 180 base pairs [50], characteristic of the apoptotic process [48, 51, 52]. In fact, most ctDNA fragments measure between 180 and 200 base pairs, suggesting that apoptosis likely produces much of the ctDNA in the circulation [53–55].

Table 1 Applications of liquid biopsy

Early detection
Assessment of molecular heterogeneity of overall disease
Monitoring of tumour dynamics
Identification of prognostic, response and resistant markers towards a targeted therapy
Evaluation of early treatment response
Monitoring of minimal residual disease
Assessment of evolution of resistance in real time by longitudinal analysis

Applications of ctDNA

It has been demonstrated that ctDNA can also be useful while monitoring the tumour burden [43, 57–59]. Given the short half-life (approximately 2 h), ctDNA allows the assessment of tumour evolution in hours than weeks to months [55]. Also, given the specificity of ctDNA for an individual's tumour without any bias while undermining the somatic cancer mutations that would be reported in matched normal DNA. Several studies in melanoma, ovarian, breast and colon cancers have established the potential utility of ctDNA to precisely define the tumour dynamics during the therapy for patients with advanced disease [43, 55, 60–62].

Analysis of ctDNA in plasma samples obtained before and after treatment by NGS can ultimately provide an evolutionary picture of the molecular events in a patient's tumour. The molecular events include the dynamic changes in the mutation profile which can be tracked before, during and after the therapy along with the heterogeneity that emerges as a result of the therapeutic selective pressure. This understanding of the mechanisms of acquired resistance to targeted agents at the molecular level can be used to plan combinatorial treatments with drugs that will suppress the expansion of the clones that are responsible for most of the current failures of medical treatment [63]. The prior knowledge of resistance mechanisms could result in the early adoption of alternate therapies (see Table 1).

Methods to Assess ctDNA

ctDNA was first identified by Mandel and Metais in 1948 [64]. However, due to the lack of association with any disease the concept of liquid biopsy remained dormant for approximately another 30 years. In 1977, Leon et al., found ctDNA in plasma of patients affected by lung cancer [65]. cfDNA was identified in the peripheral blood of healthy individuals. However, patients with cancerous tumours have higher quantities and the detection is associated with poorer prognosis [61, 66]. In the view of identifying and establishing the potential role of ctDNA in clinical set up, several sensitive PCR-based techniques have been developed from the start of this century. However, in the past decade, owing to the drop in genomics cost and exponential progress made with NGS technology, the innovation has picked up the pace and many techniques have seen the day of the light. Few of the techniques developed in detecting the ctDNA

include the amplification refractory mutation system (ARMS) [67], pyrophophorolysis-activated polymerization (PAP) [68], pyrosequencing [69], Sanger sequencing [70], beads, emulsion, amplification and magnetics (BEAMing) [71], modified semi-nested or nested methylation-specific PCR [72], tagged-amplicon deep sequencing (TAM-Seq) [43], CAPP-Seq [73], droplet digital PCR (ddPCR) [74], and Guardant360 [75].

ctDNA-Based Clinical Trials and Research

Clinical trials have been carried out by different laboratories around the world, showing the significance of analyzing the ctDNA in predicting the beneficial treatment regimens. In metastatic breast cancer, acquired ESR1 mutations are a major mechanism of resistance to aromatase inhibitors (AIs). A study on 171 patients with advanced breast cancer by ultrahigh-sensitivity multiplex digital PCR showed a major mechanism of resistance in ctDNA. The study showed that patients with ESR1 mutations have shorted progression-free survival (PFS) and more common in metastatic disease patients [76]. In the BOLERO-2 trial the authors demonstrated that patients treated with everolimus (an mTOR inhibitor) achieved a 3.1 months PFS benefit who had reportedly an ESR1 mutation D538G in their ctDNA [77]. Over a period of 3 years a team from Cambridge, UK, used the ctDNA of a patient with metastatic ER-positive and HER-positive breast cancer, to evaluate the metastatic heterogeneity in real time. They analysed both tumour and plasma samples using exome and targeted amplicon sequencing and established that the mutation levels in the plasma samples reflected the clonal hierarchy of tumour biopsies; thus, confirming the efficiency of ctDNA in real-time sampling [78]. Pre-clinical studies have shown that inhibiting the PI3K pathway may be a viable treatment option for women with advanced hormone receptor (HR)–positive breast cancer that become resistant to endocrine therapy. A recent phase III BELLE-2 clinical trial analysed blood samples of 587 patients for the PIK3CA mutation status. Patients with PIK3CA mutations in their ctDNA have been reported to have a PFS longer (7.0 months) when they received the combination therapy versus fluverstrant alone (3.2 months). This suggests the role of ctDNA in selecting the patients for beneficial treatments [79]. Many other laboratories showed the role of ctDNA in tracing the disease progression. While screening the patients, a translational clinical trial [80] using cell lines and patient-derived xenograft models showed an understanding of the distinct patterns of oncogene addiction in highly amplified cancers and demonstrated the importance of clonality in predicting response to targeted therapy along with why screening for amplification in ctDNA could be a viable approach. The Japan Breast Cancer Research Group (JBCRG) is currently initiating a translational research study involving ctDNA analysis in triple negative breast cancer (TNBC) patients as well. Sequencing of paired tumour–normal biopsies and blood samples has identified millions of protein-altering somatic mutations in thousands of patients [81]. However, functional characterization and clinical decision-making are restricted by neutral ‘passenger’ mutations that greatly outnumber pathogenic ‘driver’ mutations [82]. Many newer functional impact scores predict pathogenic variants using supervised

modeling [83–85]. The JBCRG study plans to integrate the genomic data and prediction algorithms to describe variants and interpret their pathogenic along with their clinically actionable status. The current ongoing clinical trials and future studies on liquid biopsy along with the massive interest not only from academic laboraties but also from the industry, liquid biopsy has been shown to be the way going forward to identify or discover any potential prognostic and response biomarkers. As is expected of the genomic data to have high-level quality reads of DNA regions and samples, high-level data management languages also would help in answering the biological and clinical questions with simple, powerful, orthogonal abstractions which can be easily translated and can be used by a physician while making a treatment decision. A brief background of how artificial intelligence and machine learning could help genomics has been addressed in the next section.

Artificial Intelligence and Machine Learning

Remarkable innovations in the NGS technologies are used in generating the omics data which in the past decade has swiftly created a profound effect on the cost of genome analysis that costs approximately $1000 per genome. The spiraling cost has led to the creation of vast amounts of data. The challenge of how to harness this rich clinical genomic information, and from it the resulting targetable therapeutics, has been thrust to the forefront of advanced analytics. It is anticipated that in the upcoming decade genomics data itself will require computing resources [86]. Faced with the computing challenge, new ways of advanced algorithms and scientific knowledge are needed to transform, store and share the reliable genomic and transcriptomic information into an advanced understanding of disease pathogenesis and pharmacogenomic-driven treatment response. NGS assays produce millions of data points ranging from variations to mutations to gene expression levels to methylation status and many more genomic aberrations. These hierarchies of data are part of a complex molecular and cellular biological systems forming intricate networks, pathways, and natural biological structures. Hence, powerful but reliable computationally scalable tools are required that can assess and potentially integrate different types of biological and clinical information to ultimately produce interpretable and knowledge-rich information, that can be used for patients' treatment benefits (Fig. 3).

Because of the amount of 'omics data that have been and will be generated, some of the challenges include storage, collaboration, computational capacity, integration of different tools and assays to generate interpretable and reliable results from deep analyses of complex data, which ultimately help in generating knowledge that can inform decision making. Given the myriad but known challenges encountered in the 'omics analytics space, the following key strategic and technical aspects could be considered.

Different machine learning algorithms may provide different performance advantages depending on the nature of the data and complexity of the genomics or transcriptomics data (eg, linear vs nonlinear). Hence, choosing the algorithm that answers the underlying question is significant. Efficient algorithms should take into

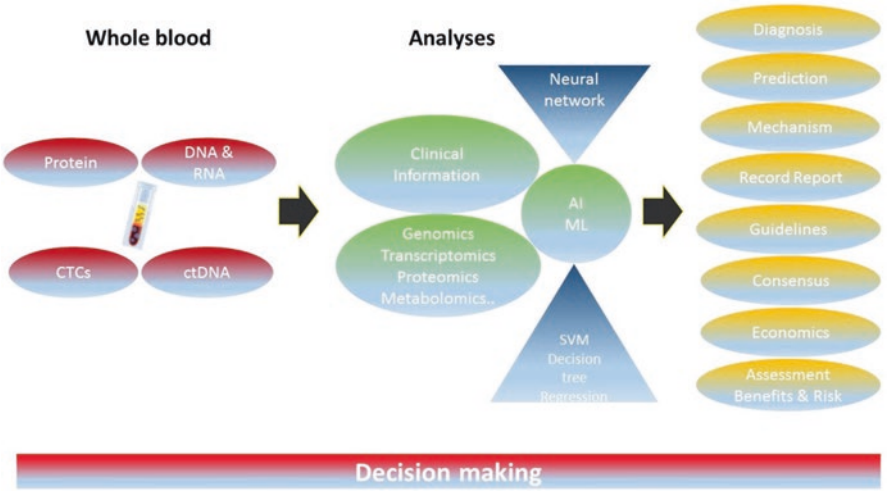

Fig. 3 Comprehensive representation of different steps involved in assisting in efficient health care decision making

account and leverage the broad range of existing prior knowledge from different publicly available databases on genomic or transcriptomic or clinical contexts [56]. It helps if the results of the complex analyses are displayed in an interpretable and intuitive manner such as a Manhattan plots, graphical representations or survival curves. Algorithms should be computationally scalable and allows for the integration of different components of a complex biological and clinical data (Fig. 4) to effectively interpret the structure, function and the meaning of the genetic information that can be translated into effective therapeutics.

Doctors have been using the concept of precision medicine for over a century [87]. The idea for a real-time feedback to the doctors while offering the unbiased and individualised treatment decisions by using artificial intelligence and machine learning techniques is to improve the quality and value of care the patients receive. Cancer being a heterogeneous and complex disease, understanding how to learn to read the cancer genome becomes more crucial. Though humans are good with recognizing audio visual patterns, unless one is trained, we are not equipped with understanding the genomic data that is made with alphabets, underlying mechanisms and numerous interacting pathways. To help make a head start, laboratories across the world have been working on machine learning techniques that can be used to interpret the genome. Researchers across the biological, computational, mathematical and clinical fields are advocating the need to develop more reliable machine learning techniques to handle the large data [88]. The idea behind developing machine learning techniques is that the models would help interpret, infer, predict and explain the observed data. When the machine is trained with a known data set (training set), it can use the model to make interpretations of the future data (validation set).

The fact that the underlying relationship between the genotype and the phenotype are extremely complex in a tumour's environment with constant evolutionary

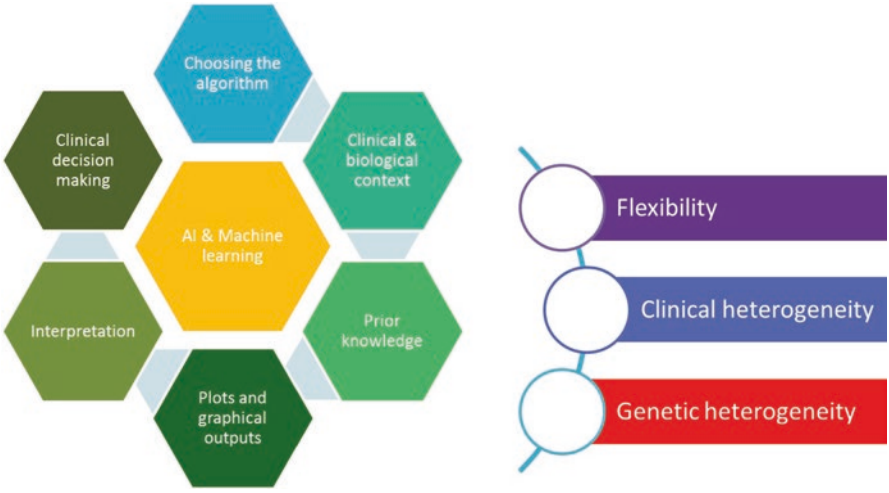

Fig. 4 Different biological and clinical aspects that could be taken into consideration while designing and choosing an algorithm

changes through intricate and interconnected biophysical and biochemical processes and hidden variables like interaction of the genome with the different kinds of food one consumes and the nature they live in makes it a far cry to achieve the near human predictions using machine learning techniques. Hence, considering the "variables" that best fit the training models could address human-like situations and deal with ever-changing data. While using predictive modeling applications is useful, some values may be missing. For instance, in case of, patient data often have missing diagnostic tests that would be helpful for estimating the likelihood of diagnoses or for predicting the effectiveness of a treatment. To overcome the issue of missing values in multiple logistic regression (MLR), an alternative decision tree (ADTree) model was developed for accurately predicting diagnostic and treatment outcomes in primary breast cancer [89]. A recent multi-factorial, principal component analysis (MFA) study in breast cancer patients indicated that the expression was the strongest indicator of sensitivity for paclitaxel, and copy number and expression were informative for gemcitabine [90]. The study used support vector machines (SVMs) to combine the factors and predicted cell line sensitivity to paclitaxel with 82% accuracy. Likewise, when the copy number profiles of three genes (*ABCC10, NT5C, TYMS*) were factored in, along with the expression of seven genes (*ABCB1, ABCC10, CMPK1, DCTD, NME1, RRM1, RRM2B*), the model predicted gemcitabine response with 85% accuracy. However, when the nucleic acid integrity was taken into account, the gemcitabine SVM exhibited only 62% prediction accuracy for the tumour blocks. Establishing the nucleic acid integrity and the isoform abundance might help improve the model and prediction accuracy along with the sensitivity. Considering and including the variables that affect the ability to obtain high quality gene expression measurements from the FFPE samples from older tissue blocks might help improve the SVM model performance. Taking into account the

interaction between the platelets and tumour cells, using RNA sequencing and machine learning techniques across different cancer types and healthy individuals, the study reported a 71% accuracy in locating the primary tumour and 96% while distinguishing patients' with localised and metastasized tumours from healthy individuals [91]. The study identifies the limitations such as mRNA degradation, influence of other symptoms on platelets and the heterogeneous nature of the cohort could all influence the accuracy and sensitivity. Recently, a team [92] from Harvard and MIT used millions of training patches to train a deep convolutional neural network to make patch-level predictions to discriminate tumour-patches from normal-patches. They showed that by combining the deep learning technique predictions with human pathologist's diagnoses represented an approximately 85% reduction in human error rate while carrying out the task of whole slide image classification and tumour localisation in metastatic breast cancer sentinel lymph node biopsies. By using natural language processing (NLP) algorithms, which help in automatically extracting mammographic and pathologic findings from free text mammogram and pathology reports, the authors demonstrated that patients with estrogen receptor-positive tumours were more likely to have speculated margins, and those with tumours that overexpressed HER2 were more likely to have heterogeneous and pleomorphic calcifications [93]. These results demonstrate the power of using deep learning and different machine learning techniques to produce significant improvements in the accuracy of pathological diagnoses.

With the obvious and increasing demand of machine learning techniques in a clinical set up, taking into account the variables that may affect the tumour microenvironment, tumour cell interactions, genetic and epigenetic influence, liquid biopsy timing, quality of the tumour and somatic cell genomes, life style changes, health risks, patient information, medications, therapy, dose schedules, response, adverse effects, various sequencing platform limitations and unbiased ability to consider different variables while using machine learning techniques might help achieve therapeutic choices that could improve the patients' health. While considering the single cell genomics and machine learning techniques, first and foremost it is important to choose a technology that is unbiased while isolating the single cells, to rule out any spurious biological conclusions after the analyses. In the past few years single-cell isolation technologies where the trade-offs in accuracy, throughput, reproducibility and ease of use were reviewed [94–96]. Other aspects like minimising the PCR artefacts like mutations, introduction of false positives, amplification bias, genome loss and chimeras have been taken into account while developing the whole genome amplification technologies. The type of genomic interrogation (DNA or RNA analyses) also needs to be carefully considered in the context of the questions one can ask to seek clinically relevant answers. Though evolutionary trees can be constructed using tumour biopsies, techniques that also can identify and correlate the origins of circulating tumour cells [97, 98] or circulating tumour DNA [99], efforts are still needed to accurately predict the prognostic and response biomarkers, which can be used in selecting the best treatment option available to the clinician.

While this can be achieved with an extensive collaboration between a biologist, mathematician, computer expert and a clinician, it is also important to test the models

that mislead the interpretations, in order to know which model fits the study design with the highest accuracy. Also, it is quint essential that the interpretability aspect also improves hand in hand with different machine learning techniques. For instance, a data analyst should be able to ask a researcher to test a prediction experimentally which would give more confidence to the clinician while making a decision, than relying on the previous literature, which most models are trained with. Another aspect that can be taken into account while developing different models is "conservation". Current models seem to emphasise more on evolutionary conservation. However, it is also important to take functional conservation into account, as not all evolutionarily conserved sequences or motifs or domains are functionally conserved across different genomes [100, 101].

Technical aspects that need to be considered while using the artificial intelligence (AI) and machine learning (ML) could include how flexible the algorithm is. Algorithms used should be able to accommodate typical outcomes in clinical trials like continuous, binary, and time-to-event. AI and ML algorithms should be trained to handle covariates, a critical consideration in analysis of clinical trials. Also, many clinical trials have populations with diverse genetic ancestry. Hence, algorithms that take clinical and genetic heterogeneity into consideration should also be developed (Fig. 4).

Acknowledging the significance of AI & ML in the modern day clinics, IBM and the Broad Institute of MIT and Harvard have launched a five-year, $50 million research collaboration whose aim is to discover the basis for drug resistance in cancers. The teams will use IBM Watson's [102] computational and machine learning methods to study drug resistance in thousands of different tumours types. While the Broad institute will focus to generate tumour genome sequence data from patients who initially respond to treatment but then become drug-resistant. IBM scientists use Watson to analyze the data and identify genomic patterns that may help researchers and clinicians predict drug sensitivity and resistance. "The key will be learning from clinical experience, so that we know cancer's moves in advance and can plan strategies to cut off its escape routes. Knowing how cancers can become resistant will ultimately require learning from hundreds of thousands of patients' experiences" Eric Lander, founding director of the Broad, said in a statement.

A probabilistic approach to machine learning that provides a framework for modelling uncertainty, deep learning techniques and computational models to predict measurable intermediate cell variables to train the models is the way going forward to find the best therapeutic options. With such big data, the issue of "confidentiality" is an extremely important ethical issue that needs to be taken care along with the consent of the study participant, whether his/her data can be used to further improving the model and design of the future studies. The ultimate hope of marrying technology and human big data is that the data being generated using next generation sequencing technology and analysed using artificial intelligence will complement each other. Thus, leading to sharing and incorporating the data sets into large publicly available open networks databases like TCGA and ICGC which inform, educate, and help cancer treatment and research. The American Society of Clinical Oncology (ASCO) has developed CancerLinQ, which proposed an idea to incorporate data of patients with cancer in the United States into one large data col-

lector. This would not only capture cancer data on 100% of patients with cancer other than the 3% who are entered into various clinical trials, and thereby accelerate new information, knowledge, and discoveries that could enhance the therapeutic benefits [103]. As, however, uncertain, the nature and human evolution are, as long as the right questions are asked in priority, artificial intelligence, machine learning techniques and big data could only help us achieve the expected results while keeping a tap on cancer evolution. There are excellent reviews [82, 104–106] and studies [108, 109] which reviewed and investigated the cancer resistance mechanisms against different treatments and use of technology which assisted in making informed clinical decisions for the patient's wellbeing. Contrary to the Darwinian evolution, during 2016 a review [107] and a study offered an insight into an initial outburst of mutational events and then a stable clonal expansion of cancer [110]. This offers an exciting path ahead to understand the cancer evolution and the underlying resistance mechanisms for a better future and treatment option while integrating artificial intelligence.

Harnessing the Immune System Using Liquid Biopsy, ML & AI

From the times before Steven Rosenberg's interest in harnessing the immune system [111] to fight cancer slowly but the dream is becoming a reality. Many inspiring and breakthrough stories of tumour regression and terminal illnesses going into remissions, backed by clinical data have led to an exponential interest and billions of dollars of investments in the rapidly growing field of immunotherapy. Research institutes across the globe, Pharmaceutical companies, philanthropists and the different government initiatives, one like the "cancer moonshot" programme have been granting funds to develop immune treatments. Numerous clinical trials in different countries have been initiated involving immunotherapy, alone or combined with other treatments, for nearly every type of cancer. A network of cells, tissues and biochemicals which form the integral part of the immune system that defends the body against viruses, bacteria and other invading molecules. However, cancer often finds it's ways to hide and overcome the immune system or block its ability to fight. Though cancer immunotherapy achieved a remarkable success in treating melanoma patients [112, 113], efforts have been made to discover prognostic predictors in other types of cancer [114–118]. A comprehensive whole genome analysis of a large breast cancer cohort was used to suggest that substitutions a particular type could be more effective in triggering an immune-response [118]. A recent study across [118] tumour types, provided evidence for immunoediting and uncovered genetic amplifications and immuno suppressive factors such as PDL1/2 in tumour-intrinsic resistance to cytotlytic activity [119]. While the recent studies massively contributed in better understanding of cancer immunoediting [120], there is still much to learn about the potential tumour-immune system interaction and its impact and outcome in the patients'. Likewise, though the role of tumour infiltrating immune cells have established the role of cytotoxic T cells (CTLs) and tumour-associated macrophages (TAMs) in some

cancers, the clinical role of other immune cells in many cancers still remains poorly understood. Hence, it is the need of the hour for a more in-depth and comprehensive genomic and translational analyses of the tumour immune system to enhance the understanding of the multi-dimensional anti-tumour response and guide breast cancers towards effective immunotherapies. Also, investing the time and funds to develop prediction of the breast cancer treatment, particularly in line with anti-PD-1, PD-L1 antibody might also yield potential therapeutic opportunities. The idea in developing an immunotherapy is to try and to help the immune system recognize cancer as a threat, and attack it. Investigators and researchers are now focused on two promising approaches of immunotherapy. The first approach being, to create a new, personalised treatment for each patient by removing some of the person's immune cells, altering them genetically to kill the cancer and then infusing them back into the bloodstream to fight the cancer metastasis. The second broader approach involves mass-production of drugs that do not have to be tailored to each patient personally. The drugs meant to free the immune cells to fight the cancer by blocking a mechanism called a checkpoint, which cancer uses to shut down the immune system. This approach helps in overcoming the resistance, by combining with other therapeutic modalities. Chemotherapy and radiotherapy are known to modulate and increase the sensitivity of tumor cells to immune therapy.

Different tumour types have been characterized by a large number of mutations in their DNA, and work over the past few years has demonstrated that the 'neoantigens' encoded by these mutated genes form a major driving force behind current cancer immunotherapies [121]. Given the hurdles to face while isolating and culturing tumour infiltrating lymphocytes (TIL) access to the fresh tumour material could ease and present an efficient and a shortcut for obtaining neoantigen-specific T cells from the study participants. The strategy to identify the tumor-reactive T cell population in peripheral blood is to focus on the cytotoxic CD8+ T cell population that expresses the surface molecule programmed cell death 1 (PD-1), a reliable and well-known marker for previously activated or exhausted T cells [122]. Gros and colleagues [122] in their study included only four people with melanoma, and they were able to isolate mutation-specific T cells from the blood of three of them. Another work [123] carried out in six different tumour types, few of breast cancer participants had to be excluded as the low mortality rate was not informative. However, the possibility of isolating mutation specific T cells from the blood of three melanoma patients can only encourage to explore and develop sensitive and reliable liquid biopsy isolation and detection methods in which the breast cancer study participants are subjected to immunotherapy. One possible strategy could be to isolate and expand the PD-1+ T cell subset from the patients' peripheral blood, and then to reinfuse it into the patient. However, like any best available opportunity, the limitation of the above strategy is the low frequency of tumor-specific T cells among the PD-1+ in the blood draw. We believe that with ever growing advances in high-throughput screening along with the most advanced ML algorithms and AI can only add value of liquid biopsy to develop tumor-specific T cell products along with improved understanding of the breast cancer resistance mechanism to various treatments.

Conclusion

Since we are still in the nascent stage of exploring and exploiting liquid biopsy potential in establishing as a potential biomarker, the advances in technology can assist a physician run one or a handful of cheaper, simpler but established tests to determine therapy strategy. Liquid biopsy based biomarkers can act as companion or complementary tests to the traditional diagnostics that are available now. For patients having metastases or advanced diseases, the therapeutic strategy needs to be considered based on to their disease biology. In the future, tumour-host interaction as well as tumor cell genomic analyses should be incorporated with disease diagnosis and prognosis. Inter-tumoural heterogeneity between metastatic lesions should be well examined further by integrating the liquid biopsy analysis with tumour biopsy testing. In the case of therapeutic resistance, testing the liquid biopsy for a specific or a panel of resistance biomarkers could be required than the efforts or cost of comprehensive sequencing panels.

Multiple efforts are underway to translate the liquid biopsy technology into a standard-of-care tool for guiding the treatment of cancer patients world-wide. More interventional clinical utility studies among academia, industry and public can pace up and demonstrate how targeted sequencing of the liquid biopsy approach can improve patient outcomes across multiple types and stages of cancer and in better understanding of the mechanisms underlying treatment resistance. Along with resistance to EGFR, FGFR, HER2 targeted therapies and combination therapy in hormone responsive tumors, the promising role of liquid biopsies for monitoring drug efficacy and detecting the genomic alterations in resistance mechanisms needs to be established by clinical trials.

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