International Journal of Clinical Oncology (2019) 24:131–140
https://doi.org/10.1007/s10147-018-1373-5

REVIEW ARTICLE

Clinical target sequencing for precision medicine of breast cancer

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Received: 1 August 2018 / Accepted: 19 November 2018 / Published online: 2 January 2019

Abstract
Precision medicine can be defined as the customization of medical treatment based on the individual genetic profile, which enables one to identify patients who respond to therapies while sparing side effects for those who do not. Breast cancer patients have been treated based on subtyping, which is considered a prototype of precision medicine. Furthermore, the development of multigene panel testing has resulted in a paradigm shift in the treatment of breast cancer. The knowledge generated from the Human Genome Project, and subsequently The Cancer Genome Atlas, has provided the concept of precision medicine, in which cancer patients can be sub-classified based on actionable driver mutations that can be selectively targeted by molecular targeted drugs and treated by appropriate molecular targeted therapies. Development of next-generation sequencing has both dramatically advanced genomic sequencing technology and revealed actionable driver mutations for individual cancer patients when applied to a clinical setting. Clinical target sequencing by next-generation sequencing enables one to formulate treatment strategies, not only by selecting a subgroup of patients who are expected to experience more effectiveness of each drug, but also by revealing patients with drug resistance based on their actionable driver mutations.

Keywords Precision medicine · Breast cancer · Next-generation sequencing · Targeted therapy · Clinical target sequencing · Drug resistance

Introduction

Breast cancer is the most common cancer in women, representing one quarter of all cancers [1]. In the United States, there were nearly 252,710 estimated new cases and 40,610 estimated deaths in 2017 [1]. Treatment for breast cancer has advanced remarkably during the past 4 decades. In the 1980s, an effect of tamoxifen as an estrogen receptor (ER) antagonist was discovered in breast cancer patients, leading to the development of pharmacological endocrine therapy, which can be considered the first "molecular targeted therapy" for breast cancer [2]. In the late 1990s, human epidermal growth factor receptor 2 (HER2) overexpression was validated as a target and was shown to be a predictive biomarker for the efficacy of trastuzumab [2]. More recently, there have been an increasing number of molecular targeted drugs based on the rapid advancement of genomic studies. Therefore, understanding the functional mechanisms behind the molecular targeted therapies is crucial for clinicians to utilize those complicated drugs in efficient ways.

Precision medicine is an emerging approach that allows for the customization of medical treatment and disease prevention based on the genomic profile of an individual or a disease, allowing for the tailoring of treatments specific to the patient's genome or disease. Over the past 2 decades, it has become apparent that malignancies with identical pathological diagnosis may, in fact, include a number of genetically different cancers. Due to technological advancements, next-generation sequencing (NGS) now enables us to

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decipher entire individual cancer genomes at a rapid pace, revolutionizing genomics research and enabling scientists to study biological systems at a level that was never before possible. Clinical target sequencing of large-scale gene panels, facilitated by NGS, identifies genetically-based drivers of disease to implement and develop new, more effective molecular targeted drugs. Such an approach not only allows for the selection of patients expected to have a better treatment response, but also reveals possible mechanisms of resistance to targeted therapies based on actionable driver mutations. Taken together, clinical target sequencing allows for the differentiation of cancers based on the expected therapeutic response and may ultimately be the driving force in the realization and widespread implementation of precision medicine.

In this review, we summarize the concept of precision medicine and introduce the utility of clinical target sequencing of large-scale gene panels in both the identification of resistance mechanisms and the selection of molecular targeted therapies with a focus on breast cancer.

What is precision medicine?

Precision medicine is defined as the customization of medical treatment based on the genomic profile of an individual or disease. Using this approach, patients are classified into subpopulations on the basis of their susceptibility to a particular disease, the biology of those diseases they may develop, or their response to a given treatment [3]. As a result, preventive or therapeutic interventions are able to focus on the patients who will benefit, sparing the expense and side effects for those who will not [4]. Although the methodology seems novel, the concept has been used to guide transfusion therapy based on blood typing for more than a century.

In the 2015 State of the Union Address, then United States President Barack Obama announced a research initiative that aimed to promote progress in precision medicine, stating, "Tonight I'm launching a new precision medicine initiative to bring us closer to curing diseases like cancer and diabetes and to give all of us access to the personalized information we need to keep ourselves and our families healthier [5–7]." By way of a $215 million annual federal budget dedicated exclusively to researching the genetic causes of cancer, precision medicine, specifically apropos of oncologic care, was brought to the forefront of the US national dialogue [8].

When compared with the traditional stratified medicine approach, the objective behind a precision medicine strategy is increasing the efficacy of anticancer agents through the reliance upon individuals' genomes and molecular profiles, while concomitantly minimizing toxic side effects typically associated with nonspecific therapeutic modes of action [9, 10]. However, it should be noted that precision medicine is vastly different from the notion of personalized medicine, the latter of which implies an inefficient and cost-intensive approach for designing unique treatments for individual patients [3]. The focus of precision medicine is to explore how treatment approaches can be developed based on the combination of genetic, environmental, and social factors of a particular group and subsequently targeted to individuals or populations. Moreover, precision medicine enables innovations in prevention strategies based on risks predicted by genetic signatures in each subgroup analysis. For example, identifying cancer predisposition genes, such as *BRCA1/2* in breast cancer patients, permits screening programs to find patients at higher risk for cancer development and helps them to make decisions with regard to individual risk modification measures [11]. Taken together, precision medicine can be applied, not only to cancer patients, but also to healthy individuals for risk stratification.

Paradigm shift in management of breast cancer

Currently, breast cancer is categorized by the expression of ER, progesterone receptor (PR), and HER2 protein, given the availability and efficacy of specifically tailored therapies to each [10, 12, 13]. Such a treatment strategy based on the subtyping of disease is considered one of the earliest and most successful prototypes of precision medicine, warranting an in-depth review of its development in the overall schemata of breast cancer therapy.

Up until 3 decades ago, indications for chemotherapy for breast cancer were determined exclusively by tumor size [14, 15] (Fig. 1). However, it soon became apparent that ER-positive tumors were less responsive to cytotoxic chemotherapies than ER-negative tumors [16]. Thus, a need emerged for additional metrics, such as receptor expression, in the breast cancer treatment algorithm when deciding the need for adjuvant therapies [17–19] (Fig. 1). More recently advances in the understanding of genetic signatures and their downstream signaling pathways have enabled clinicians to develop entirely new paradigms to determine which breast cancer patients should receive adjuvant therapies, including hormone therapy, molecular targeted therapy, and conventional chemotherapy [20–22] (Fig. 1). In recent years, targeted therapies have become established as an essential part in the armamentarium of breast cancer treatment paradigms, signified not only by an exponential increase in the number of approvals of molecular targeted drugs by the United States Food and Drug Administration, but also by dramatic and clinically significant improvements in patient outcomes. One of the most successful models of such an approach is

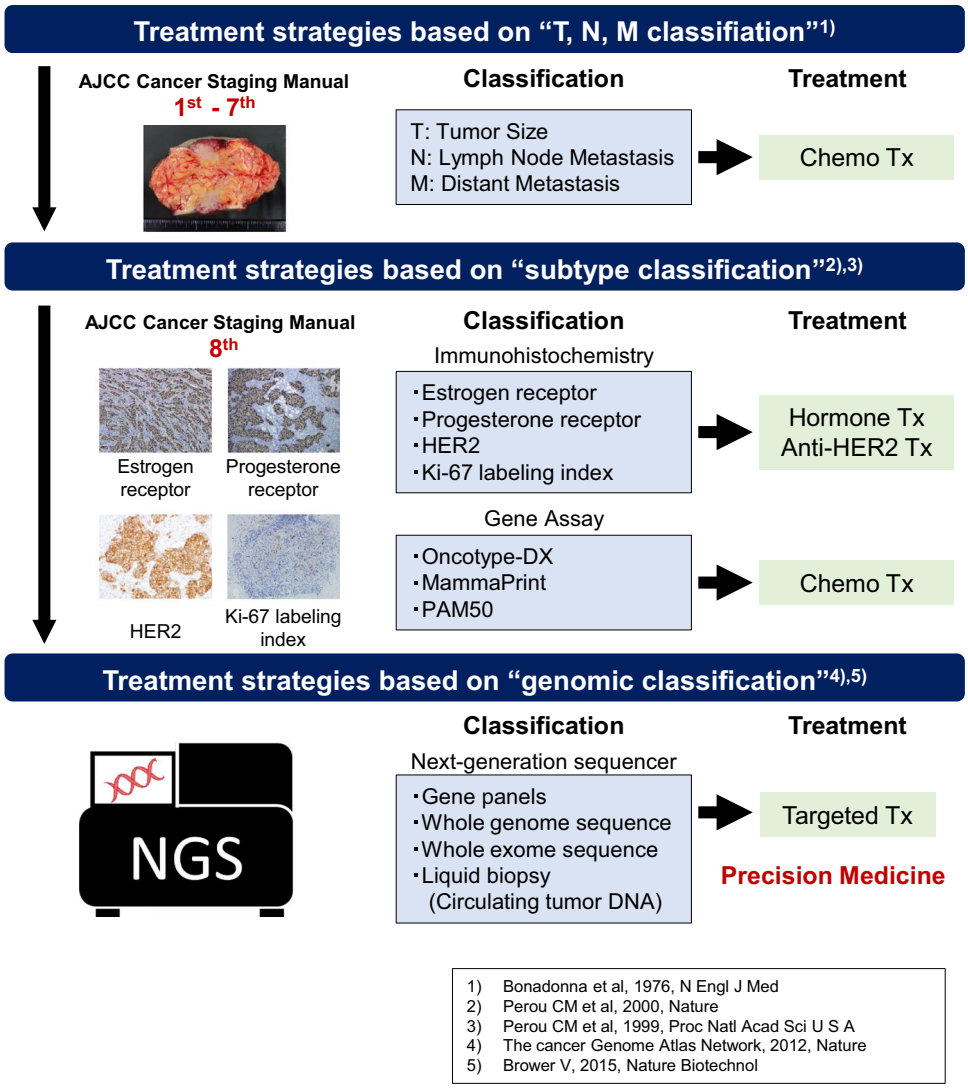

Fig. 1 Paradigm shift of breast cancer treatment. Initially, indications for chemotherapy for breast cancer were determined exclusively by tumor size, lymph node metastasis and distant metastasis. Currently, breast cancer is categorized by the expression of estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2 (HER2) protein, and Ki-67 labeling index, given the availability and efficacy of specifically tailored therapies to each. More recently, gene assays predict the benefit of chemotherapy. Lately, genomic test utilizing next-generation sequencer enables one to select patients who are expected to respond better to each drug. *Ad* adjuvant; *AJCC* American Joint Committee on Cancer; *Tx* treatment

the use of anti-HER2 therapies. HER2 overexpression, once thought to confer a worse prognosis due its association with biologically high malignant potential, is now in fact considered a favorable prognosticator for breast cancer patients due to the advent of targeted anti-HER2 treatments. Nonetheless, cancers have shown the ability to develop resistance to targeted therapies, and as a result, complete remission remains an elusive goal. Thus, understanding the mechanisms of resistance has become a topic of great interest.

The 21-gene Oncotype DX breast cancer assay is now standard of care in the United States [22]. Using reverse transcriptase polymerase chain reaction (RT-PCR), this genomic test quantifies the expression of 21 genes, 16 cancer-related genes and five reference genes, in a tumor sample following removal via surgery or biopsy [23–25]. It delivers a Breast Recurrence Score (BRS), which predicts the magnitude of chemotherapy benefit in patients with node-negative, ER-positive breast cancer, as well as the risk of recurrent cancer for patients diagnosed with early-stage invasive breast cancer [26]. The overwhelming evidence of the test's efficacy prompted both the National Comprehensive Cancer Network (NCCN; National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology: Breast Cancer, version 3; 2017) and American Society of Clinical Oncology (ASCO) [27] to include Oncotype DX BRS analysis in their recommendations for testing node-negative, ER-positive, HER2-negative breast cancer patients [22].

Similarly, MammaPrint is a breast cancer recurrence assay that measures the mRNA expression of 70 genes predictive of breast cancer's ability to metastasize, including those associated with regulation of the cell cycle, invasion, and angiogenesis [28, 29]. The genetic signature stratifies patients into low- or high-risk prognostic groups, which help physicians determine whether a patient will benefit from chemotherapy. As such, women with low-risk results can safely forgo chemotherapy without a negative impact on disease-free survival.

Predictor Analysis of Microarray 50 (PAM50) measures the expression of 50 genes that help identify intrinsic biological breast cancer subtypes [16, 30, 31]. The assay develops a proliferation score based on a subset of genes related to cell cycle progression, as well as a prognostic risk of relapse score based on the PAM50 genetic signature, intrinsic subtype, tumor size, nodal status, and proliferation score. Three categorical risk groups are defined to determine the overall risk of breast cancer metastases [32].

Until the last edition of the AJCC Cancer Staging Manual, breast cancer stage had been determined only by classical anatomical factors, T, N, and M [33–39]. Considering the recent rapid progress in biology, diagnostics, and therapeutics of breast cancer, however, the Expert Panel for the AJCC Cancer Staging Manual 8th edition considered it necessary to incorporate basic biomarkers, including the molecular subtyping based on the status of ER, PgR and HER2, and Oncotype Dx as a multigene molecular profiling, into the staging system [40]. This revision is quite significant since the AJCC decided to consider not only anatomical factors, but also the non-anatomical basic biomarkers as a stage modifier, which will provide a paradigm shift in the field, not only of breast oncology, but also other solid tumors [40]. AJCC 8th edition had included only Oncotype Dx among the multigene molecular profiling tests into the staging system, since it was determined based on the evidence by 2016, and Oncotype Dx only showed Level I evidence at that point [40]. However, the Expert Panel already admired the importance of other multigene molecular profiling tests in the introduction of the staging manual [40], and the next edition is expected to include more tests considering evidence since 2016 [41], which will be more suitable for the era of precision medicine.

The development and commercialization of the aforementioned multigene panel test have provided a paradigm shift in treatment strategies for patients with solid tumors including breast cancer [22]. However, despite the utility of molecular diagnostics for ER-positive breast cancer patients as described above, triple-negative breast cancer (TNBC) patients appear to have been omitted, mainly because addressable molecular targets have yet to be discovered [10]. Although TNBC frequently expresses other hormone receptors, such as prolactin and androgen, cells with a luminal phenotype are rarely observed [42]. Nonetheless, there have been attempts to subcategorize patients with TNBC to identify appropriate therapies for each. For instance, a cluster analysis identified six subtypes of TNBC: basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen receptors [43]. The analysis of the TNBC subtypes has shown differing clinical outcomes and varying responses to therapy, both in the neoadjuvant and adjuvant settings [43]. Furthermore, new treatment strategies considering the differences in TNBC subtypes have resulted in more appropriately targeted treatment [44].

Foundation of precision medicine utilizing NGS

Development of NGS has both dramatically advanced genomic sequencing technology and revealed important genomic alternations for individual cancer patients. Prior to the advent of NGS, the first successful sequencing of the human genome was completed by the Human Genome Project in 2003 [45]. The Project relied heavily on an approach known as shotgun sequencing, in which genomic DNA is randomly broken down into smaller segments and cloned into vectors. Segments of sequence are subsequently aligned and re-assembled using overlapping ends of different vectors to eventually generate the complete overall sequence. The concept of shotgun sequencing contributed heavily to the development of NGS [46–48].

The completion of the Human Genome Project marked the first step to fully understanding the biology of cancer, with the reference genome established by the Project used as a foundational tool to shed light on disease pathogenesis. The Project revealed that genetic alterations are responsible for many different types of human malignancies [49] and inspired similar projects, such as the National Institutes of Health's (NIH's) The Cancer Genome Atlas (TCGA), to further the scientific understanding of cancer.

TCGA Project, a large-scale collaboration overseen by the United States National Cancer Institute's (NCI's) Center for Cancer Genomics and the National Human Genome Research Institute, generated high-throughput "omics," i.e. genomic, epigenomic, and proteomic, data from primary samples of 32 different cancer types, including over 1000 diverse breast cancers [50]. Using NGS technology, researchers revealed genomic alterations in cancer, not only at the DNA level (point mutation, copy number change, and epigenetic modification), but also at the RNA level (messenger RNA [mRNA], non-coding RNA, and microRNA [miRNA]). TCGA Project also uncovered protein expression in breast cancer (both protein and phosphoprotein analyses), which was subsequently analyzed in the context of DNA and RNA status. The integration of these data identified subtype-specific genetic, epigenetic, and proteomic alterations, and provided a potential functional interpretation of the underlying biology.

Further, TCGA Project can be used to characterize the association between survival and specific patterns of gene expression utilizing its robust clinical data set, which may allow for the prediction of survival impact of therapeutic targeting of identifiable gene patterns [50–53]. Thus, a

therapeutic roadmap for precision medicine is emerging and setting priorities for clinical trial development [54].

Application of precision cancer medicine for clinical trials

As previously described, precision medicine refers to the classification of individuals into subpopulations that differ in their susceptibility to a particular disease or in their response to a specific treatment [3]. The discoveries put forth by TCGA Project have posited that precision medicine may be at the forefront of cancer treatment if patients are categorized by actionable driver mutations and subsequently treated via the appropriate molecular targeted therapies. Recently, the concepts behind precision medicine have undergone robust and rigorous testing by way of clinical trials.

Two novel structures of clinical trials, umbrella and basket trials, have been developed to gather specific patient populations to test the efficacy of a precision medicine approach. Umbrella trials comprise multiple treatment arms under the "umbrella" of a single trial of a cancer type [55]. In this approach, patients with a given type of morphologically defined cancer are assigned to a treatment arm based on the genetic mutations detected in their tumors [10, 56, 57]. One immediate advantage of this methodology is the ability to draw meaningful conclusions specific to a tumor type, which are, therefore, less prone to chance tumor heterogeneity present within a cohort trial [58]. Furthermore, when the researchers randomize patients within cohorts to targeted versus non-targeted treatments and particularly when a marker-negative cohort is included, they can more exhaustively evaluate the drug's purported mechanism of action and can empirically distinguish prognostic versus predictive marker effects [58].

One example of an umbrella design is the BATTLE (Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination) trial conducted on 255 pretreated lung cancer patients reported in 2011, which was the first completely prospective, biopsy-mandated, biomarker-based adaptively randomized study of its kind. Following an initial equal randomization period, patients with advanced-stage non-small cell lung cancer refractory to prior chemotherapy were optimally randomized to several groups with different molecular targeted drugs, including vandetanib, erlotinib, erlotinib plus bexarotene, or sorafenib, based on molecular biomarkers examined by core needle biopsy. As a result, it was shown that there was a benefit from sorafenib among patients with mutant-KRAS. More importantly, however, the trial established the feasibility of a new paradigm for the precision medicine approach to cancer clinical trials [59].

Basket trials test the effect of a single drug on a molecular alteration in a variety of cancers, potentially including those not otherwise studied in clinical trials of targeted therapies [60]. With such a "histology-agnostic" (without knowledge of the histology) approach, patients with different cancer types are divided into separate study arms, or "baskets," allowing for separate analysis of individual responses by cancer type, as well as an assessment of a drug's impact on the entire group of patients in aggregate [61]. A major advantage of this study design is the ability to identify molecular alterations in tumors and subsequently select the patients who would most likely benefit from a particular targeted therapy [62]. A basket trial is also beneficial when the cancer type or gene mutation is rare, as it enables researchers to test therapies for cancers that would otherwise be severely underrepresented in clinical trials [61]. One major limitation, however, is its reliance on the notion that molecular profiling may be sufficient to replace histological findings; in several cases, tumor histology has been found to better predict treatment response when compared with biomarkers or mutations comprising the basket study cohorts [63].

The NCI-MATCH (Molecular Analysis for Therapy Choice), another example of a basket approach trial currently underway by the NCI, is designed to explore treating patients based on the molecular profiles of their tumors. Structured as a multi-arm phase 3 study, it plans to screen 3000 adult patients with advanced cancer, lymphomas, or myeloma refractory to standard treatments or for which there is no therapy, with the ultimate goal to enroll 1000 patients [55]. Biopsy specimens from enrollees will be analyzed for more than 4000 different variants across 143 genes; using the results of genomic profiling, the individual patients will be assigned treatment with 1 of 24 drugs with either FDA-approved or investigational-based actionable mutations [61, 64]. Each treatment arm will act as a single-arm open label trial within the confines of one larger trial and will not be accompanied by a control arm [61]. The primary endpoint for each arm will be the objective response rate, defined as percentage of patients whose tumors have a partial or complete response to treatment; treatments will be considered promising for future studies if at least 16 % of patients in a given study arm are observed to have tumor shrinkage [65–67]. The hope is that the results from the NCI-MATCH trial will contribute evidence towards the clinical validation and utility of molecular information in guiding a precision medicine-based approach to care.

NGS technology plays an important role in detecting genetic alterations in cancer patients not only in research endeavors, including TCGA and other projects, but also in clinical settings, such as the trials described above. In TCGA Project, whole-exome sequencing (WES) by NGS was

performed to determine entire genomic alterations for more than 20,000 genes. Nonetheless, WES may not be feasible in clinical practice due to its high cost, shallow sequencing depth, and its tendency to provide excessive data on variants or genes of unknown significance [68]. Recently, we reported that clinical target deep sequencing with cancer-associated gene panels would identify all actionable genomic driver mutations and further determine mutational burden in cancer patients, both of which enable development of personalized treatment strategies. Currently, clinical sequencing with large gene panels based on NGS technology is widely used for clinical trials evaluating the utility of precision medicine. As NGS continues to develop, NGS enables us to assign patients to matched treatments based on multiple driving mutations, biomarkers, or pathways [69].

Clinical benefit of NGS for precision medicine

Increasing numbers of molecular targeted drugs allow for therapeutic intervention directed to the mutations detected by DNA sequencing with NGS [55, 70]. Clinical sequencing with a large number of gene panels utilizing NGS enables the detection of "actionable" mutations in each cancer patient to select subgroup of patients who are expected to be more responsive to each drug based on their actionable driver mutations [55, 71–73] (Fig. 1). We reported that clinical sequencing captures broad actionable mutations in advanced colorectal cancer patients [74]. For instance, HER2-positive colorectal cancer patients are candidates for HER2-targeted therapy even though the therapy was originally developed for breast cancer. Shimada et al. reported that the results of HER2 status were identical between immunohistochemistry and comprehensive genomic sequencing. Comprehensive genomic sequencing has the potential to facilitate precision medicine [75]. Furthermore, multigene panel testing revealed that right-sided colorectal cancer is more likely to harbor genetic alterations associated with resistance to anti-EGFR therapy than left-sided colorectal cancer [76]. We also reported the clinical utility of genomic sequencing with a multigene panel in gastric cancer. The actionable gene-based classification using comprehensive genomic sequencing creates a framework for further studies for realizing precision medicine in gastric cancer [77].

Patients who lack the target of a drug will not only fail to benefit, but can also be harmed by the drug's "off-target" side effects when used without information on the resistance [78]. Clinical target sequencing reveals the drug resistance mechanisms of cancer in each patient by identifying certain actionable mutations related to signaling pathways involved in the resistance of molecular targeted therapies [79]. For instance, several studies have identified alterations in genes downstream of EGFR, such as *RAS* and *RAF*, as likely indicators of resistance to anti-EGFR antibody therapies in colorectal cancer patients [80]. We previously found that there were patients who progressed while on anti-EGFR therapy, and clinical sequencing with NGS revealed that the patients had previously unidentified mutations downstream of EGFR, emphasizing that single gene testing alone is inadequate in guiding therapeutic strategies [74].

As for breast cancer, recent investigations have identified alterations in genes downstream of HER2 which can cause drug resistance to anti-HER2 therapies [81] (Fig. 2). We experienced a HER2-positive breast cancer patient who was resistant to anti-HER2 therapies, including trastuzumab, pertuzumab, TDM-1 and lapatinib. HER2-positive breast cancer tumor tissue obtained from the patient was analyzed using the NGS-based large panel test (Table 1). Interestingly, there were deletions of *CDKN2A* and *CDKN2B*, which are known to upregulate the cyclin-dependent kinase (CDK) pathway. There was also deletion of *STK11*, which activates the mTOR pathway. Moreover, the patient showed a mutation of *TP53*. In general, anti-HER2 therapy is used to suppress the proliferation signal of a HER2-positive breast cancer. Considering that both the mTOR and CDK pathways are downstream of the ERBB2/EGFR pathway [82–85], it is possible that the mTOR or CDK pathway may contribute to the cell proliferation signal for this patient even when the ERBB2 pathway was suppressed. Indeed, this patient showed resistance to anti-HER2 therapy, presumably due to alterations in the mTOR and CDK pathways. Clinical target sequencing with large gene panels can indicate appropriate molecular targeted drugs by revealing actionable mutations related, not only to sensitivity to the drugs, but also resistance to them.

One of the ultimate aims of precision medicine is to improve the cost effectiveness of cancer treatment. However, there has not yet been enough evidence to reveal the cost effectiveness of precision medicine [86]. Precision medicine is expected to show the cost effectiveness in multiple ways, including: (1) subgrouping of patients for appropriate molecular targeted therapy to avoid wastefulness of the resource [87]. (2) Prevention of the disease by identifying high-risk groups, which includes screening or surveillance, and medical intervention, such as prophylactic mastectomy [88, 89]. For the implementation of precision medicine, it will be necessary to prove its cost effectiveness by accumulating appropriate clinical data, systematically cooperating with bioinformaticians, as well as biostatisticians.

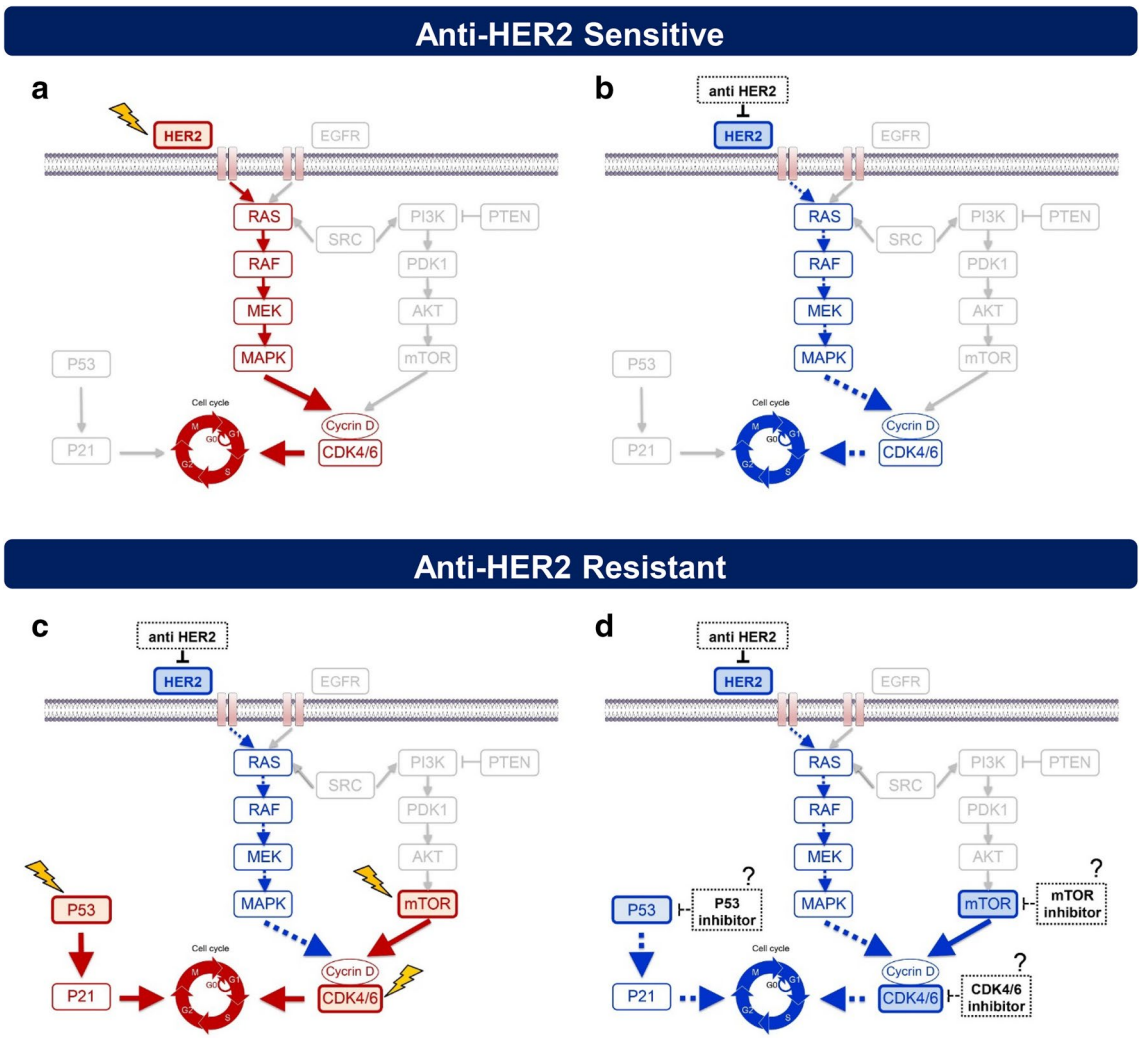

Fig. 2 Resistance mechanism of human epidermal growth factor receptor 2 (HER2)-positive breast cancer patient. **a** HER2 is a crucial cell proliferation signal of HER2-positive breast cancer. **b** Anti-HER2 therapy suppresses the cell proliferation signaling pathway of a HER2-positive breast cancer. **c** Even when the ERBB2 pathway was suppressed by anti-HER2 therapy, resistance may be acquired when the patient has (i) activation of the cell proliferation signaling pathways rather than the ERBB2 pathway, such as the PI3K pathway due to an alteration of mTOR, (ii) activation of the CDK pathway due to alteration of CDK4/6, or (iii) dysregulation of the cell cycle due to alteration of the P53 pathway. **d** These pathways may become treatment targets for patients with resistance to anti-HER2 therapy

Conclusion

The knowledge from the Human Genome Project followed by TCGA has established the concept of precision medicine, in which cancer patients can be sub-classified based on the actionable driver mutations and treated by appropriate molecular targeted therapies. One of the ultimate aims of precision medicine is to improve the cost effectiveness of cancer treatment. Clinical target sequencing with large gene panels enables one to select subgroups of patients who are expected to have more effectiveness of each drug, while at the same time identifying the resistance mechanisms of cancer.

Table 1 Gene alterations detected in the patient with HER2 resistance and relevant therapies to the alterations

Pathway	Gene, variant	Relevant therapies	Status of development
ERBB2	*ERBB2*, amplified	Trastuzumab Ado-trastuzumab	Approved therapy
CDK	*CDKN2A*, deleted	Palbociclib	Unapproved therapy
CDK	*CDKN2B*, deleted	Palbociclib	Unapproved therapy
P53	*TP53*, T125P	Investigational	Under development
mTOR	*STK11*, deleted	Everolimus	Unapproved therapy

The gene alterations were determined by NGS-based gene panel test (CANCERPLEX, KEW Inc., MA)

Acknowledgements This project was supported by funding from Denka Co., Ltd. This work was supported by the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research Grant Numbers 16K19888 for J.T., 18K19576 for M.N., and 16K15610 for T.W. M.N. was also supported by Takeda Science Foundation. K.T. was supported by NIH/NCI grant R01CA160688 and Susan G. Komen Investigator Initiated Research Grant IIR12222224. J.T. and M.N. were supported by Tohoku Cancer Professional Training Promotion Plan.

Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest.

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