Genomic studies have shown that large numbers of candidate targets are observed in breast cancer. Nevertheless, only a few of them are validated as relevant targets in clinical studies. Estrogen receptor (ER) and HER2 expressions could be associated with a level 1 evidence. Beyond ER and HER2, BRCA and PIK3CA mutations (when targeted with alpha-specific PI3K inhibitors) could be considered as promising targets in breast cancer since they have been associated with objective responses in phase I/II trials. In addition to these four molecular alterations, several others have shown promising results in preclinical studies and are being investigated in clinical trials. These genomic alterations include AKT1, ERBB2, and ESR1 mutations. These considerations highlight the lack of evidence for using multiplex technologies to individualize therapy in metastatic breast cancer. Sequencing multiple genes to treat metastatic breast cancer is very promising but should be done in the context of clinical trials, either to enrich phase I/II trials in patients with genomic alterations or to show medical usefulness of new biotechnologies like next-generation sequencing (NGS). Although most current approaches of precision medicine are aiming at targeting drivers, additional applications could be developed in the future. This includes the identification of DNA repair deficiencies, mechanisms of immune suppression, and identification of minority lethal subclones. Finally, one of the very promising applications of genomics for metastatic breast cancer is the identification of pathway activation or defects at the individual level. For example, gene expression and single nucleotide polymorphisms (SNP) signatures are being developed to detect kinase (such as mammalian target of rapamycin [mTOR]/CDK4) activations or DNA repair deficiencies.

Molecular studies have shown that breast cancer includes a large number of subgroups defined by the presence of a specific genomic or protein alteration. ER expression was the first validated target in breast cancer, leading to the optimal development of endocrine therapy.1 In the late 1990s, HER2 overexpression was validated as a target and was shown to be a predictive biomarker for the efficacy of trastuzumab.2 During the 2000s, genomic analyses based on gene expression arrays suggested that breast cancer could be divided into four different subgroups: luminal A, luminal B, HER2-enriched, and basal-like.3–5 Further studies have suggested that basal-like cancers can be subdivided into six subgroups.6 More recently, studies on NGS have suggested that approximately 40 genomic alterations can be found in primary breast cancers.7,8 Overall, this introduction emphasizes that each single breast cancer presents a specific molecular profile and specific molecular mechanisms of cancer progression. Parallel to the advances in the understanding of disease biology, several advances in technology could dramatically change patient care. Indeed, it has been shown that high-throughput DNA sequencing together with comparative genomic hybridization (CGH; copy number analyses) can be performed robustly in the clinical practice.9,10 This has led to the development of precision medicine that involves multiplex molecular analyses to identify molecular mechanisms of cancer progression in each individual in order to improve treatment. In the following review, we present the current state of this approach in metastatic breast cancers (mBC). Several technologies are available to identify genomic alterations in individuals. First, sequencing allows for the detection of mutations. Sanger sequencing, the original method of sequencing, is an approach that allows analysis of only a few genes at the same time. NGS allows sequencing large number of DNA bases in a single run. This latter technology allows multigene sequencing for precision medicine purposes. In some specific platforms and conditions, NGS can also allow quantifying gene copy numbers, although CGH array or fluorescence in situ hybridization analyses are usually better for this purpose. Finally, gene expression array and reverse transcription polymerase chain reaction quantify gene expression. More recently, RNA sequencing is being developed to assess gene expression, translocations, and mutations.

LEVEL OF EVIDENCE SCALE FOR DRUG TARGETS

NGS and other high-throughput technologies analyze several hundred or thousands of genes in the same assay. These technologies can identify many genomic alterations in each patient. Nevertheless, only a few of them are truly implicated in the disease progression. A level of evidence scale that ranks...
targets according to their relevance has been developed to facilitate interpretation of these high-throughput assays, better communicate results to patients, and prioritize research needs. Level I evidence includes molecular alterations that have been shown to be validated targets, either based on phase III trials or several large phase I/II trials. Level II evidence includes molecular alterations associated with response to treatment in small or unique phase I/II trials. Level III molecular alterations include those that have been considered as promising targets based on preclinical studies. Finally, Level IV evidence includes genomic alterations that are selected based on bioinformatics analyses only, without biologic studies to support them. This level of evidence scale also makes a difference according to whether the molecular alteration has been investigated in the same disease and whether studies included negative controls (evidence that patients without the alteration do not derive benefit from the targeted therapies). This level of evidence scale does not evaluate the medical usefulness of targeting the molecular alteration (benefit as compared with standard of care), but the antitumor activity obtained by targeting it. In the next sections of this article, we will use this level of evidence scale to classify molecular alterations in breast cancer. From this analysis, we will further discuss the current positioning of multiplex assay to treat patients with breast cancer.

MOLECULAR TARGETS AND THEIR RESPECTIVE LEVEL OF EVIDENCE IN BREAST CANCERS

Molecular alterations can be divided between drivers, mechanisms of resistance, mutational process and DNA-repair defects, immune alterations, cell death, angiogenesis, and metabolism. These latter three systems will not be discussed in the present article. The list of the most investigated molecular alterations and their level of evidence are reported in Table 1.

Oncogenic drivers can be defined as molecular alterations involved in malignant transformation and cancer progression. Targeting a driver is expected to lead to tumor shrinkage (known as oncogene addiction). As mentioned in the introduction, the two historic drivers include ER expression and ERBB2-amplification. These two targets are associated with level I evidence. Additional candidate molecular alterations are being investigated in breast cancer. These alterations should be divided between DNA-based assays and pathway-based assays. At the DNA level, there are between 10 to 20 genomic alterations that are currently the targets of drug development.

PIK3CA mutations are observed in approximately 25% of breast cancers, mainly those with ER or HER2 expression. PI3K activates AKT1 that subsequently activates mTOR. AKT1 also interact with pathways that do not relate with mTOR, including FOXO, BAD, and GSK3. Several drug families target PI3K/AKT/mTOR pathways, including mTOR inhibitors, AKT inhibitors, nonselective PI3K inhibitors, and alpha-selective PI3K inhibitors. In an ancillary study of the BOLERO2 trial, PIK3CA mutations were not predictive for the efficacy of mTOR separate inhibitors in ER-positive/HER2-negative breast cancer. Nonselective PI3K inhibitors target most of the PI3K subunits and thus present a narrow therapeutic index. These drugs can therefore achieve modest PI3K inhibition. This could explain why nonselective PI3K inhibitors have shown mitigated results in phase II randomized trials. Conversely, alpha-selective inhibitors are very potent inhibitors of alpha subunit, a major player of PI3K activity in cancer. Interestingly, phase I studies using alpha-selective PI3K inhibitors have shown extremely encouraging results in patients who with PIK3CA mutations, suggesting that PIK3CA mutation could be a relevant target in mBC. This genomic alteration should be ranked level IIa. Activating AKT1 mutation is the other genomic alteration located in

| TABLE 1. Potential Applications of Genomics for Metastatic Breast Cancers |
|---------------------------------|-------------------|-----------------|-----------------|
| **Application of Genomics**     | **Optimal Technology** | **Targets** | **Level of Evidence Associated with the Target** |
| **Drivers (DNA)**              | Next-generation sequencing if multiple genes validated | ERBB2 amplification | I |
|                                 |                   | PIK3CA mutations | II |
|                                 |                   | AKT1 mutations | III |
|                                 |                   | ERBB2 mutations | III |
| **Drivers (RNA/Proteins)**     | Gene expression Phosphoprotein assays | ER expression | I |
|                                 |                   | mTOR activation | ND |
|                                 |                   | CDK4/6 activation | ND |
| **Lethal subclone**            | Ultra-deep sequencing Circulating DNA | ESR1 mutations | III |
| **DNA repair**                 | Targeted sequencing Whole-exome sequencing SNP arrays | BRCA1/2 mutations | I/II |
| **Immune system**              | Whole-exome sequencing RNA sequencing | PD-L1 overexpression | ND |
|                                 |                   | Neoantigen | |

Abbreviations: ER, estrogen receptor; mTOR, mammalian target of rapamycin; ND, not determined; PD-L1, programmed death ligand 1.

KEY POINTS

- Only a few molecular alterations are validated as targets in breast cancer (specifically ER and HER2 expression).
- Driver identification in breast cancers includes DNA-based analyses but also detection of pathway activation and dependency (e.g., ER, mTOR, and CDK4).
- Driver identification is not the sole application of genomics to personalize therapy for metastatic breast cancer.
- There is no evidence that using multiplex genomic testing for metastatic breast cancer improves outcomes.
- Ongoing trials are evaluating the medical usefulness of next-generation sequencing in metastatic breast cancers.

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the pathway. First, AKTi mutations occur in approximately 3% of breast cancers. These alterations are oncogenic in preclinical models and have been associated with objective response to mTOR inhibitors in molecular screening programs.10 Ongoing studies are investigating the efficacy of AKT inhibitors in patients with this genomic alteration. Although some retrospective analyses suggest oncogene dead-
p
diction in patients with AKTi mutations, this target is still considered to be a level III target.

FGFR1 amplifications occur in approximately 10% of breast cancers, mainly those with ER expression. This alter-
at
tion has been associated with very promising results in pre-
classical studies.17 Nevertheless, treatment with highly specific
and bioactive FGFR inhibitors failed to demonstrate antitu-
mer activity in phase I trials.18 Interestingly, multikinase in-
hibitors like lucitanib were associated with promising antitumor activity in patients presenting FGFR1 amplifica-
tions,19 but whether this antitumor activity relates to FGFR
inhibition is unclear. FGFR1 amplification could be associ-
ted with level III evidence as a target. CCND1 is amplified in
approximately 15% of breast cancers. This amplification is
not clearly associated with CCND1 expression, and clinical
studies have failed to validate that CCND1 amplification are
relevant targets in breast cancer.20 Finally, the last interesting
target located on tyrosine kinase is ERBB2 mutations. These
mutations have been shown to be activating mutations and to
be associated with antitumor activity of neratinib, a HER2
inhibitor.21 Phase II trials are ongoing. This target is cur-
cently considered as level III evidence but could jump to level
I if consistent studies report high levels of antitumor activity
for neratinib in this genomic segment.

Besides alterations at the DNA levels, assessing pathway ac-
tivation and dependency could provide relevant information
about driving forces of cancer progression. To illustrate, ER
expression drives cancer progression in ER-positive breast
cancer, although no alteration is usually detected at the DNA
level. This emphasizes the relevance of assessing pathway ac-
tivation in breast cancer. The Cancer Genome Atlas data
have suggested that PI3K/AKT/mTOR and CDK4/Rb path-
ways are the two most relevant targetable pathways in breast
cancer. Gene expression signatures could provide informa-
tion about activation status of these pathways, together with
assessment of phosphoproteins. For example, in the BO-
LERO3 trial, biomarker studies showed that activation of
mTOR (specifically PS6K) is associated with high sensitivity
to everolimus.

Emergence of lethal subclone is a well-described mechanism
of resistance to targeted therapies. In patients with lung cancer,
it has been well documented that T790M mutations, although
the minority at the time of diagnosis, become predominant after resistance to EGFR inhibitors.22 In breast cancer, a similar phe-

omenon is being observed with ESR1. ESR1 is the gene that
encodes for ER. Although less than 1% of early breast cancers
present mutations, it is considered that between 10% to 30% of
breast cancers resistant to aromatase inhibitors will have a hot-
spot mutation thus leading to ligand-independent activation of
the receptor.23 24 It has been suggested from preclinical works
that high-dose fulvestrant could present some antitumor activ-
ity in this subset of patients. Several new ER degraders like
GDC-0810 are being developed in this setting (NCT01823835).
Until now, this target is classified level III. There are several
questions surrounding this genomic alteration that could have
some clinical influence. First, as opposed to T790M, this muta-
tion has not yet been reported in a minority subclone in a pri-
minary tumor. Finding the ESR1 mutation in a minority clone in
a primary tumor would open the path for the development of
ultra-deep sequencing to detect them and potentially treating
them very early during the disease course. Second, the use of
circulating DNA could help detect these mutations during the
disease course and treat them early. Finally, one study has sug-
gested that ESR1 mutations could be associated with very poor
outcome. If validated, this finding would suggest that this
 genomic segment would deserve some fast-track approvals
based on phase II data.

The third application for genomic tests is the identification of
DNA repair defects and mutational processes at the individual
level. Identifying DNA repair defects could lead to administra-
tion of personalized synthetic lethality strategies or specific
genotoxic agents. The best example in breast cancer is provided
by BRCA1 and BRCA2 mutations. When biallelic, BRCA1 and
BRCA2 mutations and/or loss lead to homologous recombi-

nation deficiency and genomic instability. BRCA1/2 mutations
have been associated with sensitivity to PARP inhibitors (syn-
thetic lethality) and DNA alkylating agents (genotoxic).25 Inter-

estingly in these trials, patients without BRCA1/2 alterations did
not present a similar degree of antitumor activity as compared
with patients with mutations. Based on these consistent data
from phase III and large phase II programs, BRCA1/2 mutations
drug targets have a level I evidence.11 The controversy in this
area is more about how to position each therapeutic strategy
(PARP1 inhibition and DNA alkylating agents) and to show
medical usefulness over standard of care, rather than whether
BRCA1/2 mutations constitute a target per se. The second con-
troversy is about whether some functional tests evaluating ho-

omologous recombination deficiency (HRD) could have better

performance than detecting BRCA1/2 mutations. HRD tests
could have better performance either by selecting the right pa-
tient with the BRCA1/2 mutation or by identifying patients with
BRCA1/2 wild-type who present with HRD. When assessed ret-

rospectively, the HRD test developed by Myriad was not associ-
ated with a differential sensitivity between platinum and
docetaxel. The HRD test developed by Clovis has been associ-
ated with sensitivity to the PARP inhibitor rucaparib, even in the
absence of BRCA mutation.26 An ongoing phase II trial (RUBY)
is testing whether rucaparib could present antitumor activity
in patients with BRCA1/2 wild-type who present a high HRD.
Beyond HRD and BRCA, assessing other DNA repair genes or
pathways could allow expanding the array of patients who could
be eligible for synthetic lethality strategies. ATM and ATR mu-
tations are observed in approximately 2% of breast cancers and
could define a subset of patients eligible for synthetic lethality
approaches. In terms of pathways, several studies have sug-
gested that the mutational pattern detected by whole-exome se-

quencing could allow for defining which DNA repair pathway is
altered in each individual.\(^{27}\) This could potentially lead to the use of whole-exome sequencing to individualize synthetic lethality approaches for patient treatment.

Finally, the fourth potential application field of genomics to individualize therapy is immunology. Genomics could allow detecting neoantigens and expression of ligands for immune checkpoints,\(^{28}\) but also test the competence of the cancer cell to present antigens and to be killed. Recent data obtained in metastatic triple-negative breast cancer suggest that anti-PD-1 antibody could present some antitumor activity.\(^{29}\) Finally, genomic tests could evaluate whether the host could generate an antitumor immune response following immunogenic cell death.\(^{30}\) For example, TLR4 polymorphisms confer lack of immunogenicity and have been associated with resistance to anthracyclines in patients with breast cancer.\(^{30}\)

**CLINICAL DEVELOPMENT OF PRECISION MEDICINE IN METASTATIC BREAST CANCER: WHICH TECHNOLOGY? WHICH SAMPLES? WHICH TRIALS?**

As discussed in the previous section, there are large numbers of applications for genomics to better treat patients in the metastatic setting. Each application deserves a specific technology and it is important to define which technology will be developed for which purpose. Current approaches of precision medicine aim at identifying drivers at the DNA level. For this purpose, sequencing is the best technology. Whether sequencing should be based on Sanger technology or should consist in NGS depends on the number of genes to be tested. An important aspect of breast cancer is the high number of copy number alterations that could potentially drive cancer progression. Assessing gene copy numbers requires fluorescence in situ hybridization technology for a single or a few genes, or CGH/SNP arrays for a large number of genes. Interestingly, some centers can now robustly assess copy number using NGS technology.\(^{9}\) This therefore makes this technology a preferred choice for clinical research programs that aim to identify drivers in mBC. Alternatively, circulating DNA could be useful when biopsies are not feasible. As mentioned previously, assessment of pathways activation could be done by gene expression arrays, reverse transcription polymerase chain reaction, or phosphoprotein assays. Other technologies could be dedicated to specific purposes of clinical research. First, ultra-deep sequencing could be an interesting approach to detect minority clones, and circulating DNA could be an interesting approach to detect the appearance of resistance. SNP array could be interesting to quantify HRD. On a long-term perspective, one could argue that the best approach to personalize therapy will be to apply whole-exome sequencing to hard-to-treat mBC. Whole-exome sequencing offers the advantage of detecting both drivers, DNA repair defects and neoantigens.\(^{28}\) Finally, RNA sequencing could offer the advantage of detecting pathway activation and target expression.

One field for controversy is which sample should be used for target identification in patients. Primary tumors offer the advantage of being accessible and not requiring additional biopsies. Nevertheless, several studies have shown that targets can be lost or gained during the disease evolution.\(^{31}\) More recently, evidence has been reported that targets acquired during the disease course—although not trunk alterations—could drive cancer progression.\(^{32}\) This emphasizes the need to assess genomic and molecular targets at the time of treatment decision and start. Assessing cancer biology at the time of treatment decision would need to perform biopsies of metastatic sites. Whenever this is feasible, biopsy of metastatic sites should be the priority since it allows assessing genomic alterations but also RNA and protein expression together with immune markers. Nevertheless, if the tumor site is difficult to biopsy or in case of bone disease, circulating DNA could be a possible alternative.

Until now, there has been no data to support the use of DNA- or RNA-based technologies in daily practice for patients with mBC. Several nonrandomized trials have been performed but they did not provide a clear picture about the potential medical usefulness of precision medicine for breast cancer. In the SAFIR01 trial,\(^{10}\) approximately 28% of the patients presented evidence of antitumor activity but only 10% had an objective response. These numbers align with phase I/II trials that tested drugs without molecular selection. In a clinical trial testing gene expression, Von Hoff et al reported that a group of patients had their progressive-free survival prolonged under genomic-based therapy.\(^{33}\) Nevertheless, this trial did not have a control group with patients treated with the same treatments but not driven by genomics. Outside of these two trials, there is no large study reporting efficacy of genomics in mBC, and therefore, with the exception of prospective clinical trials, multiplex approaches cannot be recommended for routine practice.

The next question is how to provide evidence that the use of genomics improves outcomes in patients with mBC. There are two possible strategies to address this question. The first strategy consists of prospectively validating each target in large clinical trials. This strategy is currently being used in most of the clinical programs in breast cancer. For example, HER2 inhibitors are being developed in patients with ERBB2 mutations, PI3K inhibitors are being developed in patients with PIK3CA mutations, AKT inhibitors in patients with AKT1 mutations, and so on. This approach will lead to the clinical validation of several genomic alterations. Once the number of such genomic alterations is large enough, companion diagnosis will very likely switch from Sanger single-gene sequencing to NGS. The second approach will consist of the clinical validation of a multiplex approach. In this design, the trial does not aim to validate each genomic alteration, but to test the hypothesis that using a multiplex technology improves outcomes. For example, the SAFIR02 trial (NCT02299999) is testing the hypothesis that use of NGS and CGH arrays improves outcomes compared with standard of care.

**MOVING TO RATIONALE COMBINATIONS**

As previously mentioned, breast cancer is a complex disease in which each patient could present several altered genomic alterations and pathways. Therefore, there is a strong rationale to
combine drugs in the era of molecular medicine. There are three possible ways of combining drugs. First, drugs can be combined based on the presence of multiple genomic alterations. It has been suggested that multiple genomic alterations on drivers could be associated with resistance to therapy. Combining drugs that target different genomic alterations could therefore lead to antitumor activity. This is the rationale to combine HER2 and PI3K inhibitors in patients with ERBB2-amplified, PIK3CA-mutated BC. The second rationale to combine therapies would be to target molecular processes in different systems. For example, ERBB2-amplified BC is associated with a high level of PD-L1 expression and PD-L1-induced immune suppression. There is therefore a rationale to combine HER2-inhibitors and anti-PD-1 in patients presenting ERBB2-amplified and PD-L1-induced immune suppression (PANACEA; NCT02129556). Finally, other combinations will aim at avoiding cancer cell adaptation. For example, cancer cells presenting a PIK3CA mutation can further adapt to PI3K inhibitors through CDK4 or mTOR activation. This provided a rationale to evaluate triple combinations therapy.

CONCLUSIONS AND PERSPECTIVES
A high number of gene or molecular alterations could be considered as actionable in breast cancer. Nevertheless, only a few of them (ER, HER2 expressions, and—to a lesser extent—BRCA and PIK3CA mutations) are currently validated as relevant targets. There is therefore no robust evidence that using a multiplex technology in mBC improves patient outcomes. Nevertheless, this approach could allow for accelerated drug development by detecting patients with genomic alterations and driving them to phase I/II trials. The current approach to validating genomics for mBC consists of testing drug efficacy in each genomic segment by validating each molecular alteration one by one. Since most of these genomic alterations are rare, there is a need to develop very large molecular screening programs. Several international screening programs have been set up recently including the AURORA program, developed by Breast International Group. Alternative development of precision medicine could consist of evaluating the overall effect of sequencing technologies in the whole population of patients with breast cancer, independently of each single alteration. This approach is being used in the SAFIR02 trial. Finally, one of the major challenges in the future will be optimally to implement these new technologies to secure access to innovations for all patients. To achieve this goal, the Institut National du Cancer has set up 28 public genomic centers in France that have a goal to offer access to innovations for all patients. To achieve this goal, the Institut National du Cancer has set up 28 public genomic centers in France that have a goal to offer access to innovations for all patients. To achieve this goal, the Institut National du Cancer has set up 28 public genomic centers in France that have a goal to offer access to innovations for all patients.

Disclosures of Potential Conflicts of Interest

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