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Abstract

Purpose of review

Breast cancer heterogeneity constitutes a significant investigational and therapeutic challenge. Here we review recent findings on breast cancer heterogeneity, focusing on its extent across the distinct molecular subtypes, the degree of spatial and temporal intra-tumor heterogeneity, and possible approaches to dissect and counteract it.

Recent findings

Recent massively parallel sequencing studies have solidified the notion that estrogen receptor (ER)-positive and ER-negative breast cancers have divergent genetic landscapes. Numerous studies have addressed the origins of heterogeneity and the challenges it poses for patient management; however, its dynamic evolution in the light of novel targeted therapies is yet to be fully understood.

Summary

Tumor heterogeneity poses diagnostic and therapeutic challenges. Implementation of novel methodologies, such as single cell sequencing and analysis of cell-free DNA, might afford us the means to comprehend intra-tumor heterogeneity with greater precision, and to overcome the diagnostic and therapeutic challenges posed by it.

Keywords

Breast cancer Heterogeneity Molecular subtypes Estrogen receptor Therapeutic implications

Introduction

Massively parallel sequencing (MPS) studies have deepened our understanding of the genetic landscape of human cancers and have offered the opportunity of matching specific therapeutic agents with the genetic alterations a given tumor harbors [1], allowing us to offer rational personalized therapies to cancer patients [2]. Nevertheless, precision medicine still faces important challenges, such as the ones posed by the substantial heterogeneity between and within tumors [3, 4, 5]. A burgeoning body of evidence has accumulated throughout recent years supporting the vast heterogeneity of breast cancer, evident at the morphologic, genomic, and transcriptomic levels, which translates into dissimilar clinical behaviors and responses to therapy [6, 7, 8].

Notwithstanding current state-of-the-art technologies, our understanding of tumor heterogeneity, its causes, and clinical implications is still preliminary. Inter-tumor heterogeneity, which refers to differences between tumors from different individuals, poses formidable challenges for the delivery of precision medicine, given that in breast cancer highly recurrent targetable genetic alterations are scarce (Fig. 1a). Moreover, the dynamic and evolving coexistence of different clones within a tumor, with dissimilar genetic alterations and drug sensitivities (i.e., intra-tumor heterogeneity), as well as the emergence of resistant cancer cell populations, also pose diagnostic and therapeutic challenges, adding complexity to the management of breast cancer patients [9] (Fig. 1b). Albeit the therapeutic hurdles raised by tumor heterogeneity are numerous, novel clinical trial designs and therapeutic opportunities are emerging, based on the concepts stemming from the analysis of heterogeneity [10].

In this review, the phenomena of inter- and intra-tumor heterogeneity in breast cancer are discussed, with a special emphasis on their origins, implications in tumorigenesis, the diagnostic and therapeutic challenges they pose, and the potential strategies to circumvent them (Fig. 1c).

Tumor Heterogeneity and Tumorigenesis

Origins of Heterogeneity

It has been posited that tumorigenesis follows Darwinian evolutionary dynamics, in which the interplay between mutation generation and clonal selection shapes the genome of a tumor. Different cancer subclones coexist in a cooperative or competitive manner [11, 12], and while most somatic mutations have little impact in cell fitness, driver mutations result in an evolutionary advantage, allowing the cells harboring them, as well as their progeny, to prosper, illustrating branched clonal evolution [13]. Importantly, early truncal mutations may disappear throughout tumor progression, and conversely, subclonal mutations may be present at low

frequencies in different regions of a tumor, and therefore their detection in distinct tumor areas should not define them as truncal [14, 15].

In contrast to the traditional dogma that regards tumorigenesis as a gradual process [16], catastrophic phenomena, such as chromothripsis, chromoplexy, and kataegis, can lead to substantial modifications of the evolutionary course of a tumor. Menghi et al. [17] analyzed the tandem duplicator phenotype (TDP), a chromotype characterized by the presence of a high proportion of head to tail duplications of DNA segments, across a variety of tumors, and uncovered its enrichment in triple-negative breast cancer (TNBC). Notably, TDP was found to confer sensitivity to cisplatin in triple-negative (TN) cell lines and patient-derived xenografts (PDX) [17]. Loci of kataegis, a common phenomenon in breast cancer associated with chromosomal rearrangements and localized hypermutation [18], were found to be concentrated in genomic areas containing genes and functional regulatory elements, and to be more frequent in chromosomes 8, 17, and 22, while rare in chromosomes 2, 9, and 16 [19]. In line with these findings, a study in mouse models suggested that breast malignant transformation can be induced in an expeditious manner, requiring the introduction of a sole mutation targeting KRAS [20].

A study of the copy number evolution in TNBC opposed a gradual copy number alterations (CNAs) acquisition model, and instead suggested that CNAs occur in brief bursts early in the life of a tumor, after which they remain stable throughout clonal expansion [21]. Importantly, this punctuated evolution model implies that assessment of CNAs at an early stage could forecast the aggressiveness of a tumor, and therapy could be tailored accordingly. Along the same lines, a recent study of 904 tumors across 14 tumor types showed that 323 of them evolved in a neutral fashion, implying that clonal selection occurs early in the life of a tumor, rather than in late subclonal populations, whereas non-neutral tumors display continuous clonal selection, with heterogeneity being the result of passenger mutations [22].

Mutational Signatures

The mutational processes that sculpt the genomes of tumors leave an imprint on it, recognized as mutational signatures, which can be regarded as surrogates of the DNA damage and altered repair processes that engendered them [23]. In most cases, breast tumors display a combination of mutational signatures [24]. A study across 30 different types of cancer unveiled 21 mutational signatures with distinct underlying mechanisms. Some signatures are related to endogenous mutational processes (signature 1 A/B), whereas others are the result of the exposure to exogenous mutagenic agents, such as UV light (signature 7), tobacco (signature 4), and anticancer therapies (signature 11). Aberrant DNA repair processes, such as defective DNA

mismatch repair (signature 6), or altered homologous recombination (signature 3), as well as somatic immunoglobulin gene hypermutation (signature 9), leave mutational scars in the genome. Abnormal functioning of DNA-modifying enzymes or error-prone polymerases, such as AID/APOBEC cytidine deaminases, also results in mutational signatures (2 and 13). Nonetheless, the mechanism behind many signatures remains unknown [25].

Whole-genome sequencing of a cohort of 560 breast cancers led to the identification of 12 base substitution signatures and 6 rearrangement signatures. The latter were found to be a result of tandem duplications or deletions, and to be related to defective DNA repair through homologous recombination [24]. Of note, a recent transcriptomic analysis of 266 breast tumors undertaken to understand how substitution signatures translate at the transcriptomic level showed a positive correlation between the number of substitutions and the expression of cell cycle related genes, regardless of the specific mutational signature. Importantly, this study showed an association of signatures 3 and 13 with a gene expression tumor infiltrating lymphocytes signature. This feature correlated also with a high extent of lymphocytic infiltration and better prognosis [26].

Inter-Tumor Heterogeneity and Therapeutic Implications

Tumor Heterogeneity Within Molecular Subtypes

In the past decade, several studies [27, 28, 29] have led to the molecular classification of breast cancer into the so-called intrinsic subtypes (i.e., luminal A, luminal B, HER2-enriched, and basal-like). Since then, additional molecular subclasses have been described, such as the claudin-low [30] and the molecular apocrine subtype [31], all with distinct clinico-pathologic features, responses to therapy, and outcomes [32]. Importantly, striking heterogeneity can be observed within each one of these subtypes [33], and their stability on the basis of research versions of the assays for their identification is questionable [34, 35, 36].

Seminal studies by Lehmann et al. [37] showed marked inter-tumor heterogeneity within TNBC and recognized seven subtypes: basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), luminal androgen receptor (LAR), and an unstable group. The clinical relevance of this taxonomy has been confirmed in independent analyses of retrospective cohorts of patients treated with neoadjuvant chemotherapy [38, 39]; those studies have revealed that BL1 tumors display the highest pathologic complete response (pCR) rates following neoadjuvant chemotherapy, whereas BL2 and LAR less frequently evolved to pCR. A more recent study by the same group has demonstrated that the likeliest most parsimonious number of TNBC transcriptomic subtypes is four, given that the transcriptomic

features of immunomodulatory and mesenchymal stem-like subtypes likely derive from inflammatory and mesenchymal cells, respectively, rather than from tumor cells [39]. Based on RNA profiling, Burstein et al. [40] defined four TNBC subtypes with distinct potential actionable targets. Tumors of the luminal androgen receptor (LAR) subtype could be therapeutically targeted with androgen receptor antagonists or MUC vaccines; the mesenchymal (MES) subgroup may benefit from IGF or PDGFR inhibitors; immune therapies might prove effective for basal-like immune-suppressed (BLIS) tumors, whereas basal-like immune-activated (BLIA) tumors could respond to Ipilimumab. Taken together, these results demonstrate that TNBCs comprise a remarkably heterogeneous group of tumors, that there might be at least three distinct subtypes (basal-like, mesenchymal and LAR), and that immune response likely plays a pivotal role in the biology of the disease.

HER2-positive tumors, as defined by conventional immunohistochemistry and fluorescence in situ hybridization (FISH) [41], display marked molecular heterogeneity, encompassing all intrinsic molecular subtypes [42], and have genetic landscapes which vary according to estrogen receptor (ER) status and intrinsic subtype [43]. The molecular heterogeneity of HER2-positive disease is paralleled by its clinical behavior and response to therapy, as a considerable proportion of patients with HER2-positive tumors develop primary or secondary resistance to anti-HER2 therapies [44]. Efforts have been made to identify biomarkers that could predict response to anti-HER2 therapies. Recently, Pogue-Geile et al. [45] classified HER2-positive breast tumors using PAM50 through the nCounter platform and observed clinical benefit upon treatment with trastuzumab across all intrinsic subtypes. In contrast, Perez et al. [46] applied the PAM50 Prosigna algorithm to stratify HER2-positive tumors across different intrinsic subtypes and observed that patients with non-basal tumors benefitted from treatment with trastuzumab in terms of recurrence-free survival, whereas patients with basal-like tumors did not. The different methods to define intrinsic subtypes used in these studies [45, 46] could have had an impact on the classification of individual tumors; however, these studies suggest that stratification of HER2-positive disease could potentially be used to fine-tune therapy in this subgroup [47].

In addition, based on the integration of gene expression and genome-wide CNA data, the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) devised a classification of breast cancer into ten integrative clusters (IntClusts) [48, 49], with prognostic implications. Subsequently, Pereira et al. [50] showed that CBFB mutations were more common in IntClust3 and IntClust8, whereas GATA3 mutations had higher frequencies in IntClust1 and IntClust8. IntClust7 showed frequent alterations in genes of the MAP kinase cascade, IntClust3 in genes related to tissue organization, IntClust1 in AKT pathway-related genes, and IntClusts 9 and 10 in DNA damage response genes; in addition, IntClust10 displayed frequent alterations in genes linked to cell cycle regulation and ubiquination [50]. As further evidence for the clinical relevance of

this classification, the analysis of 7544 breast cancers showed that IntClust10 tumors displayed the highest rates (37%) of pCR, whereas none of the tumors belonging to IntClust2 subtype reached pCR [48]. Interestingly, analysis of the cancer cell fractions of mutations in driver genes varied across the different IntClusts. IntClusts 3, 7, and 8, which are associated with better clinical outcomes, have a lower frequency of clonal mutations in driver genes, compared to IntClusts 2 and 10, which carry a worse prognosis [50].

The Mutational Landscape of Breast Cancer

The Cancer Genome Atlas (TCGA) project [51] revealed a limited number of highly recurrently mutated genes in breast cancer. Indeed, solely TP53, PIK3CA, and GATA3 were found to be consistently mutated in more than 10% of unselected breast cancers, with the remaining genes being affected in less than 7.7% of cases, and a high number of them being altered in less than 1% of cases [51]. Nonetheless, a recent analysis of over 2000 breast cancers revealed additional genes mutated in >10% of cases, including MUC16, AHNAK2, SYNE, and KMT2C [50], and additional significantly mutated genes, such as transcription regulators, including TBX3, CBFB, and RUNX1, and chromatin function-related genes, such as KMT2C, ARID1A, NCOR1, CTCF, KDM6A, PRBM1, and TBL1XR1 [50]. Mutations also vary according to the histologic subtype. For instance, pathogenic loss-of-function mutations affecting the CDH1 gene have been confirmed to be one of the defining features of lobular carcinomas, and mutations affecting PTEN, TBX3, and FOXA1 have also been shown to be significantly more frequent in invasive lobular carcinomas than in invasive ductal carcinomas of no special type [52].

Co-occurrence and mutual exclusivity of somatic mutations have helped define the potential significance of specific somatic genetic alterations. In primary breast cancer, mutations affecting genes within the same signaling pathway are, in some instances, mutually exclusive, such as mutations targeting PIK3CA and those affecting AKT1, PIK3R1, and FOXO3, illustrating epistatic interactions within the AKT signaling cascade [53]. In contrast, PTEN inactivating mutations co-occur with PIK3CA and PIK3R1 mutations, and CDH1 is frequently co-mutated with PIK3CA, TBX3, and RUNX1 in lobular carcinomas [50, 52]. Whether these patterns of mutual exclusivity and co-occurrence are maintained in advanced breast cancers following systemic therapy remains to be investigated.

Mutational Heterogeneity Across Different Molecular Subtypes and in Relation to Estrogen Receptor Status

Heterogeneity is evident in the pattern and type of mutations of the different breast cancer intrinsic subtypes; however, there is no single hotspot mutation or highly recurrently mutated gene that defines the individual “intrinsic” subtypes. Basal-like tumors are enriched for nonsense and frameshift TP53 mutations, whereas luminal A and luminal B cancers more frequently harbor TP53 missense mutations [51]. Similarly, the TCGA study has shown that GATA3 intron 4 hotspot deletions were found solely in luminal A tumors, whereas seven of nine frameshift mutations in exon 5 were found in luminal B breast cancers [51].

MPS studies have reinforced the notion that ER-positive and ER-negative breast cancers represent two molecularly distinct entities. Mutations in ER-positive tumors affect mainly PIK3CA (40.1%), MAP3K1 (11.0%), MAP2K4 (5.6%), GATA3 (13.8%), MLL3 (7.6%), CDH1 (8.5%), and AKT1 (3.1%), whereas TP53 (84.5%) leads the list of recurrently mutated genes in ER-negative breast cancer [51, 54]. The study of Pereira et al. [50] uncovered driver tumor suppressor genes not previously found to be altered in the TCGA dataset; ER-positive tumors showed mutations affecting FOXO3, CTNNA1, FOXP1, MEN1, and CHEK2, whereas CDKN2A, KDM6A, and MLLT4 were altered in both ER-positive and ER-negative tumors. ER-positive and ER-negative tumors harbored ERBB2 mutations at comparable rates; however, mutations at codon 755 were more frequent in ER-positive disease. Similarly, ER-positive tumors had PIK3CA mutations affecting the helical domain, whereas in ER-negative breast cancers, PIK3CA was mutated predominantly in the kinase domain [50].

Mutations in driver genes show association with breast cancer-specific survival. MAP3K1 and GATA3 mutations were found to be related to better survival in patients with ER-positive tumors. Conversely, mutations affecting SMAD4 and USP9X were associated with shorter survival [50]. These findings are in line with data showing that decreased SMAD4 expression and altered TGF-beta signaling portend a poor prognosis for breast cancer patients [55].

Taken together, these studies show how the dissection of breast cancer into different subgroups is of paramount importance to overcome the challenges posed by the marked heterogeneity of this entity, and can allow us to better comprehend and make use of the prognostic value of certain genetic alterations.

Intra-Tumor Heterogeneity and Therapeutic Implications

The vast intra-tumor genetic heterogeneity of breast cancer, which is clear at the histologic level, has been illustrated by multiple lines of evidence at the molecular level [23, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66] and poses important challenges in the management of breast cancer patients.

Histologic and genetic differences can exist between geographically separated areas within a tumor, between a primary tumor and a metastatic outgrowth, or even between two or more different metastatic foci (spatial heterogeneity) [66] (Fig. 1b). Moreover, as tumors evolve over time, their genomic landscapes undergo modifications (temporal heterogeneity; Fig. 1b). These changes are particularly evident in three instances during the life of a tumor: (i) progression of in situ to invasive disease; (ii) temporal evolution of a primary breast cancer; and (iii) development of metastasis [32]. Key to the understanding of the therapeutic implications of spatial and temporal intra-tumor heterogeneity is the awareness that only a minority of the mutations is essential for cancer development and progression, whereas most of them lack any significant biological impact or might theoretically even have negative effects on tumor fitness [67, 68].

Spatial Heterogeneity

Yates et al. [69] performed a whole-genome and targeted sequencing study of different regions of 50 breast cancers, with an in-depth analysis of 8 different regions of 12 breast tumors. Most (8/12) of these displayed spatial heterogeneity in terms of somatic mutations, whereas two cases showed intra-tumor heterogeneity in terms of copy number, and two cases showed minor variations. Importantly, not only “passenger” alterations were found to be subclonal, given that a third of the cases had subclonal mutations targeting driver genes, such as TP53, PIK3CA, and BRCA2. In addition, this group also observed temporal heterogeneity possibly triggered by therapy in 5 of 18 cases, as residual tumor following neoadjuvant chemotherapy harbored subclones that were not present in the pre-treatment specimen.

The observation that driver genetic alterations can also be heterogeneously distributed within a given breast cancer is corroborated by the bioinformatics inferences made by Shah et al. [60], where TP53 mutations were found to be subclonal in a small but substantial subset of TNBCs, and by a study led by our team that has provided direct evidence that intra-tumor genetic heterogeneity may affect even HER2 gene amplification itself in breast cancers classified as HER2-positive using the ASCO/CAP guidelines [41]. In those HER2-positive but heterogeneous cases, the components lacking HER2 gene amplification were found to harbor alternative driver genetic alterations, including activating HER2 somatic kinase domain mutations (i.e., a clear example of a convergent phenotype [53]), BRF2 and DSN1 amplifications [70].

It should be noted that some biological phenomena appear to be less prone to intra-tumor spatial heterogeneity than somatic mutations. A recent study has demonstrated that distinct assays for the assessment of homologous recombination DNA repair deficiency in breast cancer show limited spatial heterogeneity [71].

Temporal Heterogeneity and Therapeutic Selective Pressure

Multiple studies have been carried out to understand the temporal evolution of tumors. Ding et al. [72] performed the genomic analysis of a primary basal-like breast cancer, and its corresponding brain metastatic outgrowth and PDX, and uncovered a SNED1 missense mutation, a silent FLNC mutation, and a MECR deletion occurring de novo in the metastatic focus. Interestingly, the PDX displayed all the mutations of the primary tumor and a mutation enrichment pattern similar to the brain metastasis illustrating how metastases may stem from a minority of cancer cells of the primary tumor [72].

Therapy leads to selective pressure that can further sculpt a tumor genome (Fig. 1b). Murtaza et al. [73] analyzed the clonal evolution of a HER2-positive/ER-positive metastatic breast cancer in a patient who received targeted therapy, via the study of multiple biopsies of the primary tumor and metastatic foci, as well as circulating tumor DNA in plasma (ctDNA) collected throughout 3 years. They observed that truncal mutations displayed the highest levels in plasma, followed by branch and private mutations. Most importantly, they found that changes in the circulating levels of subclonal private somatic mutations correlated with the divergent responses to treatment observed in the various metastatic foci, as revealed by imaging. As another example, in an autopsy study, Juric et al. [74] analyzed 14 metastatic foci of a patient with breast cancer with an activating PIK3CA mutation, who developed resistance to BYL719, a PI (3)Kα inhibitor. While all metastatic foci showed loss of one copy of PTEN, foci that were refractory to treatment had additional but different PTEN mutations, resulting in a complete PTEN loss of function, illustrating an instance of a convergent phenotype [53] driving therapeutic resistance.

The neoadjuvant setting also offers the opportunity to study temporal heterogeneity and therapeutic selective pressure. In a study of patients with HER2-positive breast cancer who had received neoadjuvant chemotherapy and subsequent adjuvant trastuzumab, Janiszewska et al. [75] tracked changes in intratumoral heterogeneity by the concurrent analysis of somatic mutations and CNAs in single cells of breast cancers and observed modifications in the frequency and geography of cancer cell subpopulations, leading to the

selection of cancer cells harboring PIK3CA mutations, a known genomic determinant of resistance to anti-HER2 targeted therapy [76].

Studies addressing the temporal heterogeneity between primary breast cancers and their metastases have been limited so far. Most of the studies focused solely on “hotspot” mutations or key cancer genes and revealed limited but important differences between primary breast cancers and their metastatic deposits [77]. For instance, the study of 79 paired breast primary and metastatic cancers showed that metastatic foci harbored mutations affecting KRAS (15%), PTPN11 (8%), and SMAD4 (8%), which were absent in the matching primary tumors [77], and an analysis of 15 cases of metastatic breast cancer to the brain revealed TP53 mutations at higher frequency in the metastatic outgrowths (58.5%) than in the primary tumors (38.9%) [78]. Importantly, out of the differences observed between primary and metastatic breast cancers, one that is immediately actionable is the presence of somatic ESR1 mutations in relapses of patients with ER-positive breast cancer treated with aromatase inhibitors or subjected to estrogen deprivation [79, 80, 81, 82]. While mutations affecting the ligand binding domain of ESR1 are found in <1% of primary breast cancers, these mutations are observed in up to 54% of relapses in patients treated with anti-estrogens and estrogen deprivation [81].

Additional important aspects of intra-tumor heterogeneity are to be considered, such as the fact that a given mutation can change from passenger to driver under selective pressure [53]. Moreover, it has recently been suggested that subclones of inferior selective advantage can promote tumor growth by triggering changes in the microenvironment, and that the elimination of these subclones may result in tumor collapse [83].

Novel Sequencing Approaches to Overcome Tumor Heterogeneity

Traditional cancer genome sequencing studies, performed on DNA extracted from the tumor bulk [66, 84], provide results of average mutant allele fractions and average allelic copy number, hindering the detection of minor subclones, which may potentially be accountable for therapeutic resistance [85, 86]. Since the implementation of MPS in single cells [57], an array of methodologies have been developed with the attempt to offset the technical difficulties associated with single cell sequencing [87, 88, 89].

The application of single cell sequencing approaches for the study of breast cancer might represent a way to dissect the heterogeneity of this disease. For instance, the study of an ER-positive tumor and a TNBC through the integration of single cell whole-genome and exome sequencing with copy number analysis in single nuclei

showed that aneuploid rearrangements are early events in the life of a tumor, whereas point mutations occur gradually contributing to heterogeneity [90]. As another example of the potential of this methodology, a highly multiplexed single-nucleus sequencing method was used to study the temporal evolution of CNAs in TNBC [21], a tumor with high levels of aneuploidy [91], and identified no intermediate copy number profiles, but rather evolution of genomes from diploid to aneuploid, in support of a punctuated copy number evolution and clonal stasis model in TNBC [21]. Nonetheless, the clinical relevance of single cell sequencing is still unclear, as sequencing data derived from a limited number of single cells per cancer might not provide direct information on the remaining tumor cell population [66].

In the context of spatial heterogeneity, the use of in situ methodologies for assessment of gene CNAs and protein expression of genes targeted by genetic hits would allow for detailed topographical genotyping and inference of clonal structure. Novel high definition in situ techniques have been introduced, both at the DNA and RNA level [92, 93, 94], where genetic aberrations found in the bulk of the tumor or in single cells can be traced back to the tumor topology [66]. For instance, specific-to-allele PCR-FISH [75], a novel method based on the combination of in situ PCR [95] and FISH, constitutes an appealing integrative approach which can be used in formalin-fixed paraffin-embedded samples, allowing for simultaneous detection of point mutations and CNAs at the single-cell level.

Generous tissue sampling through the biopsy of multiple tumor sites is another strategy to overcome the challenges posed by intra-tumor heterogeneity. In the neoadjuvant setting, multiple biopsies are preferred, as they provide more information about the tumor and facilitate treatment planning [96, 97, 98] (Fig. 1c), and could resolve discordances between the mutational repertoires of primary tumors and corresponding metastatic outgrowths [72, 99].

Other means to study the mutational landscape of tumors overcoming sampling bias have been developed, such as the assessment of circulating tumor cells, circulating cell-free plasma DNA (cfDNA), and circulating cell-free cerebrospinal fluid DNA [100], which are indicators of disease burden and may serve as surrogates for spatial heterogeneity as well as markers for temporal heterogeneity [66] (Fig. 1c). In a proof-of-principle study, temporal heterogeneity of breast tumors was traced assessing specific PIK3CA and TP53 in cfDNA from breast cancer patients [101]. This analysis suggested that monitoring of cfDNA is a viable, sensitive, and real-time surrogate for tumor burden [101].

Interestingly, ESR1 mutations have been detected in cfDNA of patients with metastatic disease after progression on endocrine therapies [102]. Recently, Chandarlapaty et al. [103] conducted the analysis of the most prevalent ESR1 mutations in cfDNA of patients with ER-positive metastatic breast cancer treated with aromatase inhibitors included in the BOLERO-2 trial, and showed that the presence of either Y537S and D538G ESR1 mutations predict shorter survival.

Finally, Garcia-Murillas et al. [104] have monitored cfDNA to estimate the minimal residual disease (MRD) in early breast cancer patients following completion of neoadjuvant therapy, and were able to predict metastatic relapse on the basis of increases in the mutant allele fractions in plasma, a surrogate of the total amount of cell-free tumor DNA within the total amount of cell-free plasma DNA. In fact, this assessment provided a shorter median lead time for the detection of metastatic disease of 7.9 (range 0.03–13.6) months, compared to the methods currently employed in standard of care. Targeted capture MPS of cfDNA using an extended gene panel revealed additional somatic mutations, not detected in the primary tumor but present in the metastatic lesions, providing evidence to demonstrate that MPS of cfDNA may provide an accurate assessment of the repertoire of somatic genetic alterations found in MRD, and predict the genetic profile of the metastatic relapse with more accuracy than through sequencing of the primary tumor [104]. Adjuvant therapeutic interventions could be therefore tailored to the genetic events present in the MRD.

Therapeutic Strategies to Overcome Tumor Heterogeneity

Crosstalk between signaling pathways, as well as their redundancy, limits the success of targeted monotherapies [105]. Seminal studies by Goldie and Coldman [106, 107] proposed the use of combinatorial anticancer therapies to overcome therapeutic resistance. Nowadays, the limitations of this approach are being recognized, as cancer cells are able to circumvent therapeutic efforts by various mechanisms. For instance, clonal interference [108], which refers to the coexistence of different mutations as tumor drivers, restrains the expansion of different tumor clones, but at the same time increases the number of targets needed to be aimed at for successful tumor eradication [32].

Another feature of tumors that limits treatment success is their plasticity, which allows them to adapt their signaling circuitries under therapeutic selective pressure. Duncan et al. [109] assessed changes in the kinome in TNBC cells and genetically engineered mice after MEK inhibition and found that this treatment led to ERK suppression, followed by c-Myc degradation and subsequent activation of different receptor tyrosine kinases. Most importantly, prevention of proteosomal c-Myc degradation inhibited kinome reprogramming.

These data illustrate how anticancer monotherapy with single kinase inhibitors may be ineffective and showed how the analysis of a kinome-resistance signature might be worth exploring as a means to guide therapy [109].

Traditionally, therapeutic efforts focused solely on targeting mutations present in the modal tumor population; however, the importance of targeting subclonal mutations is being increasingly recognized. Importantly, the cancer subclone that may ultimately become dominant and lethal might be displayed at low frequency at the time of diagnosis [79, 80, 81, 82] (Fig. 1b). These data imply that targeting rare subclones is of paramount importance for tumor eradication and prevention of metastasis.

Conclusions

Inter- and intra-tumor heterogeneity poses enormous challenges to both the diagnosis and treatment of this disease. Novel technologies, such as single cell sequencing and analysis of ctDNA, and bioinformatics tools for the analyses of the sequencing results will undoubtedly provide greater opportunities to dissect the evolving genomic landscape of tumors with increasing accuracy. Likewise, an array of therapeutic strategies is being implemented to counteract the obstacles raised by tumor heterogeneity. Despite this wealth of knowledge, the clinical implications of this phenomenon are only now beginning to be clarified and open questions remain on how to overcome the hurdles of breast cancer heterogeneity and the means to take advantage of it for therapeutic success.

Compliance with Ethical Standards

Conflict of Interest

Fresia Pareja, Caterina Marchiò, Felipe C. Geyer, Britta Weigelt, and Jorge S. Reis-Filho declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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FIGURES

Fig. 1

Tumor heterogeneity and methods to tackle it. a Inter-tumor heterogeneity refers to genetic and/or phenotypic differences between tumors of distinct individuals. Precision medicine has the potential to overcome inter-tumor heterogeneity and improve response to therapy. b Intra-tumor heterogeneity, which refers to differences between different areas of the same tumor, or between the primary tumor and metastatic focus of the same individual, can be classified as spatial and temporal. Therapeutic intervention based on the genomic analysis of a sample representative of the modal population can lead to clonal selection and to changes in the clonal composition of tumors. c Clinical assessment of intra-tumor heterogeneity. A single biopsy might not detect the subclonal population that may lead to metastatic disease, whereas strategies such as multiregional biopsies or cell-free DNA analysis might be able to detect clonal and subclonal populations and allow successful combinatorial therapeutic intervention. Furthermore, assessment of cell-free DNA has the potential for detection of subclinical recurrence