Cancer Research

Mutation Profiling of Key Cancer Genes in Primary Breast Cancers and Their Distant Metastases

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Abstract

Although the repertoire of somatic genetic alterations of primary breast cancers has been extensively catalogued, the genetic differences between primary and metastatic tumors have been less studied. In this study, we compared somatic mutations and gene copy number alterations of primary breast cancers and their matched metastases from patients with estrogen receptor (ER)–negative disease. DNA samples obtained from formalin-fixed paraffin-embedded ER-negative/HER2-positive ($n = 9$) and ER-, progesterone receptor (PR-), HER2-negative ($n = 8$) primary breast cancers and from paired brain or skin metastases and normal tissue were subjected to a hybridization capture-based massively parallel sequencing assay, targeting 341 key cancer genes. A large subset of nonsynonymous somatic mutations (45%) and gene copy number alterations (55%) was shared between the primary tumors and paired metastases. However, mutations restricted to either a given primary tumor or its metastasis, the acquisition of loss of heterozygosity of the wild-type allele, and clonal shifts of genes affected by somatic mutations, such as *TP53* and *RB1*, were observed in the progression from primary tumors to metastases. No metastasis location-specific alterations were identified, but synchronous metastases showed higher concordance with the paired primary tumor than metachronous metastases. Novel potentially targetable alterations were found in the metastases relative to their matched primary tumors. These data indicate that repertoires of somatic genetic alterations in ER-negative metastatic breast cancers may differ from those of their primary tumors, even by the presence of driver and targetable somatic genetic alterations.

Significance: Somatic genetic alterations in ER-negative breast cancer metastases may be distinct from those of their primary tumors, suggesting that for treatment-decision making, genetic analyses of DNA obtained from the metastatic lesion rather than from the primary tumor should be considered. *Cancer Res; 78(12); 3112–21. ©2018 AACR.*

Introduction

Despite the advancements in our understanding of breast cancer and the introduction of novel therapies for patients with breast cancer, metastatic breast cancer remains incurable. The survival of patients with metastatic breast cancer has shown significant but only incremental increases over the past three decades (1, 2). This is particularly a challenge for patients with estrogen receptor- (ER), progesterone receptor- (PR), and HER2-negative (i.e., triple negative) breast cancers [triple-negative breast cancers (TNBC)] and HER2-driven breast cancers, which have been shown to have a higher incidence of brain metastases than ER-positive/HER2-negative cancers (3).

Massively parallel sequencing analyses of primary breast cancer have revealed that breast cancers have a complex repertoire of somatic genetic alterations, and that these vary according to ER status (4, 5). As a group, primary breast cancers have been shown to harbor few highly recurrently mutated genes, namely *TP53*, *PIK3CA*, *GATA3*, *MAP3K1*, and *CDH1*, and a large subset of genes is rarely mutated. In this subset of rarely mutated genes, targetable genetic alterations have been identified, including driver somatic genetic mutations affecting *HER2* (6), *ESR1* (7), and *AKT1* (8). In addition, recent studies have demonstrated that a large subset of breast cancers displays intratumor genetic heterogeneity, and that the level and type of intratumor genetic heterogeneity may differ between ER-positive and ER-negative disease (9, 10).

Given the intratumor genetic heterogeneity described in breast cancers, it is plausible that in the progression from primary to metastatic disease, the metastatic process itself as well as the therapeutic interventions administered in the adjuvant setting may result in clonal selection (11). In this context, the analysis of primary versus metastatic breast cancer would result in the identification of genes whose somatic genetic alterations are restricted to or are enriched for in the metastatic lesions as compared with their respective primary tumors. Consistent with this notion, massively parallel sequencing analysis of primary and metastatic breast cancers (11–14), as well as analysis of breast cancers, plasma DNA, and metastatic lesions (15, 16), has revealed that a varying proportion of somatic mutations, even those affecting known driver genes, such as *PIK3CA*, *SMAD4*, and *TP53*, are either restricted to or enriched for in the metastatic lesion as compared with the primary tumor. These studies, however, either focused on hotspot mutations or on a limited number of breast cancers of different subtypes.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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In this study, we sought to define the extent to which metastatic breast cancers differ from their respective primary tumors in their repertoire of somatic genetic alterations. We focused on TNBCs and ER-negative/HER2-positive disease, and on brain and skin metastases to minimize confounding variables stemming from the distinct biology of ER-positive versus ER-negative breast cancer subtypes (10) and to factor in the notion that metastatic deposits to distinct anatomic sites may differ in terms of their biology and genetics.

Materials and Methods

Patients

Samples of primary and metastatic breast cancers were selected from an existing database entailing material from 400 patients from the Department of Pathology of the University Medical Center Utrecht (Utrecht, the Netherlands) diagnosed between 1986 and 2014 (17). For the purpose of this study, we selected TNBC and ER-negative/PR-negative/HER2-positive primary breast cancers from patients with brain or skin metastases, for which sufficient primary and metastatic tumor tissue and normal tissues were available. Representative formalin-fixed paraffin-embedded (FFPE) blocks from 17 primary–metastatic breast cancer pairs were retrieved, comprising eight triple-negative (4 with skin metastases, 4 with brain metastases) and nine ER-negative/HER2-positive breast cancers (4 with skin metastases, 5 with brain metastases). Normal tissues were obtained from blocks containing normal breast ($n = 4$) or lymph node tissues ($n = 13$). In the normal breast tissue, we have primarily retrieved DNA from stroma and adipose tissue, avoiding the terminal duct lobular units. All cases were centrally reviewed by a pathologist with an interest and expertise in breast cancer (P.J. van Diest) and graded following the Bloom and Richardson histologic grading system (Table 1; ref. 18). Immunohistochemical analysis of ER, PR, and HER2 was initially performed for patient management, but centrally reviewed for the inclusion of cases in this study following the American Society of Clinical Oncology/College of American Pathologists guidelines (19, 20). TNBCs were defined as tumors lacking ER (<1%), PR (<1%), and HER2

Table 1. Clinicopathologic characteristics of the primary breast cancers and matched metastases subjected to targeted massively parallel sequencing

Characteristics	Subgroup	All ($n = 17$) *n* (%)	ER-negative/HER2-positive ($n = 9$) *n* (%)	Triple-negative ($n = 8$) *n* (%)	*P*
Age at diagnosis (years)	Range	31–61	37–61	31–55	
	Mean	48	50	46	ns
Tumor cell content primary tumor (%)	Range	10–80	20–80	10–80	
	Mean	62	59	66	ns
Histologic type	Ductal	17 (100)	9 (100)	8 (100)	
	Lobular	0 (0)	0 (0)	0 (0)	ns
Histologic grade (Bloom & Richardson)	1	0 (0)	0 (0)	0 (0)	
	2	7 (41)	4 (44)	2 (25)	
	3	10 (59)	5 (56)	6 (75)	ns
Mitotic activity index (per 2 mm^2)	Range	4–68	4–56	12–68	0.016
	Mean	31	21	45	
Tumor diameter (cm)	Range	1.3–6.5	1.4–6.5	1.3–4	
	Mean	3.1	3.2	2.9	ns
Lymph node status	+	11 (65)	8 (89)	3 (38)	
	−	5 (29)	1 (11)	4 (50)	
	Unknown	1 (6)	0 (0)	1 (12)	ns
Time between primary tumor and metastasis (days)[a]	All				
	Range	−1,595–1,919	−1,595–1,919	−26–1,496	
	Mean	605	662	541	ns
	Brain				
	Range	−1,595–1,496	−1,595–1,313	11–1,496	
	Mean	468	326	645	ns
	Skin				
	Range	−26–1,919	−21–1,919	−26–905	
	Mean	760	1,083	436	ns
Location of metastases	Brain	9 (53)	5 (56)	4 (50)	
	Skin	8 (47)	4 (44)	4 (50)	ns
Tumor cell content of metastasis (%)	Range	30–80	30–80	50–80	
	Mean	66	59	71	ns
Meta- or synchronous metastasis	Meta	14 (82)	8 (89)	6 (75)	
	Syn	3 (18)	1 (11)	2 (25)	ns
Treatment history[b]	naCT	2 (12)	0 (0)	2 (25)	
	aCT	10 (59)	5 (56)	5 (63)	
	aHT	2 (12)	1 (11)	1 (12)	
	aTT	3 (18)	3 (33)	0 (0)	
	naRT	1 (6)	0 (0)	1 (13)	
	aRT	9 (53)	3 (33)	6 (75)	
	Unknown	4 (24)	4 (44)	4 (50)	ns

Abbreviations: a, adjuvant; CT, chemotherapy; HT, hormone therapy; na, neoadjuvant; ns, not significant; RT, radiotherapy; TT, targeted therapy.
[a]Negative values represent the metastasis being diagnosed before the primary tumor.
[b]Numbers add up to more than 100% because of combination therapy.

expression [0 or 1+ by IHC, or 2+ by IHC but lacking *HER2* amplification by fluorescence *in situ* hybridization (FISH), or nonamplified HER2 by FISH] and ER-negative/HER2-positive breast cancers were classified on the basis of lack of ER expression and HER2 overexpression (3+) and/or *HER2* gene amplification.

The use of leftover material requires no ethical approval according to Dutch legislation ("opt-out"; ref. 21). We only performed analyses on somatic but not germline mutations, and the study proposal was approved by the Internal Review Board of the UMC Utrecht.

DNA extraction

A 4-μm–thick hematoxylin and eosin (H&E)–stained section from each FFPE tissue block was used to guide macrodissection and to semiquantitatively define the tumor cell content by a pathologist (P.J. van Diest). Only tumor samples containing at least 10% neoplastic cells and normal tissue devoid of any tumor cells were cut (ten 10-μm–thick sections) and subjected to macrodissection using a scalpel, and areas with necrosis, dense lymphocytic infiltrates, and preinvasive lesions were intentionally avoided. DNA extraction was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen) and quantified using a Qubit fluorometer (Thermo Fisher Scientific). In addition, to determine DNA fragmentation, a size ladder PCR was performed using the Specimen Control Size Ladder Kit (In Vivo Scribe) on a Veriti Thermal Cycler (Thermo Fisher Scientific) with a 35-cycle PCR reaction. All samples selected had $\geq$200 kB bands and were considered for downstream analysis.

Targeted capture massively parallel sequencing

DNA extracted from the 51 FFPE tissue specimens (matched primary breast tumors, distant metastases to skin or brain, and normal tissue) from the 17 patients included was subjected to targeted capture massively parallel sequencing on an Illumina HiSeq 2500 at the Memorial Sloan Kettering Cancer Center (MSKCC; New York, NY) Integrated Genomics Operation (IGO) using the MSK-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) assay, targeting all exons of 341 cancer genes harboring actionable mutations and noncoding regions of selected genes, following validated protocols as described previously (22, 23). MSK-IMPACT sequencing data have been deposited into the NCBI Sequence Read Archive under accession SRP078319.

Analysis for the MSK-IMPACT sequencing data was performed as described previously (23). In brief, paired-ends were aligned to the human reference genome GRCh37 using the Burrows–Wheeler aligner (24). Local realignment, duplicate removal, and quality score recalibration were performed using the Genome Analysis Toolkit (25). Somatic mutations were defined using MuTect for single nucleotide variants (SNV; ref. 26), and Strelka and VarScan2 for small insertions and deletions (indel; refs. 27, 28). Variants with a mutant allelic fraction (MAF) of <1% and/or variants supported by <5 reads and/or covered by <10 reads at a given locus were disregarded (23). In addition, variants whose MAF in the tumor was <5 times that in the matched normal sample were disregarded, as were variants that were present at >5% minor allele frequency in dbSNP (Build 137; ref. 23). Mpileup files generated from SAMtools mpileup (version 1.2 htslib 1.2.1; ref. 29) for each sample were used to determine whether each mutation detected from the pipeline exists in the BAM file of the corresponding metastasis or primary tumor.

Copy number alterations and loss of heterozygosity (LOH) were identified using FACETS (30) as described previously (31). LOH was determined using the lesser copy number estimate of each segment for genes harboring a somatic mutation. Copy number profiles of the primary tumor of patient 7 and of both primary tumor and metastasis of patient 19 were excluded in the analyses performed due to insufficient sequencing depth for the primary tumor of patient 7 and the normal sample of patient 19, despite repeated rounds of sequencing of these samples.

The cancer cell fraction (CCF) for each mutation was inferred using ABSOLUTE (32) as described previously (31). Mutations whose clonal probability was >50% or whose lower bound of the 95% confidence interval of its CCF was >90% were classified as clonal (31).

A combination of mutation function predictors was employed to define the potential effect of each nonsynonymous SNV, as described previously (23, 31, 33), and mutation hotspots were defined according to Chang and colleagues (34). The Drug Gene Interaction database (http://dgidb.genome.wustl.edu) was employed to identify potentially "druggable" genes (exact targetable hotspots were not taken into account). The treatment implications of specific genetic alterations were further defined using OncoKB (35).

Comparison of pathogenic mutations to METABRIC data

Mutational data from primary breast cancers ($n = 2{,}000$) of the METABRIC trial (36) were downloaded from cBioPortal (37). Data were filtered for triple-negative and ER-negative/HER2-positive breast cancers and the 341 genes in MSK-IMPACT. Mutations and clinicopathologic characteristics of the METABRIC dataset were compared with characteristics of our primary breast tumor samples to assess whether they would be comparable and could be extrapolated to a larger group (Supplementary Table S1).

Differentially altered genes per clinical subtype and metastasis location

We defined somatic genetic alterations that were differentially altered between the metastases and paired primary tumors. Selected alterations met the following conditions: metastasis only variants, variants that were subclonal in the primary tumor and clonal in the paired metastasis, genes that were amplified or homozygously deleted in the metastasis or LOH present in the metastasis, but not in the primary tumor and were used for pathway analysis. The genes most significantly differentially altered between primary tumors and paired metastases per subgroup were defined using ToppGene with Bonferroni–Holm correction for multiple comparisons (http://toppgene.cchmc.org).

Validation of *TP53* mutations using Sanger sequencing

The *TP53* mutations identified by targeted capture sequencing were validated using Sanger sequencing in 14 primary tumor–metastasis pairs, for which sufficient DNA was available (for primer sequences see Supplementary Table S2), as described previously (31). Sequencing was performed on an ABI-3730 capillary sequencer (Thermo Fisher Scientific), and sequences were analyzed with Sequence Analysis Software 6 (Thermo Fisher Scientific) and mutations assessed with Mutation Surveyor Software (Soft Genetics).

Statistical analyses

Nonpaired analyses between clinicopathologic characteristics of patient groups were computed using the Mann–Whitney U test. Paired analyses between primary tumors and metastases were performed using the Wilcoxon signed rank test. Kaplan–Meier

survival curves were computed for metastasis-free survival, defined as the time in days between the diagnosis of the primary breast tumor and the diagnosis of the metastasis. Low concordance between variants in the primary tumor compared with the paired metastasis was defined as <30% and high concordances as >30% shared variants. Log-rank test was used for comparison of survival between groups. $P < 0.05$ was considered statistically significant. All statistical calculations were done using IBM SPSS Statistics 21 and visualized with GraphPad Prism 6 and R software (version 3.2.5).

Results

Mutational landscape of metastatic primary ER-negative/HER2-positive breast cancer and TNBCs

Targeted capture massively parallel sequencing yielded a target coverage of 283× (range, 60×–539×) and 169× (range, 36×–381×) in primary breast cancers and normal tissue, respectively (Supplementary Table S3). A median of 5 (range, 1–25) and 4 (range, 2–10) somatic mutations in the primary ER-negative/HER2-positive and TNBCs was found, respectively (Supplementary Table S4). When assessing nonsynonymous somatic mutations, a median of 3 (range, 1–22) for HER2-positive and 3.5 (range, 2–8) for TNBCs was found.

The mutational profiles of our primary breast tumors were compared with those of 474 primary breast cancers from the METABRIC study ($n = 335$ for triple negative, $n = 139$ for ER-negative/HER2-positive primary breast cancers; Supplementary Table S1; ref. 36). With the exception of *PIK3CA* (e.g., H1047R) and *TP53* (e.g., R248Q and R306*), no specific mutations were shared between both databases. This may be partly explained by the fact that primary tumors from the METABRIC database had higher histologic grades for the ER-negative/HER2-positive tumors (Supplementary Table S1), but mainly underscores the intertumor genetic heterogeneity observed in primary breast cancer (5).

Consistent with previous reports (5, 10), *TP53* (14/17, 82%) and *PIK3CA* (5/17, 29%) were the two most frequently mutated genes in the primary tumors included in this study (Fig. 1A; Supplementary Table S4). Although *PIK3CA* and *TP53* mutations were numerically more frequent in ER-negative/HER2-positive and in TNBCs, respectively (Fig. 1B), no statistically significant differences were observed in regards to the mutation frequencies and the number of total variants in ER-negative/HER2-positive and TNBCs (Fig. 1C). All *TP53* variants detected by targeted capture massively parallel sequencing in the 22 samples of 14 patients were validated by Sanger sequencing (Fig. 1D).

Of the recurrent amplifications identified in more than one primary tumor (Fig. 1A; Supplementary Table S5), *MYC* amplifications were among the most prevalent affecting 3 of 7 (43%) and 4 of 8 (50%) of ER-negative/HER2-positive and triple-negative primary breast cancers, respectively (Fig. 1A). In addition, *ERBB2* and *CDK12* amplifications were found in all 7 ER-negative/HER2-positive primary breast cancers, for which copy number analysis could be performed (see Materials and Methods). *RECQL4* was amplified in 38% (3/8) of triple-negative and mutated in 11% (1/9) of ER-negative/HER2-positive primary breast cancers. In addition, *RB1* alterations were found in both the ER-negative/HER2-positive (nonsense mutation; 1/9; 11%) and the triple-negative primary breast cancers (2/8 missense mutations, 25%; 2/8 homozygous deletions, 25%).

The ER-negative/HER2-positive and triple-negative primary breast cancers with distant metastases to the skin or brain analyzed in this study were, akin to other primary breast cancers of these clinical subtypes (5, 10), heterogeneous at the genetic level with *TP53* being the most commonly mutated gene.

Somatic genetic alterations in primary breast tumors and their matched distant metastases

Targeted capture massively parallel sequencing analysis of primary ER-negative/HER2-positive and TNBCs and their respective distant metastases revealed a total of 249 somatic mutations, of which 189 were nonsynonymous (Fig. 2A; Supplementary Table S4). The number of somatic mutations in the metastatic lesions (median 6, range, 2–55) was significantly higher than in their respective primary cancers (median 4; range, 1–25; $P = 0.020$; Wilcoxon signed rank test); this did not reach statistical significance when only focusing on nonsynonymous mutations between the primary tumors (median 3; range, 1–22) and metastases (median 4; range, 2–42; $P = 0.058$; Wilcoxon signed rank test; Supplementary Table S4). In total, 20 mutations (15 nonsynonymous mutations) were found in the primary tumors only, 85 mutations (56 nonsynonymous) in the metastases only, and 72 mutations (59 nonsynonymous) were concordant between all primary and matched metastases (Fig. 2A). Thus, 45% of individual variants (45% of the nonsynonymous variants) were shared between primary tumor and metastasis, with a range of 23% to 100% for nonsynonymous variants per patient. The mutations found to be discordant between matched primary tumors and metastases could not be explained by copy number alterations affecting the discordant genes targeted by SNVs and indels. It should be noted, however, that some of the discordances may have stemmed from low sequencing coverage in some of the samples studied.

Akin to the mutational profiles, also the gene copy number profiles of primary tumors and their matched metastases showed many similarities (Fig. 2B; Supplementary Table S5). However, unlike somatic mutations, the number of gene copy number alterations or the percentage of the genome affected by gene copy number alterations did not differ between primary cancers and their respective metastatic lesions (Fig. 2B). Thirteen amplified genes and 1 homozygously deleted gene were found to be restricted to the primary breast tumors (mean 0.82 alteration/patient; range, 0–4), 36 amplified genes and three homozygously deleted genes restricted to the metastasis only (mean 2.29 alteration/patient; range, 0–15), and 60 amplified genes plus four homozygously deleted genes were concordant between primary tumors and matched metastases (mean 3.76 alterations/patient; range, 1–12). In total, 55% of individual amplifications and 50% homozygous deletions were shared between primary tumors and metastases (Fig. 2C).

In all 7 ER-negative/HER2-positive samples, which could be assessed for gene copy number alterations (cases 7 and 19 were excluded due to low coverage, see Materials and Methods), *ERBB2* overexpression or amplification previously determined during routine diagnostic workup was validated by sequencing-based copy number analysis, and amplification in the primary tumor was always retained in the matched metastasis. *ERBB2* amplification always cooccurred with *CDK12* amplification (Figs. 1A and 2D).

As an exploratory hypothesis-generating analysis, we have investigated the discordances between primary and metastatic tumors grouped according to the timing of the diagnosis of metastatic disease and the treatment the patients received. Although only three synchronous metastases were included (#14, #15, and #188), a significantly higher concordance could be detected in

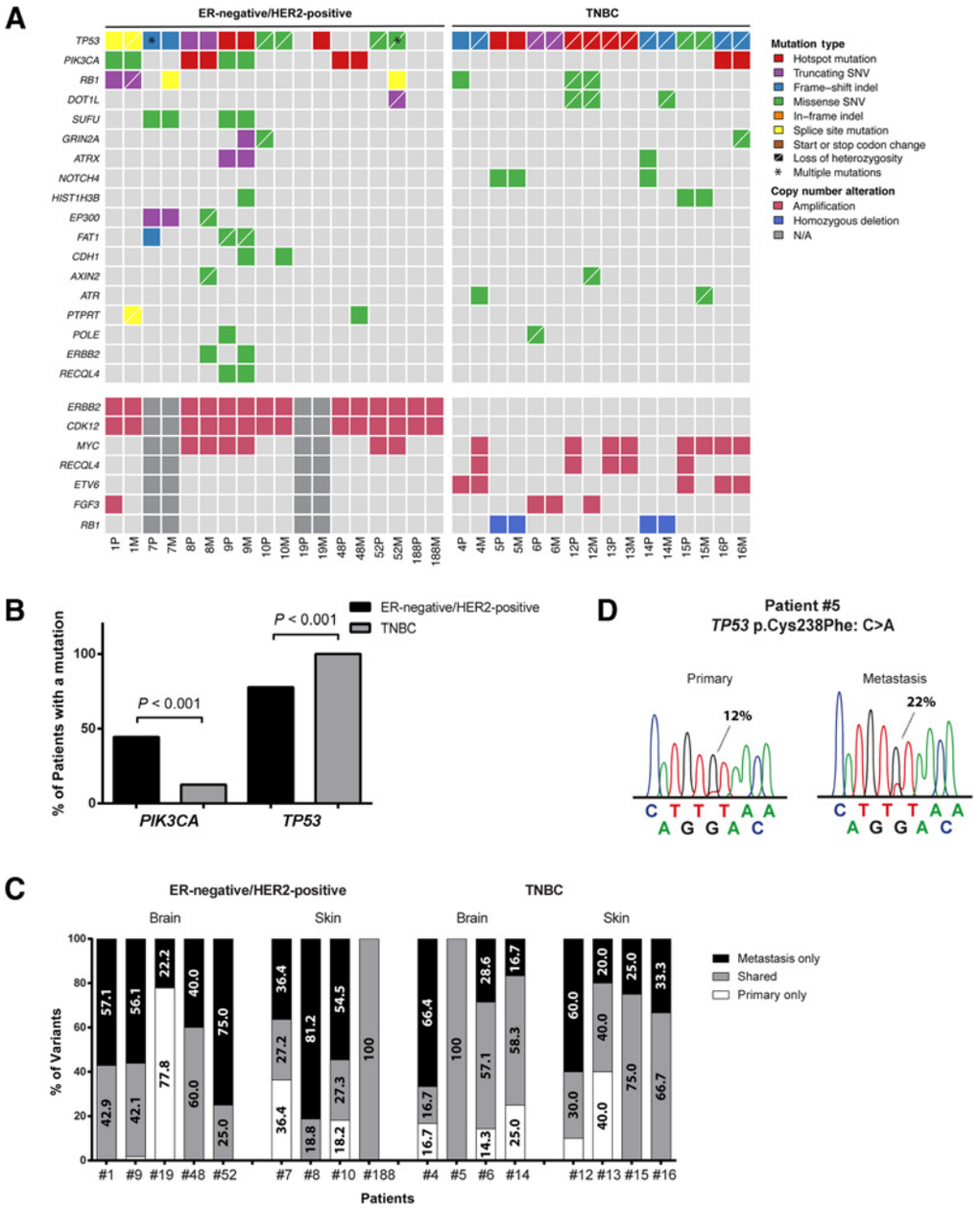

Figure 1.

Somatic genetic alterations identified in 17 ER-negative/HER2-positive and triple-negative breast tumors and matched brain or skin metastases by massively parallel sequencing of 341 cancer-related genes. **A,** Heatmap of most frequent somatic mutations and gene copy number alterations identified in this study according to subtype. Mutation types and gene copy number alterations are color-coded according to the legend. Please note that copy number alterations could not be assessed in samples from cases 7 and 19. **B,** Frequency of *PIK3CA* and *TP53* mutations according to clinical subtype of the primary tumor. **C,** Percentage of primary only, metastasis only, and shared mutations per clinical subtype and metastasis location. **D,** Sanger sequencing validation of *TP53* mutation p.Cys238Phe (exon 7, Fw primers) in patient #5 (triple-negative primary tumor with brain metastasis).

the percentage of variants between primaries and matched synchronous metastases versus primaries and matched metachronous metastases ($P = 0.038$; Mann–Whitney U test). In addition, the time between primary tumor and metastasis was not correlated with the degree of concordance ($P = 0.099$; Spearman ρ), except when excluding two outliers (case #5 and #48; $P = 0.017$; Spearman ρ; Supplementary Fig. S1A).

In this cohort, discordant genetic alterations between primary and metastasis were not correlated with a shorter overall survival time (for primary only mutations: $P = 0.883$; for metastasis only mutations: $P = 0.493$; Spearman ρ). When treatment history was taken into account, a trend for more metastasis only mutations ($P = 0.063$; Mann–Whitney U test) was observed in Herceptin-treated patients. Conversely, this trend was not observed in

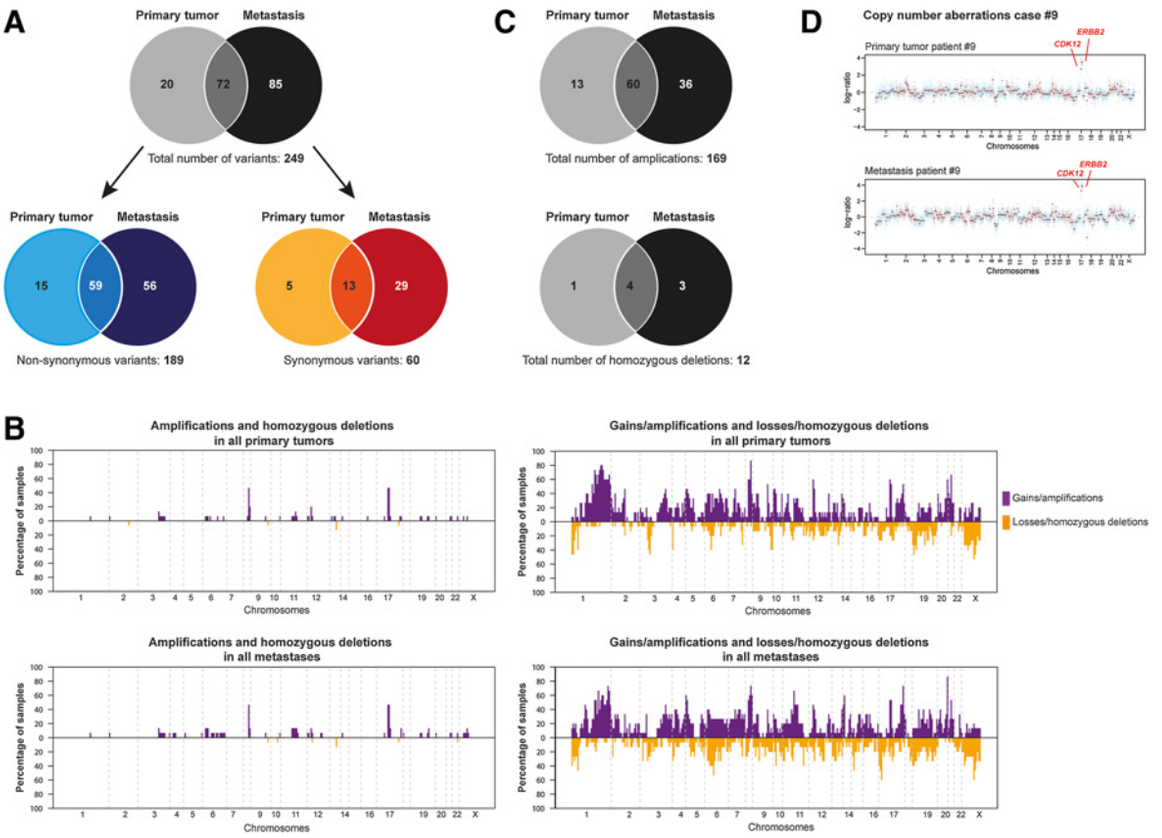

Figure 2.

Somatic mutations and copy number alterations shared between ER-negative/HER2-positive and triple-negative breast tumors and matched brain or skin metastases. **A,** Number of variants identified in the primary tumor only, in the metastasis only, and shared between paired tumors and metastases; shown are total variants and (non)synonymous variants. **B,** Gains, amplifications, losses, and homozygous deletions in all primary tumor samples and metastases. **C,** Number of amplifications and homozygous deletions in the primary tumor only, in the metastasis only, and shared between paired tumors and metastases. **D,** Copy number profiles of the primary tumor and metastasis of patient #9 (HER2-positive primary tumor with brain metastasis). *CDK12* and *ERBB2* are coamplified in the primary tumor as well as the metastasis.

patients who received chemo-, hormonal, and/or radiotherapy (Supplementary Fig. S1B). These results should be interpreted with caution, however, due to the small sample sizes and the fact that the differences in treatment are also mirrored by differences in the clinical subtypes, given that only ER-negative/HER2-positive breast cancers received Herceptin-based therapy.

Taken together, the landscape of somatic mutations and gene copy number alterations affecting key cancer genes was largely similar between primary ER-negative/HER2-positive and TNBCs and their matched distant metastases. Consistent with the notion that mutations are acquired in a gradual manner during tumor evolution, whereas gene copy number alterations are acquired in punctuated bursts of evolution (38), we observed a significantly higher mutation burden in the metastases than in their respective primary tumors, but no difference in the prevalence of gene copy number alterations.

Progression from primary breast cancers to distant skin and brain metastases and intratumor genetic heterogeneity

To assess whether the progression from the primary breast cancers to distant metastases would involve clonal shifts, we defined the cancer cell fractions (i.e., the percentage of cancer cells harboring a mutation in a given sample) of the somatic mutations identified (Fig. 3; ref. 23). In a subset of cases (not taking into account case 7 and 19, as mentioned earlier), we identified mutations that were restricted to the distant metastasis, including one clonal and one subclonal *ARID1A* mutation in the skin metastasis of case 8 (ER-negative/HER2-positive), a clonal *SMAD4* mutation associated with LOH of the wild-type allele in the brain metastasis of case 12 as well as a *RB1* mutation in the brain metastasis of case 52 (ER-negative/HER2-positive; Fig. 3). In addition, in cases 1 and 4 we found *TP53* mutations, which were associated with LOH of the wild-type allele only in the metastases, but not in the primary tumors. We also observed clonal shifts where subclonal mutations in the primary tumor became clonal in the matched metastasis, including *TP53* mutations in both ER-negative/HER2-positive (cases 1, 8, and 52) and triple-negative (case 12) cases, *NOTCH3* (case 8, ER-negative/HER2-positive) and *NOTCH4* (case 5, TNBC) mutations, and *RECQL4* (case 9, ER-negative/HER2-positive), among others (Fig. 3). These data provide evidence to suggest that primary breast cancers of ER-negative/HER2-positive and triple-negative subtypes progress to metastatic disease through a variety of processes, including clonal shifts and/or the acquisition of additional mutations and/or LOH.

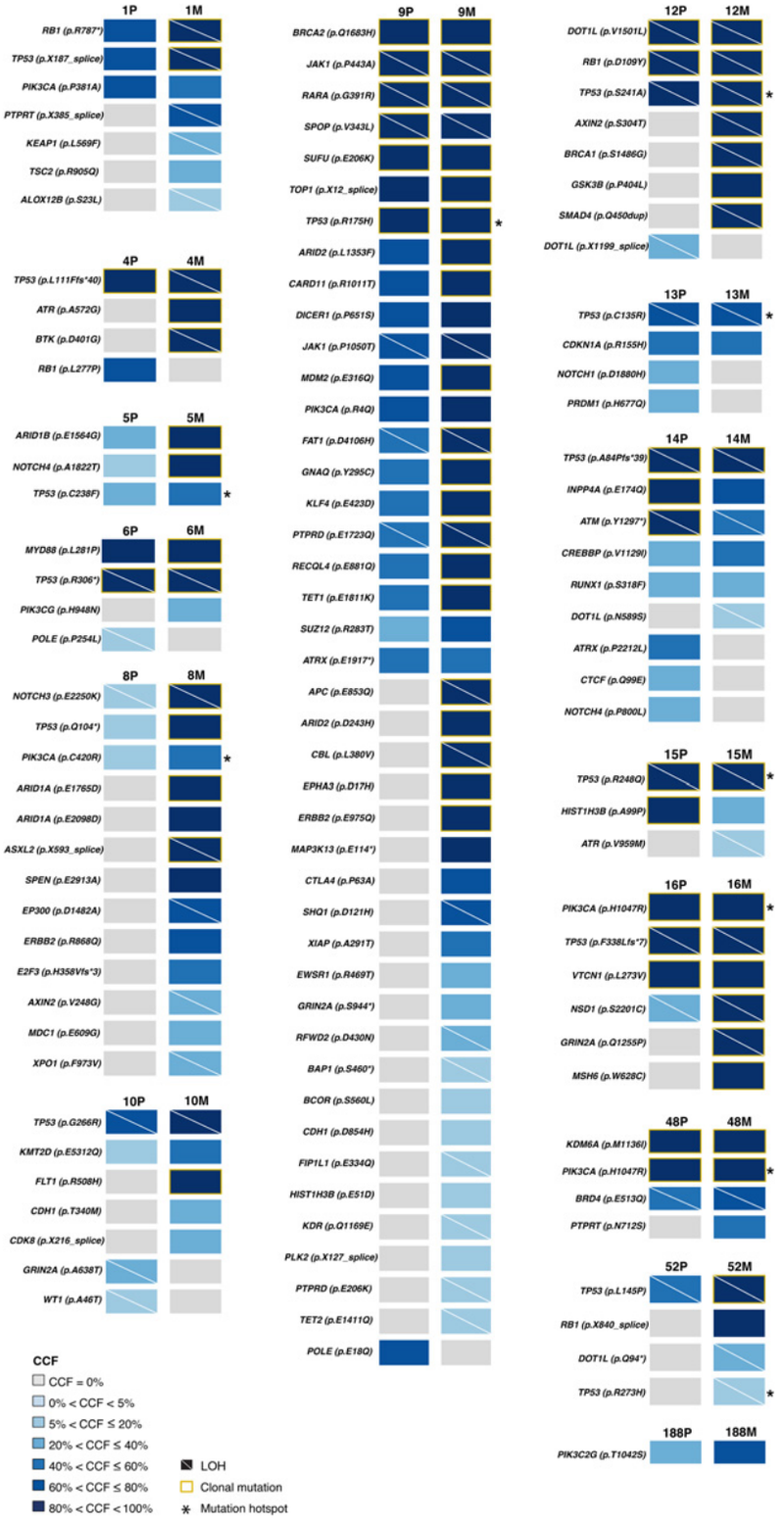

Figure 3.
Cancer cell fractions of the somatic mutations identified using ABSOLUTE for 15 ER-negative/HER2-positive and triple-negative breast tumors and matched brain or skin metastases. Color coding according to the legend. LOH is depicted by a diagonal bar, and clonal mutations by an orange box. Please note that copy number alterations and cancer cell fractions could not be assessed in samples from cases 7 and 19.

An exploratory, hypothesis-generating analysis of subclonal mutations in the primary and metastatic lesions revealed 72% (58/81) and 56% (85/151) of all somatic mutations and 70% (47/67) and 52% (57/109) of the nonsynonymous somatic mutations to be subclonal in the primary tumors and metastases, respectively ($P > 0.05$, unpaired

Mann-Whitney U test), suggesting that the metastases assessed here did not differ significantly from the primary tumors in their levels of intralesion heterogeneity. The study of larger series of matched primary breast cancers and matched brain/skin metastases, ideally with multiple sampling, using exome- or genome-wide sequencing approaches are warranted to define the extent of intratumor and intrametastasis genetic heterogeneity.

Pathways altered in primary breast cancers and distant metastases

We next endeavored to define whether there would be genetic alterations enriched in the metastases of the two clinical subtypes and in the two different metastasis locations. To do so, we subjected the somatic variants and copy number alterations that became more apparent in the metastases (metastasis only variants, variants that were subclonal in the primary tumor and clonal in the paired metastasis, genes that were amplified or homozygously deleted in the metastasis or LOH present in the metastasis but not in the primary tumor) or less apparent in the metastases to a pathway analysis. In general, a large overlap was seen between known cancer pathways more affected in metastases of ER-negative/HER2-positive compared with TNBC primary tumors (Supplementary Table S6). Tumors that disseminated to skin or brain showed no specific, but more divergent pathways that could be important in metastasis specificity.

Targetable genetic alterations in metastases relative to paired primary tumors

Finally, we sought to define whether potentially "druggable" genetic alterations would be enriched in metastases as compared with primary breast tumors. Genes were considered potentially "druggable" when they were listed in the Drug Gene Interaction database (http://dgidb.genome.wustl.edu; the exact targetable hotspots were not taken into account) and only genes that were either amplified/deleted or harbored pathogenic mutations (Supplementary Table S4) were included. Overall, 126 potentially "druggable" genetic alterations in 48 genes were present in the samples studied here. After exclusion of *ERBB2* amplifications (and *CDK12* coamplifications), 16 of 17 patients could have been stratified for targeted therapies and 30 of 39 (77%) somatic genetic alterations identified in the primary tumors (8/26 mutations, 21/67 amplifications, 1/4 deletions) remained altered in the metastases. Using this approach, in 11 of 17 (65%) metastases, novel potentially targetable genetic alterations relative to the primary tumors were identified (Supplementary Table S7). We performed a second, more stringent analysis using OncoKB (35), which provides information on treatment implications of specific cancer gene alterations. Also, this analysis identified targetable mutations affecting *TSC2*, *ERCC2*, and *ERBB2* in 4 patients (evidence levels 2A and 3A) restricted to the paired metastases and not present in the primary tumor (Supplementary Table S4).

Discussion

Here, we performed massively parallel sequencing analysis of a set of potentially actionable cancer targets in paired tumor tissue of patients with triple-negative and ER-negative/HER2-positive primary breast tumors and matched distant skin or brain metastases. We found well-known cancer drivers altered in both the primary tumors and their metastases, but also identified alterations restricted either to the primary tumor or metastasis of a given case, as well as clonal shifts and the acquisition of LOH of genes affected by mutations in the progression from primary to metastatic disease. In a panel of 341 cancer-related genes, we detected a high degree of similarity among somatic variants in matched ER-negative primary tumors and distant brain or skin metastases. Recently, Roy-Chowdhuri and colleagues reported almost similar percentages of metastasis only mutations (22%) and even a higher degree of concordant variants (77%) in 46 cancer-associated genes in 61 tumor pairs (39). However, their patient group mainly focused on ER-positive disease, and included only 2% ER-negative/HER2-positive and 25% triple-negative breast tumors.

In our cohort, the identified somatic variants were mainly observed in known cancer driver genes, including *TP53*, *PIK3CA*, and *RB1* (4, 39–41). Although *PIK3CA* mutations were more apparent in ER-negative/HER2-positive and *TP53* mutations in triple-negative primary breast cancers, enrichment of their interacting pathways was not significantly different between both subtypes. ER-negative/HER2-positive tumors presented with fewer genetic alterations than TNBCs, as shown before (39). Of interest, significantly more mutations were found in the metastases compared with the paired primary tumors. Wang and colleagues have shown before that point mutations can evolve gradually, generating extensive clonal diversity, and that many diverse mutations can occur at low frequencies (42).

Besides the diversity in somatic variants between primary tumors and matched metastases, copy number profiles were largely preserved, particularly amplifications and homozygous deletions. This is in agreement with a previous study from our research group using MLPA (43). This would be consistent with the notion that copy number alterations are acquired in punctuated bursts of evolution, whereas somatic mutations are acquired gradually (38). The functional importance of the coamplification of *CDK12* and *ERBB2* that we observed in every HER2-positive breast cancer remains to be elucidated. Recently, Mertins and colleagues showed that the kinase CDK12 is a positive transcriptional regulator of homologous recombination repair genes in breast cancer and is found to be highly active in the majority of *ERBB2*-positive tumors (44). In a genomic analysis of micropapillary carcinomas of the breast, genomic disruption of *CDK12* was found in 13% of HER2-positive breast cancers and in *in vitro* models of this disruption resulted in sensitivity to PARP inhibition (45).

MYC amplifications were more apparent in TNBCs, as reported previously (46, 47). Horiuchi and colleagues showed that aggressive breast tumors with elevated MYC levels are uniquely sensitive to CDK inhibitors, making it a possible therapeutic target in TNBCs (46). We showed that *MYC* amplifications and other potentially targetable mutations in and amplifications of *AKT2*, *CCND1*, *ERBB2*, *FGFR2*, *NOTCH4*, *PIK3CA*, and *ROS1* were largely shared between ER-negative primary tumors and paired metastases. However, large interpatient genetic heterogeneity was observed, emphasizing the clinical importance of personalized medicine (40). Interestingly, novel potentially actionable genetic aberrations in the metastases relative to the primary tumors were identified, suggesting that mutational profiling of metastatic samples would be of added value. Realistically, most of these mutations and amplifications have not been shown to predict response to therapy in breast cancer yet, but continuous research in this field to assess the efficacy of these targeted therapies in different cancer types is ongoing.

Metastases often demonstrated an increase in CCF, copy number, and novel mutations compared with the primaries,

suggesting clonal evolution. However, primary only mutations were seen as well, indicating clonal divergence within the primary tumor, either with other clones being selected for metastatic dissemination or with the metastasis branching off at an earlier time point. Importantly, spatial intratumor genetic heterogeneity should not be overlooked; differences between the primary tumor and the paired metastasis could be derived from a subclone not included due to limited tumor sampling (11, 12, 14, 48). On the other hand, the large concordance in genetic profiles and the resemblance in percentage of primary only/shared/metastasis only mutations and copy number aberrations could hardly be caused solely by heterogeneity. Also, there was a trend toward conservation of important driver mutations (or evolving convergently), while probable passenger mutations diverged between primary tumors and metastases, which implies that metastases still need these driver mutations for dissemination to and/or maintenance at distant sites. This is emphasized by the fact that also in other studies, *TP53* and *PIK3CA* were the most frequently occurring mutations in metastases (12, 39).

When assessing genetic alterations with a described role in the dissemination process of different molecular subtypes, we found a large overlap between significantly affected genes in metastases of TNBCs and ER-negative/HER2-positive tumors that have a role in general tumor-associated pathways, including angiogenesis, p53, and PI3-kinase.

Limitations of this study include the relatively small sample size given the rarity of matched primary breast cancer and metastasis tissue. It should be noted, however, that the current study represents the largest set of ER-negative matched primary breast tumors and skin and brain metastases to date. Second, given that we worked with FFPE tissues, some of which were rather small in size, we could only perform targeted massively parallel sequencing rather than whole-exome sequencing and, therefore, may have excluded genes that play a role in the progression from primary breast cancer to metastatic disease. Finally, the patients included in this study have received some form of systemic therapy prior to the biopsy of the metastatic lesion; hence, we cannot define whether the differences in the repertoire of somatic genetic alterations from primary to metastatic lesions were caused by the metastatic process itself, the therapy, or a combination of selective pressures from both phenomena. Furthermore, 77% of patients with known treatment history received (neo)adjuvant radiotherapy. It has been described previously (49, 50) that ionizing radiation generates distinctive mutational signatures, in particular in the form of deletions and balanced inversions. In the cases that have received radiotherapy, we therefore cannot entirely rule out that some of the differences in the mutational patterns identified between primary tumors and metastases may be attributed to the effects of radiation treatment. It should be noted, however, that we did not observe any significant differences in percentage of primary only, metastasis only, or concordant mutations between cases with and without radiotherapy (Supplementary Fig. S1B). Despite these limitations, this study provides a comprehensive analysis of the landscape of somatic mutations and gene copy number alterations in primary ER-negative/HER2-positive and TNBCs and their respective metastases and demonstrates that differences in the repertoire of somatic mutations, even affecting driver and potentially targetable genes, do exist. Our findings provide direct evidence to support the contention that studies seeking to define the genetic basis of therapeutic response in the metastatic setting ought to perform the genetic analysis using DNA obtained from the metastatic lesion (or plasma DNA) rather than from the primary tumor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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