Genetic heterogeneity and actionable mutations in HER2-positive primary breast cancers and their brain metastases

SUPPLEMENTARY MATERIALS

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Patients and samples

An index HER2-positive breast cancer patient subjected to warm autopsy and five additional HER2-positive breast cancer patients were included (Table 1). All patients received anti-HER2 targeted therapy as part of their systemic treatment. The tumor materials of the index patient were obtained in the autopsy program of the Vall d’Hebron University Hospital (VHUH). Formalin-fixed, paraffin-embedded (FFPE) samples of the remaining patients were retrieved from the archives of the Pathology Department of VHUH and Dexeus University Hospital, Barcelona, Spain. The study was approved by the Institutional Review Boards of both hospitals. Histologic sections were cut from representative frozen tumor tissue of the index patient and diagnostic FFPE blocks. Tumor and matched normal tissue were subjected to microdissection and DNA extraction as previously reported [1].

HER2 immunohistochemistry and fluorescence in situ hybridization

Selected primary breast cancers and brain metastases with available tumor tissue were subjected to re-assessment of HER2 status using IHC (anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody kit, Ventana Medical Systems) and/or FISH. FISH for HER2 was performed on 4 μm-thick sections using the Food and Drug Administration (FDA)-approved Vysis PathVysion HER-2 DNA Probe Kit (Abbott Molecular Inc, Des Plaines IL, USA) according to manufacturer’s instructions. Normal tissues including vessels, fibroblasts, lymphocytes or non-tumor breast tissues served as internal controls. Tumor tissues were evaluated using a 100× objective.

Targeted capture massively parallel sequencing

Tumor and normal DNA samples were subjected to targeted capture massively parallel sequencing at the Memorial Sloan Kettering Cancer Center (MSKCC) Integrated Genomics Operation (IGO). In this study, we employed a previously described customized breast cancer panel targeting all exons of 254 genes recurrently mutated in breast cancer and DNA repair-related genes (Supplementary Table 1) using custom oligonucleotides (NimblegenSeqCap) as described [2, 3]. Barcoded sequence libraries were prepared (New England Biolabs, KapaBiosystems) using 50 ng–250 ng of DNA. Massive parallel sequencing was performed on an Illumina HiSeq2000 (San Diego, CA), and bioinformatics analyses were performed as previously described [2, 3]. Allele-specific copy number alterations (CNAs) and loss of heterozygosity (LOH) of the wild-type allele in genes harboring a somatic mutation were inferred from massively parallel sequencing data using FACETS [4].

Validation of mutations by amplicon sequencing

Selected mutations found by targeted sequencing (n = 108, consisting of 104 unique mutations) were subjected to orthogonal validation using amplicon resequencing in all samples for a given patient, where genomic DNA was available in the MSKCC IGO (Supplementary Table 2). For amplicon resequencing, 5 ng of genomic DNA was amplified with primers specific for a given mutation using the AmpliTaq gold 360 master mix (Invitrogen Life Science Technologies). Amplicons were bead-purified, quantified and pooled. Pooled amplicons were subjected to standard protocol of Illumina library preparation sequencing (MiSeq). Reads were aligned to the reference human genome GRCh37 using BWA (v0.6.2) [5], and local realignment was performed using GATK (v3.1.1) [6]. Pileup files were generated using SAMtools [7]. Mutations with > 1% of mutant allele frequency (MAF) and covered by at least 50 reads were considered validated. The validation rate of the somatic mutations with sufficient coverage was 93% (100/108). False-positive variants were excluded from further analyses. Given the high validation rate, untested mutations were included in further analyses. In addition, of the 70 mutations that were not detected in a sample of a given case based on the initial targeted sequencing, 11 (16%) were subsequently detected by amplicon
sequencing and were included in the analyses, with the remaining confirmed to be genuinely absent even at a median depth of 10564× (range 272×–49532×).

**Inference of cancer cell fraction (CCF)**

ABSOLUTE (v1.0.6) [8] was used to infer the cancer cell fraction (CCF) of each mutation using the number of reads supporting the reference and the alternate alleles and the segmented Log2 ratio from targeted capture massively parallel sequencing. A mutation was classified as clonal if its probability of being clonal was > 50% [9] or if the lower bound of the 95% confidence interval of its CCF was > 90%. Mutations that did not meet the above criteria were considered subclonal.

**Identification of potentially pathogenic mutations**

A combination of MutationTaster [10], CHASM (breast) [11] and FATHMM [12] was used to define the potential functional effect of each missense somatic single nucleotide variant (SNV). Missense SNVs defined as non-deleterious/passenger by both MutationTaster [10] and CHASM (breast) [11], a combination of mutation function predictors shown to have a high negative predictive value [13], were considered likely passenger alterations. The remaining missense SNVs were defined as likely pathogenic if they were predicted to be “driver” and/ or “cancer” by CHASM (breast classifier) and/ or FATHMM [12], respectively. Frameshift, splice-site and nonsense mutations were considered likely pathogenic if they were targeted by loss of the wild-type allele or affected haploinsufficient genes [14]. SNVs, including missense and nonsense SNVs, affecting hotspot residues [15] were also considered likely pathogenic and were separately annotated. Mutations were also annotated if they affected genes included in the cancer gene lists described by Kandoth et al. (127 significantly mutated genes) [16], the Cancer Gene Census [17] or Lawrence et al. (Cancer5000-S gene set) [18]. Mutations that were neither likely pathogenic nor likely passenger were considered of indeterminate pathogenicity.

**Actionable somatic genetic alterations**

The DGIdb database [19] defines a list of 402 known and potential actionable genes (Supplementary Table 5). The clinically actionability of DGIdb is defined based on Bader Lab Genes, Caris Molecular Intelligence, Foundation One Genes, GO, Guide To Pharmacology Genes, Hopkins Groom, MSK-Impact, Russ Lampel and dGene.

OncoKB [20] is a comprehensive and curated precision oncology database with more than 3,000 unique mutations, fusions, and copy number alterations in 418 cancer-associated genes. It annotates the biologic and oncogenic effects, and provides the level of evidence that a specific molecular alteration is predictive of drug response on the basis of US Food and Drug Administration labeling, National Comprehensive Cancer Network guidelines, disease-focused expert group recommendations, and scientific literature.

**Phylogenetic tree construction**

A maximum parsimony tree was built for each case using binary presence/absence matrices based on the repertoire of somatic non-synonymous and synonymous somatic mutations, gene amplifications and homozygous deletions in the biopsies of the primary tumor and the metastatic lesion, as described by Murugaesu et al. [21]. A starting tree was constructed using the Neighbor-joining method and Hamming distance and optimized using the parsimony ratchet method implemented in the R package Phangorn [22]. Trees were rooted at the hypothetical normal where all somatic alterations are absent. Branch lengths were determined according to the ACCTRAN criterion as implemented in the Phangorn package and were drawn to scale.
Supplementary Figure 1: The mutational signatures of Case 14 were derived from the analyses of the somatic mutations in the primary breast cancer (A), mutations in the metastasis (B), mutations private to the primary (C), mutations private to the metastasis (D) and mutations shared between the primary and the metastasis (E). Below the graphs is the inferred contribution of the different signatures as defined by deconstructSigs.
Supplementary Figure 2: Genome plots of the primary tumor and matched brain metastatic lesions of the expanded cohort. Smoothed Log₂ ratios were plotted on the y-axis according to their genomic positions indicated on the x-axis. P, primary breast tumor; BM, brain metastasis.
Supplementary Figure 3: HER2 immunohistochemistry analysis of the brain metastasis # 1 and brain metastasis # 2 of case 12 revealed 2+ and 3+ respectively. HER2 FISH was performed (Figure 3) and confirmed the HER2-positive status and the clonality of HER2 gene amplification in both metastases.

Supplementary Table 1: List of 254 genes included in the targeted capture massively parallel sequencing platform. See Supplementary Table 1

Supplementary Table 2: Targeted capture massively parallel sequencing metrics
## Supplementary Table 3: List of primers used for the validation of mutations using amplicon resequencing (MiSeq)

| Gene and mutation | Primer Forward | Primer Reverse |
|-------------------|----------------|----------------|
| ABCA13_S771R      | GGAATTTTTTGAGAAATTATTGTTGC | ATTTTCCAATCCTCTTGGTC |
| AHNAK2_E201Q      | AGGTATGTTAAGCCAGCTGG | AACATAAATAAGAAGTCTCACA |
| AHNAK2_L4986R     | AGGCTTTGTGCTCCTCCC | TTGGATATGAGTCTCCTAGTC |
| AK9_K1173N        | AAAAGATCCAGCTAGAGATTTCTCA | GATTTTCCAGATGCAGAGCT |
| AKAP9_E102*       | GCTCGTGGCAGGGGGCAG | TGGCTATGAGAGTATGCTGG |
| AKT1_K377R        | CTTTTGCTAAGAACGAGCTC | TGCTAATGAGAGTATGCTGG |
| ANK3_E3429Q       | ATTTTCCAATTACCTCTAAGAGAAG | AAAAGATCCAGCTAGAGATTTCTCA |
| AOAH_R630T        | TTGAGAGAAAGAATTTGCAAGAA | AGGAGAATCCAGCTAGAGATTTCTCA |
| APOBEC3A_L55V     | TCTCGTGGCAGGGGGCAG | TGGCTATGAGAGTATGCTGG |
| APOBEC4_S25F      | TGGTACAGATGAGAGATTTCTCA | AAAAGATCCAGCTAGAGATTTCTCA |
| ARID1A_S138L      | GGAATTTTTTGAGAAATTATTGTTGC | ATTTTCCAATCCTCTTGGTC |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATRX_P711H        | CTTTTTGTGCTCCTCCC | TTGGATATGAGTCTCCTAGTC |
| BRAF_E703D        | AGGTATGTTAAGCCAGCTGG | AACATAAATAAGAAGTCTCACA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ARID1A_S138L      | GGAATTTTTTGAGAAATTATTGTTGC | ATTTTCCAATCCTCTTGGTC |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
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| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
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| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| ATM_E274          | TTCCATATATCCTCTTGGTAAG | AATTTGGAATCCAGCTAGAGATTTCTCA |
| ATR_C2150W        | GTAGGTTAGGAAAACCCCTTTGTTGAG | AAGGCTAATGATGACATTTTACCA |
| ATR_E1878Q        | TCTAGAGAGAGAAGATGTGCTATCCT | ACCCTGTAACATCTAATTCAGATAGG |
| KMT2C_R41S | GACAGCTCCGGCCCG | GACGGTGAGGCAGCCGG |
| KMT2D_E1861K | ACCCTTATACACAAAGAGGTACGG | GTGGCCATGACCCATCCT |
| LAMA5_A907T | GAGCGTGGAGCAGCCG | TACCTCCGGCACTGCAAC |
| MACF1_25308D | TCCTCAACAGCTGTCGAA | AGATGACCACTATTCCCT |
| MAP2K4_S303R | CATTGCTTCTTCTTCTGAGTAA | ATATAGTCTCTTACAGGAGGGA |
| MAP2K6_L229H | CCATTCCCGGACCTGCACCA | TCAATCCCCCAAACCTCATGTC |
| MED11_S58S | AGGAAAAAACCTAGCTGCTCCTT | GTTTCCCCCTTCTGTG6CC |
| MED12_E1861K | ACCCTTATACACAAAGAGGTACGG | GTGGCCATGACCCATCCT |
| LAMA5_A907T | GAGCGTGGAGCAGCCG | TACCTCCGGCACTGCAAC |
| MACF1_25308D | TCCTCAACAGCTGTCGAA | AGATGACCACTATTCCCT |
| MAP2K4_S303R | CATTGCTTCTTCTTCTGAGTAA | ATATAGTCTCTTACAGGAGGGA |
| MAP2K6_L229H | CCATTCCCGGACCTGCACCA | TCAATCCCCCAAACCTCATGTC |
| MED11_S58S | AGGAAAAAACCTAGCTGCTCCTT | GTTTCCCCCTTCTGTG6CC |

**Note:** The above text contains raw DNA sequence data, which is not formatted as a natural human-readable text. It appears to be a listing of gene names and their corresponding DNA sequences, possibly for a study or database entry in genomics.
Supplementary Table 4: Somatic single nucleotide variants (SNVs) and insertion/deletions (indels) present in the samples analyzed. See Supplementary Table 4

Supplementary Table 5: List of potential clinical actionable genes according to the http://dgidb.genome.wustl.edu. See Supplementary Table 5

Supplementary Table 6: List of potential clinical actionable genes according to the http://oncokb.org/api/v1/utils/allActionableVariants.txt. See Supplementary Table 6
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