Genomic mechanisms of resistance to tyrosine kinase inhibitors in HER2 amplified breast cancer

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Abstract

Though there has been substantial progress in the development of anti-HER2 therapies to treat HER2-positive metastatic breast cancer (MBC) within the past two decades, most patients still experience disease progression and cancer-related death. HER2-directed tyrosine kinase inhibitors (TKIs) can be highly effective therapies for patients with HER2-positive MBC, however, an understanding of resistance mechanisms is needed to better inform treatment approaches. We performed whole exome sequencing on 111 patients with 73 tumor biopsies and 120 cell-free DNA (cfDNA) samples to assess mechanisms of resistance. In 11/26 patients with acquired resistance, we identified alterations in previously characterized genes, such as *PIK3CA* and *ERBB2* that could explain treatment resistance. Mutations in growing subclones identified potential novel mechanisms of resistance in 5/26 patients and included alterations in *ESR1*, *FGFR2*, and *FGFR4*. Additional studies are needed to assess the functional role and clinical utility of these alterations in driving resistance.

INTRODUCTION

Human epidermal growth factor receptor 2 expressing (HER2-positive or *ERBB2* amplified) tumors account for 15–20% of breast cancers. HER2-positive breast cancers are associated with high-grade histology, higher rates of recurrence, and higher occurrence of brain metastases. In the first-line setting, combining two HER2-targeted antibodies, trastuzumab and pertuzumab, with chemotherapy is the current standard of care regimen for patients with HER2-positive, metastatic breast cancer (MBC).[1,2] In later-line settings, HER2-targeted tyrosine kinase inhibitors (TKIs) can be effective and are a widely utilized approach.[3] However, most patients eventually develop treatment resistance.

TKIs, a class of small molecules targeting the intracellular kinase domain of receptor tyrosine kinases such as HER2, have been shown to improve patient outcomes in numerous tumor subtypes.[4] The most common HER2-directed TKIs compete with ATP binding and block substrate phosphorylation, thus preventing the activation of downstream signaling pathways. Many TKIs - including lapatinib, neratinib and tucatinib - have been approved for use in patients with HER2-positive MBC and have particular benefit in patients with brain metastases.[3] While many patients experience clinically meaningful responses, most tumors will eventually develop resistance to TKIs leading to disease progression. Preclinical studies in mice xenografts and *in vitro* human breast cancer cell lines have shown that activating *PIK3CA* mutations confer resistance to lapatinib.[5] Prior work has also demonstrated that L755S, a HER2 kinase domain mutation, leads to lapatinib resistance and the activation of the downstream MAPK and PI3K/AKT/mTOR pathways.[6] While many resistance mechanisms have been demonstrated in the preclinical setting, there is limited knowledge about these mechanisms in patients.

Patients receiving HER2-directed TKIs, sampled by cell-free DNA (cfDNA) and tumor biopsy before, during and after therapy, present a unique opportunity to examine genomic alterations that may explain therapeutic resistance. In this investigation of the *in vivo* genomic landscape of mechanisms of resistance within a cohort of patients with HER2-positive MBC, we report both acquired and intrinsic

resistance mechanisms to three HER2-directed tyrosine kinase inhibitors (lapatinib, neratinib, and tucatinib).

RESULTS

Of 111 participants analyzed, 26 – the "paired cohort" – had samples taken prior to and after ≥ 6 weeks of TKI treatment; 30 – the "post-TKI cohort" – had samples collected only after ≥ 6 weeks TKI treatment; and 55 – the "pre-TKI cohort" – had samples collected only prior to ≥ 6 weeks TKI treatment (Fig. 1A**)**. All participants across the three cohorts had HER2-positive breast cancer per American Society of Clinical Oncology - College of American Pathology guidelines at time of metastatic diagnosis.[7] All paired cohort participants were HER2-positive at time of primary diagnosis except for one participant who did not undergo HER2 testing due to lack of availability of HER2 testing at the time. Detailed primary and metastatic receptor status for the paired cohort is included in **Supplementary Tables 1 and 2**, respectively. Median age at metastatic diagnosis for the paired cohort was 48 years. Most participants initially presented with stage I-III disease (66%) with the remaining presenting as *de novo* metastatic (34%). Participants were followed for a median of 4.9 years from metastatic diagnosis and the majority were female (96%) with hormone receptor-positive (HR-positive) disease (62%). Most participants had visceral disease (69%), and 65% of participants had central nervous system (CNS) involvement. Most participants in the paired cohort received lapatinib (92%) and a median number of 6 (range 1–10) lines of metastatic therapy. Median overall survival in the paired cohort was 4.51 years from metastatic diagnosis. A median number of 3 samples (2–7) per participant were collected, with a median tumor fraction of 13.2% (2.8%-53.2%). The post-TKI and pre-TKI cohorts were comparable to the paired cohort for median age at diagnosis, overall survival, follow-up time, gender, hormone receptor (HR) status, and race (**Supplementary Table 3)**.

The genomic landscape of post-TKI HER2-positive advanced breast cancer

To investigate mechanisms underlying resistance to HER2-targeted TKIs, we performed whole exome sequencing (WES) on 192 tumor and cfDNA samples from 111 participants with acquired or intrinsic resistance to anti-HER2 TKIs. Alterations in previously characterized genes that could explain treatment resistance were labeled as known mechanisms, including kinase domain mutations in *ERBB2* (L755S, D769Y, V777L) and activating *PIK3CA* mutations.[5,6,8] Potential mechanisms were identified in genes with mutations known to cause resistance in other cancer types or therapies **(**Fig. 1A**)**.

In the paired cohort, we found that eleven (42%) participants had at least one known alteration leading to resistance and 38% had no identified mechanism of resistance **(**Fig. 1B**)**. We then assessed the frequency of alterations in canonical oncogenic pathways, as well as a gene set containing *ESR1* and its transcriptional regulators (**Supplementary Table 4**), to determine whether any pathways were significantly recurrently mutated in the cohort.[9,10] The full list of genes altered in each pathway can be

found in **Supplementary Table 5** with variant classification for each mutation. RTK-RAS and TP53 were the most frequently mutated pathways with 16 (62%) participants having an alteration, followed by the WNT pathway (11 participants, 42%) and the HIPPO, NOTCH, and *ESR1* and regulators pathways (each 10 participants, 38%). The TP53 and *ESR1* and regulators pathways were both significantly mutated ($q < 0.05$) above the expected background mutation rate. We analyzed known mutational signatures and found the most frequently represented signatures were clock-like, APOBEC and capecitabine **(**Fig. 1B**)**.[11] We identified no other statistically significant differences at a pathway level between cohorts **(**Fig. 1C**)**.

In the pre-TKI cohort, RTK-RAS was the most frequently mutated pathway with 31 (56%) participants having an alteration, followed by the TP53 pathway (30 participants, 55%) and PI3K pathway (25 participants, 45%) **(Supplementary Fig. 1A)**. In the post-TKI cohort, TP53 was the most frequently mutated pathway with 23 (77%) participants having an alteration, followed by the PI3K pathway (19 participants, 63%) and RTK-RAS pathway (18 participants, 60%) **(Supplementary Fig. 1B)**. The TP53 pathway was significantly mutated ($q < 0.05$) in both the pre- and post-TKI cohorts, and the PI3K pathway was significantly mutated ($q < 0.05$) in the post-TKI cohort only.

Mutation frequencies in the most frequently altered genes were similar between the paired cohort and the pre- and post-TKI cohorts. *TP53*, the most frequently altered gene in all three cohorts, was altered in 58% of paired participants, 67% of post-TKI participants, and 53% of pre-TKI participants. *PIK3CA* was altered in 31% of paired participants, 50% of post-TKI participants, and 35% of pre-TKI participants. *ERBB2* was altered in 19% of paired participants, 17% of post-TKI participants, and 13% of pre-TKI participants. *ESR1* mutations were enriched in the paired cohort (19%) compared to both the post-TKI cohort (10%) and pre-TKI cohort (5%) (**Supplementary Table 6**).

Next, we evaluated copy number alterations. The most frequently altered genes were *ERBB2*—as expected—followed by *TP53, PTK2* and *MYC*, respectively. Six of eight participants in the paired acquired resistance cohort had focal high amplification of *ERBB2*, compared to only eight of the eighteen participants in the paired intrinsic cohort (p = 0.22, Fisher's Exact test). *TP53* biallelic inactivation—i.e., loss-of-function point mutation of one allele and deletion of the other allele—was observed across the paired cohort and occurred in 14 (54%) participants. Seven participants showed *MYC* amplifications, with one participant demonstrating focal high amplification. Five participants with intrinsic resistance did not have *ERBB2* amplification **(**Fig. 2A**)**, and lower pre-TKI *ERBB2* copy number was associated with intrinsic resistance (p = 0.0074, Wilcoxon rank-sum test) **(**Fig. 2C**)**. All five participants had clinical HER2-positive breast cancer confirmed on metastatic biopsy, a median of 842 days prior to assessed sample collection. The *ERBB2* copy number of a sample was not associated with its genomic purity or tumor fraction (Spearman's rho=-0.082, p = 0.46), suggesting that absence of *ERBB2* amplification was not a result of low purity or tumor fraction. Within the paired cohort, *ERBB2* copy number did not significantly change at a within-participant level during TKI treatment, and within-participant change did not differ significantly by HR status **(**Fig. 2B**)**. When stratifying the paired cohort by HR status, participants with HR-positive MBC had lower *ERBB2* copy number than HR-negative participants in both baseline and end-of-

TKI treatment samples (p = 0.031 and p = 0.041, Wilcoxon rank-sum test), as has previously been shown **(Supplementary Fig. 2)**.[12]

ESR1 alterations and ERBB2 copy number in HR-positive/HER2-positive MBC

Five out of the sixteen paired cohort participants with HR-positive disease had at least one activating *ESR1* hotspot mutation (D538G or Y537S). Two of these five participants had never received an aromatase inhibitor (AI) or prior hormone therapy in either the early or metastatic setting. An activating *ESR1* mutation presented in a growing subclone in four out of five participants. Three participants with an activating *ESR1* mutation did not show *ERBB2* copy number amplification per our analysis **(**Fig. 2A**).** Participants with an *ESR1* mutation had significantly lower *ERBB2* copy number relative to *ESR1* wildtype cases in pre- and post-TKI paired samples (p = 0.024 and p = 0.0096, Wilcoxon rank-sum test) **(Supplementary Fig. 3)**. When stratifying by HR status in an integrated analysis of the paired cohort and the pre- and post-TKI cohorts, participants with an activating *ESR1* mutation had significantly lower *ERBB2* copy number than both HR-negative and HR-positive *ESR1* wildtype participants in both pre- and post-TKI samples **(**Fig. 2D**)**.

Evolution of Clonal Dynamics in TKI resistance

To assess mechanisms of resistance we performed deeper evolutionary analysis on six participants with at least three serial samples collected **(**Fig. 3A**).** Known drivers of resistance were observed in multiple clones and included *ERBB2* and *PIK3CA* mutations **(**Figs. 3B, 3C, 3D**)**. Potential other mechanisms of resistance were identified as mutations in *ESR1*, *FGFR2*, and *FGFR4* **(**Figs. 3B, 3E, 3F**)**.

In participant 1, we observed two competing *ESR1* mutations in separate clones with the clone harboring a Y537S mutation, a potential driver of resistance, initially predominant during lapatinib treatment. Upon treatment switch to capecitabine with trastuzumab and eventually trastuzumab with letrozole, we observed the clone containing the *ESR1* D538G mutation outcompeting the Y537S-containing clone **(**Fig. 3B**)**. One participant with a *PIK3CA* mutation also had several missense mutations, including a truncal *ESR1* regulator pathway mutation present in *PGR*, a missense mutation in *FOXA1*, and a subclone with a *NOTCH2* mutation growing during neratinib treatment **(**Fig. 3C**)**. In another participant, growing subclones were detected with missense mutations in RTK-RAS, NOTCH, and WNT pathway genes such as *SPRED2*, *EP300*, and *TLE2*, respectively **(**Fig. 3G**).** However, for this participant, we did not observe any known or potential mechanisms of resistance, even though all participants were HER2 amplified (biopsy confirmed within 30 days of initial metastatic diagnosis) **(**Fig. 3G**)**.

WNT pathway enrichment in HER2-positive brain metastases

Thirteen (50%) participants in the paired cohort developed brain metastases. These participants more often had HR-negative breast cancer (54% of participants who developed brain metastases vs. 23% of participants who did not; p = 0.23, Fisher's Exact test). There were no significant differences in pathway mutation frequencies between participants who developed brain metastases and those who did not.

However, mutations occurring specifically in the tumor suppressor genes of the WNT pathway were significantly enriched in participants with brain metastases (p = 0.0077, Boschloo's test), with altered genes including *APC*, *CHD8*, *LZTR1, TLE1, TLE2*, and *ZNRF3.* Three of the five participants presented with subclonal WNT alterations in a cfDNA, breast or bone sample, with a median time of 903 days before the identification of any brain metastases **(**Fig. 4A, 4B**)**. Evolutionary analysis for Patient 8, with HR-negative breast cancer brain metastasis, illustrates WNT pathway involvement in the growing subclone containing a missense mutation in the tumor suppressor gene *TLE2* **(**Fig. 3G**).** In a combined analysis of the mutually exclusive pre- and post-TKI cohorts, mutations in WNT tumor suppressor genes remained significantly enriched in participants who ever developed brain metastases (p = 0.037, Boschloo's test) **(Supplementary Fig. 4)**. Of the eight participants with WNT tumor suppressor gene mutations in the combined pre- and post-TKI cohorts, two had the TSG alteration present before presentation of any brain metastases (median time of 1528 days before).

DISCUSSION

Though HER2-positive breast cancers predominantly remain dependent on the HER2 pathway, resistance develops in most metastatic tumors. Via detailed treatment data and sampling across the metastatic clinical course, we evaluated intrinsic and acquired resistance mechanisms in this cohort.

Beyond the established mechanisms - PI3K, ERBB2 and MAPK-pathway alterations - our study identified potential mechanisms of resistance driven by *ESR1*, *FGFR2* and *FGFR4* mutations. The other somatic alterations identified have biological potential to act as resistance mechanisms due to mutation type, location, and downstream signaling effects within canonical signaling pathways. However, unlike resistance to anti-EGFR TKIs in lung cancer, where approximately 50% of tumors develop acquired resistance via alteration of a single gene - *EGFR* - we observed a minority of participants (18%) with *ERBB2* mutations in all post-TKI samples.[13] These data highlight the diversity of resistance mechanisms and underscore that overcoming resistance will likely require multiple approaches. Given that we did not identify a common acquired resistance mechanism across the cohort and across different TKIs, sequential anti-HER2 TKI therapy may also be a viable treatment strategy. Further exploration in additional, larger cohorts with functional testing of mutations identified is warranted.

While less is known about WNT signaling in HER2-positive breast cancer, we observed WNT pathway alterations in well-established tumor suppressor genes in participants who had developed brain metastasis and validated this finding in an independent cohort of participants. WNT pathway alterations have previously been shown to mediate brain metastasis in lung cancer and activation of WNT signaling in glioblastoma is a known resistance mechanism to the established first-line treatment, temozolomide.[14,15] WNT pathway-mediated resistance is thought to act through over-expression of canonical ligands such as Wnt1 or through loss-of-function of tumor suppressors such as *APC* and has been shown to play a role in endocrine resistance of estrogen receptor-positive breast cancer.[16,17] Additional functional studies will be essential to further validate and characterize our intriguing finding of

enrichment of WNT tumor suppressor gene mutations in patients with HER2 + breast cancer brain metastases.

All participants included in the study had biopsy proven clinical HER2 amplification and received anti-HER2 therapy. Others have shown that HR-positive, HER2-positive breast cancers have, on average, lower HER2 copy number than HR-negative, HER2-positive tumors.[12] In our study, participants with HR-positive, HER2-positive cancers and an *ESR1* mutation were observed to have significantly lower HER2 copy number. In three of these participants, HER2 copy number was non-amplified per our genomic analysis. *ESR1* ligand binding domain mutations occur in up to 40% of patients and are a well-established mechanism of resistance to aromatase inhibitors in HR-positive breast cancers.[18] *ESR1* mutations are rare in treatment-naive tumors and are associated with estrogen pathway dependence. Pre-clinical evidence suggests that crosstalk between the HER2 and ER pathways mediates HER2 resistance, and that dual blockade may be important in effective therapy.[19–21] Our data provide further evidence of dependency on this pathway. Targeting these two pathways simultaneously was explored in the CALGB 40302 trial with clinical benefit seen with the combination of fulvestrant and lapatinib for patients with HR-positive/HER2-positive tumors.[22] However, current guidelines recommend treating HER2-positive MBC with HER2-directed therapy regardless of ER status. Our data raise the question of whether HER2-positive/ER-positive breast cancer may be not one, but at least two entities, with varying dependence on these two pathways. With the development of additional anti-estrogen therapies, such as novel selective estrogen receptor degraders (SERDs), complete estrogen receptor antagonists (CERANs), selective estrogen receptor covalent antagonists (SERCAs), and proteolysis-targeting chimerics (PROTACs), more treatment options may be available for investigation to address dual pathway blockade.

Our study is limited by the proportion (36%) of participants with evaluable samples. Most samples could not be analyzed due to tumor fraction constraints, which were selected based on prior experience demonstrating poor WES performance below these thresholds.[23] Despite this, clinicopathologic characteristics were similar across the cohort, emphasizing the representative nature of the analyzable subset. Our challenge underscores the importance of development of ctDNA sequencing approaches starting from minimal ctDNA fraction and low cfDNA input to allow for analysis of an expanded number of patient samples. Second, though all patients had clinically HER2-positive metastatic disease, a minority of tumors underwent FISH testing, a standard approach in the HER2 testing algorithm.[7] This limits our ability to correlate our findings from WES with clinical test results. Finally, our cohort is not demographically representative of patients with HER2-positive metastatic breast cancer in the United States and does not include a sufficient number of Black or Hispanic patients. Additional studies should be performed in representative patient populations with special attention to under-represented groups.

In conclusion, we conducted a genomic analysis of patients with HER2-positive MBC receiving TKIs in the metastatic setting via whole exome sequencing of tumor and cfDNA samples. We analyzed a primary cohort of participants with paired samples prior to and after TKI treatment to assess for mechanisms of resistance, followed by validation in a separate cohort of participants with comparable

clinicopathologic features. The findings in this study support previously identified pre-clinical resistance mechanisms, such as activating *PIK3CA* mutations, in a clinical cohort of patients who received anti-HER2 TKIs. Our identification of *ESR1* hotspot mutations, may add another layer to the crosstalk between estrogen receptor pathway activation and blockade of HER2 by anti-HER2 therapy. Intriguingly, we further found that mutations in tumor suppressor genes of the WNT pathway were significantly enriched in participants with brain metastases, though this finding will need to be validated in additional cohorts and characterized in functional studies. While we observed many exome-based mutations in oncogenic signaling pathways, several patients remained without a plausible mechanism of resistance. Novel methods in liquid biopsies, such as fragmentomics and epigenomics, may reveal mechanisms of resistance from activation or downregulation of pathways resulting from epigenetic alterations.

METHODS

Population and Samples

We identified participants with biopsy-proven, metastatic HER2-positive breast cancer, with and without brain metastases, with at least one cfDNA and/or tumor tissue sample collected on DF/HCC IRB-approved protocols 06-213, 09-204, 11–344, 13–198, or 17–318. Inclusion criteria, study design, and outcomes of the 06-213 (lapatinib), 11–344 (neratinib), 13–198 (tucatinib), and 17–318 (trastuzumab, pertuzumab, eribulin) clinical trials have previously been described. Participants provided informed consent for collection of blood, tumor tissue and access to clinical and genetic data for research purposes. Two Streck tubes of blood (20cc) were collected at the time of treatment change, 4–6 weeks after treatment change, and every 3 months if there was no progression and were processed as previously described.[24–27] From this cohort, we retrospectively identified participants who received ≥ 1 anti-HER2 TKI (lapatinib, neratinib and/or tucatinib) for ≥ 6 weeks in the metastatic setting. We included participants with ≥ 1 sample with tumor fraction (TFx) ≥ 9.5% and additional samples with TFx ≥ 4.5%. We identified mutually exclusive cohorts of participants with paired samples with ≥ 6 weeks of tyrosine kinase treatment, participants with samples collected pre-TKI, and participants with samples collected post-TKI. We used previously reported median progression free survival data for lapatinib, neratinib and tucatinib to inform thresholds for acquired and intrinsic resistance.[28–30] Participants who received TKI treatment for 180 days or longer were categorized as having acquired resistance. Participants who received a TKI for less than 180 days were categorized as having intrinsic resistance.

Genomic Analysis

All samples initially underwent ultra-low-pass whole-genome sequencing (ULP-WGS) via the Broad Institute Genomics Platform. Reads were aligned to the GRCh37 reference genome by the BWA-MEM algorithm.[31,32] The Picard Mark Duplicates tool identified duplicate reads.[33] The resulting genomes had an average read depth of 0.2x, with average read lengths of 146 bp. The ichorCNA tool provided estimates of the samples' TFx from ULP-WGS.[23] The TFx informed all of our subsequent analyses.

The remainder of our genomic analysis relied on whole exome sequencing (WES) data. Whole exomes were sequenced by the Broad Institute Genomics Platform. WES on cfDNA samples employed a Twist bait set; for solid tissue samples an Agilent bait set was used.[34] Reads were aligned to the GRCh37 reference using BWA-MEM with Picard Mark Duplicates. This yielded WES with average depths of 20x and average read lengths of 140 bp.

Somatic single nucleotide variants (SNVs) and indels were called via MuTect and Strelka, respectively.[35,36] Filtering steps removed potential false positives arising from FFPE and OxoG artifacts. A panel of normals filter removed potential germline events.[37] An additional filter based on BLAT re-alignment removed artifacts stemming from mismapped reads.[38] We used OncoKB to label SNVs, indels, and genes with functional annotations.[39]

Gene-level annotations of oncogene and tumor suppressor gene status were obtained from the literature.[9] However, two genes in the WNT pathway with mutations in the paired cohort—*LZTR1* and *CHD8*—were missing this annotation. Hence, we annotated these two genes as tumor suppressors based on ancillary sources of evidence: tumor suppressor status annotation in OncoKB (for *LZTR1*) and prior reports (for *CHD8*).[40]

We called somatic copy number alterations (CNAs) for each tumor sample in several steps. AllelicCapSeg generated copy number segmentations for each tumor-normal pair of whole exomes.[41] We used ABSOLUTE to compute absolute ploidy for tumors and assign allelic copy numbers to the segmentations.[42] We then translated ABSOLUTE's segment-level calls to gene-level copy number annotations. This information allowed us to annotate genes with a variety of CNAs, including gains, amplifications, deep deletions, and biallelic losses. See the Supplemental Methods for details about our criteria for calling these events.

We used PhylogicNDT to infer a clonal phylogeny for each participant from their ABSOLUTE output.[43] PhylogicNDT is a Bayesian technique that infers the phylogenetic tree of a tumor from its mutations' Cancer Cell Fractions (CCFs, i.e., variant allele fractions, adjusted for copy number and tumor purity). PhylogicNDT's output includes (i) a phylogenetic tree of clones present in the tumor; (ii) an assignment of mutations to those clones; and (iii) the CCF of each clone, in each sample.

For each participant possessing both pre- and post-TKI samples, we labeled each tumor clone as *growing*, *shrinking*, or *stable* based on whether its CCF increased or decreased between pre- and post-TKI samples. See the Supplemental Methods for details of our labeling criteria. Importantly, these three labels cannot be used to explicitly denote treatment sensitivity/resistance; for example, a clone with "shrinking" CCF may still be growing in volume/cell count. However, the labels can give evidence of clones' *relative* responses to treatment.

We used MutSig2CV to identify driver genes in our cohort.[44] MutSig2CV is a statistical model of genes' *background passenger mutation rates*, i.e., the genes' mutation rates under random chance—accounting

for gene- and patient-specific attributes. Given the observed mutations in a cohort of tumor samples, MutSig2CV yields a p-value for each gene. We aggregated these gene-level p-values into pathway-level p-values via Fisher's method, allowing us to identify *pathways* with significantly more alterations than expected under random chance.[45] We adjusted these p-values for FDR via Benjamini-Hochberg correction.[46]

Four mutational signatures were identified in our full cohort via SignatureAnalyzer's implementation of Automatic Relevance Determination Nonnegative Matrix Factorization (ARD-NMF). ARD-NMF is a Bayesian method that finds a parsimonious set of linear factors (*signatures*) that explain the mutational spectra in a population of tumor samples. These often indicate specific physical processes causing mutations in the tumors.[47,48] See the Supplemental Methods for details about our mutational signature methodology. Three of the four signatures sufficed to explain the mutations in our paired subcohort. These aligned with COSMIC3 SBS signatures for clock-like, capecitabine, and APOBEC mutational processes.[47]

DECLARATION

Prior Presentation:

This study was presented at the American Association for Cancer Research meeting, Orlando, FL in April 2023.

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Figures

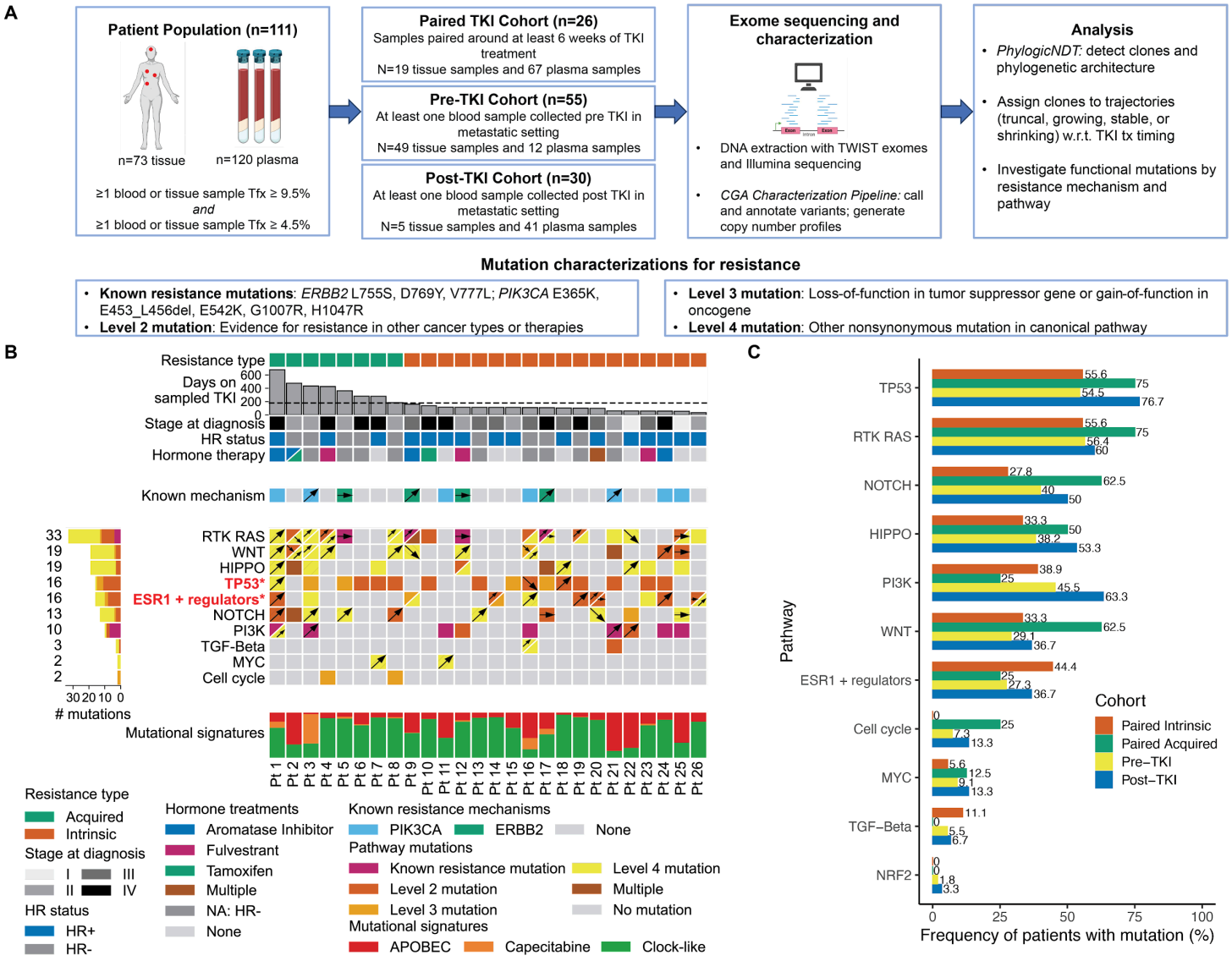

Figure 1

Study design for patient cohorts and genomic analyses

A.) Study design and mutation characterization for patient cohorts;

B.) Pathway alterations compared between acquired and intrinsic resistance paired cohort participants (n=26) depicted in decreasing frequency with known mechanisms in *ERBB2* and *PIK3CA* highlighted, TP53 and ESR1 + regulators pathways significantly mutated, and with signatures analysis identifying top three results;

C) Frequency of patients with a mutation in the oncogenic signaling pathways compared across the four cohorts

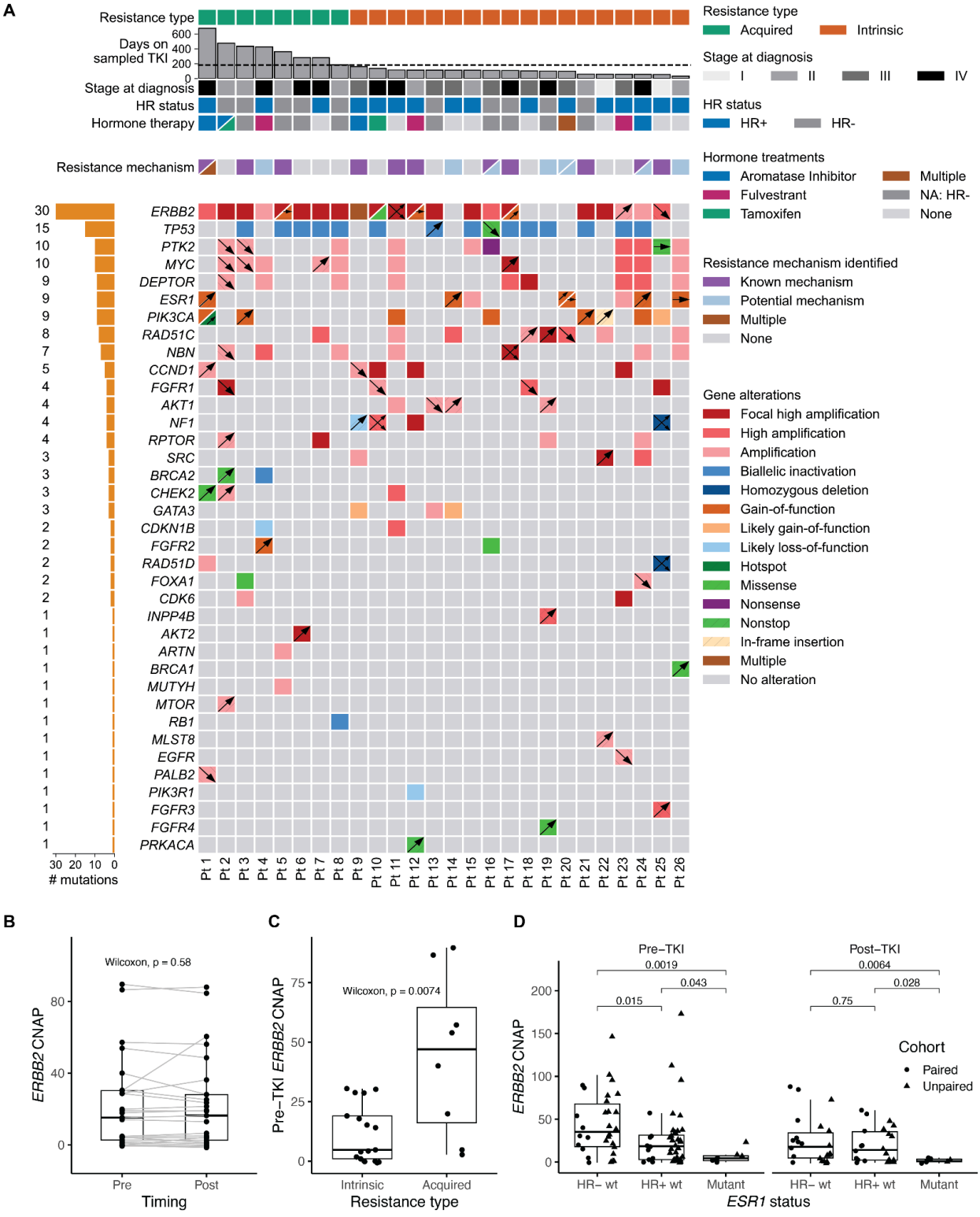

Figure 2

Genomic alterations in the paired cohort and *ERBB2* copy number dynamics

A.) Mutations and copy number alterations in selected genes;

B) Line plot showing within-participant *ERBB2* copy number change between matched pre- and post-TKI samples (n=26 participants; CNAP: copy number above ploidy);

C) *ERBB2* CNAP in pre-TKI paired cohort samples, separated by intrinsic or acquired resistance status;

D) *ERBB2* CNAP, separated by hormone receptor and *ESR1* mutation status for both paired and unpaired cohorts

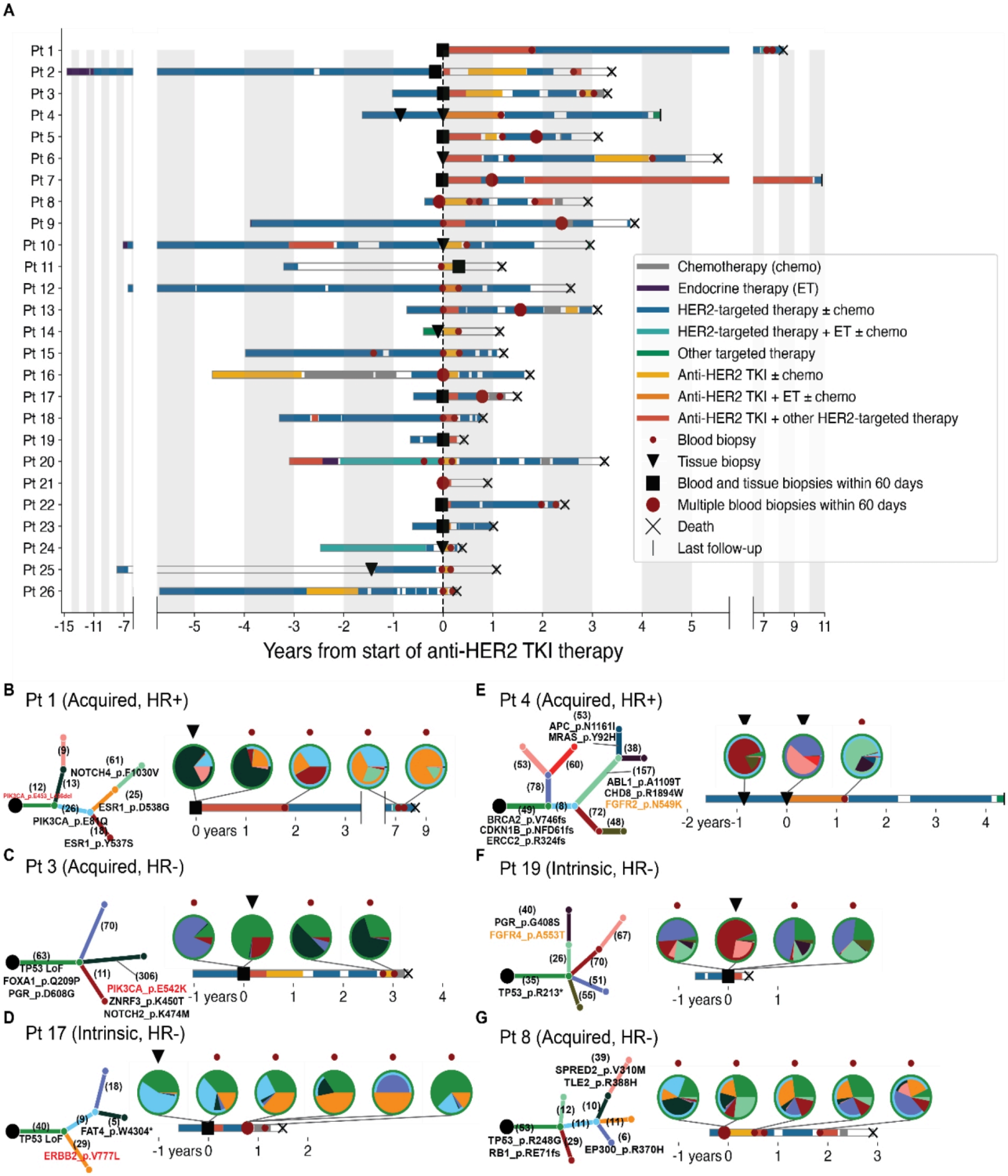

Figure 3

Phylogenetic analyses

A.) Swimmer plots for paired cohort participants (n=26) depicting treatment and sampling timing with clonal evolution of six participants demonstrating known resistance mechanisms in red and potential resistance mechanisms in yellow

B.) Competing *ESR1* mutants with known hotspot mutation Y537S growing during lapatinib treatment;

C.) Truncal *ESR1* regulator pathway mutations in *PGR* and *FOXA1*, with resistance being driven by *PIK3CA* during neratinib treatment;

D.) Growth of a clone with a known resistance mechanism (*ERBB2* V777L) during neratinib treatment;

E.) Growth of a clone with a gain-of-function mutation in FGFR2 during neratinib treatment;

F.) Growth during lapatinib treatment of a missense mutation in *FGFR4*;

G.) Truncal *TP53* and *RB1* mutations seen in pt 8 with no known resistance mechanism identified even with multiple acquired pathway mutations

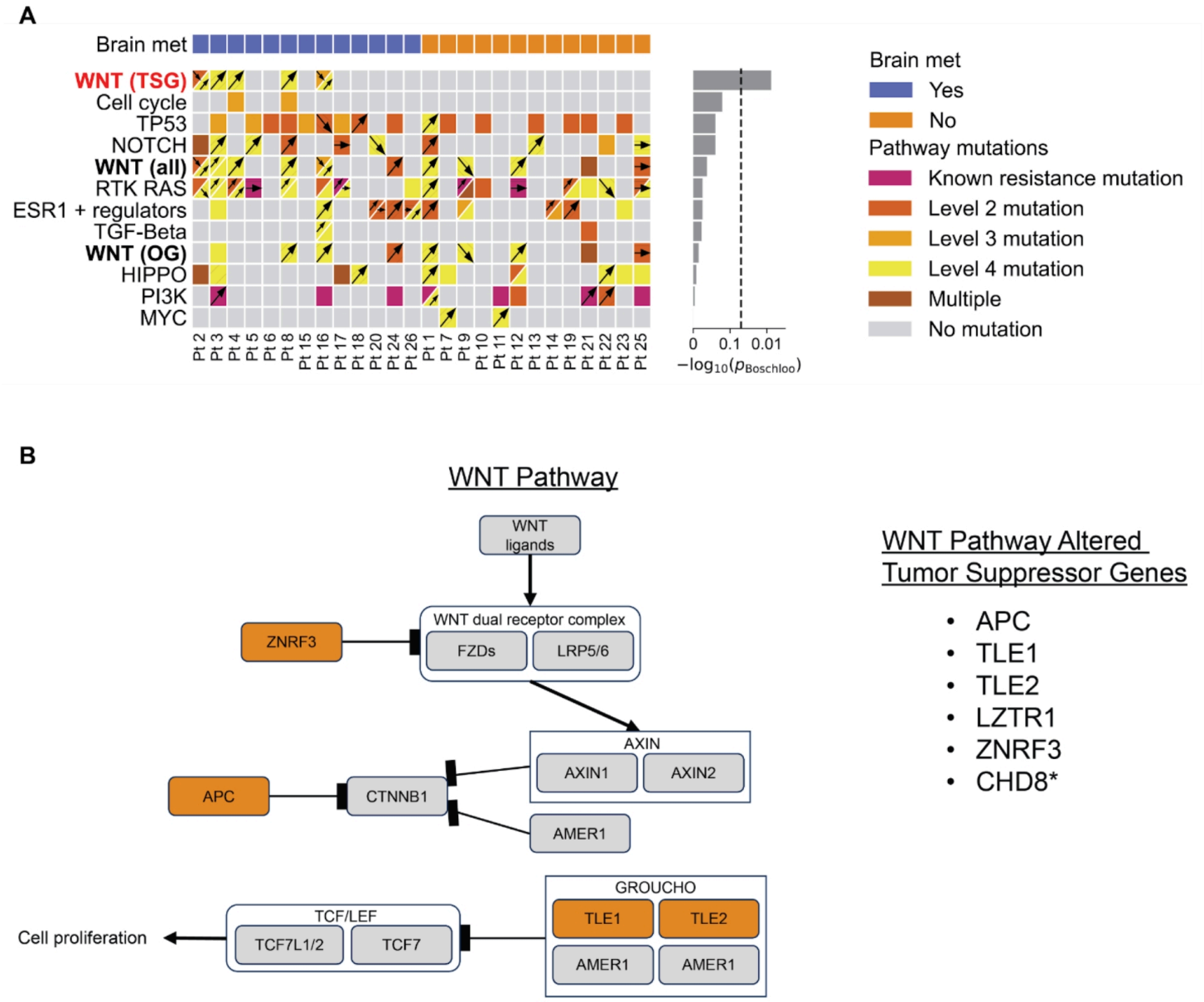

Figure 4

Genomic analysis of participants with brain metastases

A.) Comparison of pathway alterations in participants with HER2-positive MBC who developed brain metastases (n=13) vs. participants who did not (n=13). A one-sided Boschloo's test was used to assess statistical significance of enrichment for each pathway in participants who developed brain metastases;

B.) WNT pathway diagram, adapted from the Pan-Cancer Atlas, with involvement of key mutated tumor suppressor genes highlighted in orange. The diagram was generated using PathwayMapper.[49]

*All genes have been designated as tumor suppressor genes per OncoKB except for *CHD8*, which was annotated as a tumor suppressor gene based on other evidence (see Methods).

Supplementary Files

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