

# Concurrent Mutations Associated With Trastuzumab-resistance Revealed by Single Cell Sequencing

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## Research Article

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# Abstract

**Purpose:** HER2-positive breast cancer patients benefit from HER2 targeted therapies, among which the most commonly used is trastuzumab. However, acquired resistance typically happens within one year. The cellular heterogeneity of it is less clear.

**Methods:** Here we generated trastuzumab-resistant cells in two HER2-positive breast cancer cell lines, SK-BR-3 and BT-474. Cells at different time points during the resistance induction were examined by exome sequencing to study changes of genomic alterations over time. Single cell targeted sequencing was also used to identify resistance associated concurrent mutations.

**Results:** We found a rapid increase of copy number variation (CNV) regions and gradual accumulation of single nucleotide variations (SNVs). On the pathway level, nonsynonymous SNVs for SK-BR-3 cells were enriched in the MAPK signaling pathway, while for BT-474 cells were enriched in mTOR and PI3K-Akt signaling pathways. However, all of the three signaling pathways were in the downstream of the HER2 kinase. Putative trastuzumab-resistance associated SNVs included AIFM1 P548L and ERBB2 M833R in SK-BR-3 cells, and OR5M9 D230N, COL9A1 R627T, ITGA7 H911Q and ADAMTS19 V451L in BT-474 cells. Single cell targeted sequencing identified several concurrent mutations. By validation, we found that concurrent mutations (AIFM1 P548L and IL1RAPL2 S546C) led to a decrease of trastuzumab sensitivity.

**Conclusion:** Taken together, our study revealed a common pathway level trastuzumab-resistance mechanism for HER2-positive breast cancer cells. In addition, our identification of concurrent SNVs associated with trastuzumab-resistance may be indicative of potential targets for the treatment of trastuzumab-resistant breast cancer patients.

## Introduction

HER2-positive breast cancer is defined as overexpression of the HER2 protein detected by immunohistochemistry or amplification of the ERBB2 gene detected by fluorescence in-situ hybridization (FISH), taking account for around 15-20% of breast cancer [1, 2]. HER2-targeted therapies have achieved satisfactory effects in HER2-positive breast cancer [3, 4]. Trastuzumab, a recombinant humanized monoclonal antibody targeting the extracellular domain of the HER2 tyrosine kinase receptor, is commonly used in the first-line treatment for HER2-positive breast cancer, in combination with chemotherapies, such as paclitaxel and docetaxel, and/or another anti-HER2 drug pertuzumab [5-7]. However, 60-80% of the patients with an initial response to HER2-targeted therapy will acquire resistance within one year, leading to a great clinical challenge [8]. Understanding the molecular mechanism of trastuzumab-resistance is critical to improve the management of HER2-positive breast cancer patients.

Considering HER2 is a transmembrane receptor tyrosine kinase, proposed trastuzumab-resistance mechanisms include intrinsic HER2 alterations, activation of downstream pathways and bypass signaling by other kinases [9, 10]. Among these the most commonly reported is activating alterations of the PI3K/AKT/mTOR pathway, which is in the downstream of HER2 signaling and activated by HER2

phosphorylation [11]. However, only a few specific mutated genes leading to trastuzumab-resistance in this pathway have been reported, including PIK3CA and PTEN genes [12-14], more potentially targetable mutation sites need to be discovered. What's more, acquired trastuzumab-resistance is a long-term accumulation process [15], which alteration occurs first and how different alterations accumulate are still unknown, requiring a sequential monitoring.

Although several genomic studies focused on molecular mechanism of trastuzumab-resistance have already been reported [16-18], cellular heterogeneity on the trastuzumab-resistance is not known. Especially whether two or more mutations can occur concurrently in the same cell or one mutation occurs in part of cells and the other occurs in other cells. Studying at single cell level can solve this problem. In 2012 a single cell whole genome amplification method called multiple annealing and looping-based amplification cycles (MALBAC) was developed [19]. MALBAC uses quasilinear pre-amplification instead of traditional PCR to reduce the accumulation of amplification biases caused by exponential amplification. By using MALBAC we can achieve up to 93% genome coverage and ~1% allele dropout rate, making it an ideal method for single nucleotide variation (SNV) detection.

In this study, we intended to first monitor changes of genomic alterations at different time points during the process of trastuzumab-resistance induction in two HER2-positive breast cancer cell lines (SK-BR-3 and BT-474) by bulk exome sequencing. We then chose putative trastuzumab-resistance associated SNVs as targets and used MALBAC to perform single cell targeted sequencing in both two cell lines to find concurrent SNVs. Our findings would shed lights on understanding the molecular mechanism of trastuzumab-resistance and guiding treatment for acquired trastuzumab-resistant breast cancer patients.

## Materials And Methods

### Construction of acquired trastuzumab-resistant cell lines

HER2-positive breast cancer cell lines SK-BR-3 and BT-474 were purchased from the Chinese Academy of Medical Sciences (Beijing, China) and were cultured in RPMI-1640 medium (Gibco, Waltham, MA, USA) with 5%CO<sub>2</sub> at 37°C. Trastuzumab-resistant SK-BR-3 (SK-BR-3 HR) and BT-474 (BT-474 HR) cell lines were constructed as previously described by continuously exposing cells to 4µg/ml trastuzumab (MCE, Monmouth Junction, NJ, USA) for three months (Supplementary Fig. S1) [20].

The trastuzumab-resistance of SK-BR-3 HR and BT-474 HR cells were confirmed by comparing survival rates of SK-BR-3 HR and SK-BR-3 cells or BT-474 HR and BT-474 cells under different concentrations of trastuzumab treatment. For SK-BR-3 and SK-BR-3 HR cells, trastuzumab was added to the medium to final concentrations of 0, 4, 10, 50, 100, 200 and 300µg/ml for 72 hours. For BT-474 and BT-474 HR cells, final concentrations were 0, 25, 50, 75, 100, 125, 175 and 200µg/ml. The CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) was used in accordance with manufacturer guidelines. All experiments were repeated three times and data were shown as mean ± s.e.m..

## **Bulk exome sequencing of genomic DNA from different time points during resistance induction**

Cells after one, two and three months of trastuzumab treatment during the generation of trastuzumab-resistant cells and cells without trastuzumab treatment in both SK-BR-3 and BT-474 cell lines were collected for bulk exome sequencing. QIAamp DNA Micro Kit (QIAGEN, Germantown, MD, USA) was used for genomic DNA extraction. Exome libraries of genomic DNA were constructed using Agilent SureSelect Human All Exon V6 kit (Agilent, Santa Clara, CA, USA) following manufacturer's recommendations. The quality of exome library was checked by qPCR and fragment analysis using LabChip GX Touch 24 Nucleic Acid Analyzer (PerkinElmer, Waltham, MA, USA). Qualified exome libraries were sequenced by Illumina NovaSeq 2×150bp.

## **CNV calling based on bulk exome sequencing data**

Quality control of raw sequencing data was performed by discarding reads containing adapter contamination, low-quality nucleotides and unrecognizable nucleotides. The clean data was then mapped to the reference human genome (UCSC hg19) by Burrows-Wheeler Aligner (BWA) software [21]. After that SAMtools [22] and Picard were used to sort the files and perform duplicate marking, local realignment and base quality recalibration. We used cells without trastuzumab treatment as control to call differential copy number variations (CNVs) by Control-FREEC [23].

## **SNV calling and analyses**

Samtools mpileup and bcftools were used to do variant calling and the differential SNVs were detected by MuTect [24] using cells without trastuzumab treatment as control. ANNOVAR was used to perform functional annotation [25]. Nonsynonymous differential SNVs were selected. Gene ontology (GO) analysis of nonsynonymous SNVs was performed by Metascape on KEGG pathway with a P value cutoff of 0.05. We defined nonsynonymous SNVs also existing in COSMIC or KEGG pathways in cancer / ErbB signaling pathway / PI3K-Akt signaling pathway as putative trastuzumab-resistance associated SNVs. Interpretation of these SNVs with protein annotations was done by MutationMapper.

## **Single cell isolation and whole genome amplification of trastuzumab-resistant cells**

SK-BR-3 HR and BT-474 HR cells were also used for single cell isolation by mouth pipetting under microscope. A total of 96 and 50 single cells in SK-BR-3 and BT-474 cells were isolated. After single cell lysis, whole genome amplification was performed by MALBAC following the published paper [19]. In detail, nine cycles of quasilinear amplification with random primers containing 27-nucleotide sequence and 8 variable nucleotides were first performed. Then 20 cycles of traditional PCR targeting the common sequences were done. The quality of amplified DNA for each single cell was detected by qPCR with eight pairs of primers targeting random copy number conservative regions across the genome. Single cells having no less than six sites with Ct value less than 30 passed the quality control. A total of 94 and 50 single cells in SK-BR-3 and BT-474 cell lines passed the quality control.

## **Single cell targeted sequencing**

Targeted mutation sites were selected based on bulk exome sequencing data. Nonsynonymous SNVs with mutation frequency  $\geq 10\%$  in both SK-BR-3 and BT-474 cell lines were used for functional impact prediction by three methods: Mutation Assessor, SIFT and Polyphen-2. SNVs predicted to be damaging by no less than two methods were chosen for single cell targeted sequencing. A total of 21 and 22 mutation sites were selected for SK-BR-3 and BT-474 cell lines, and the detailed mutation information and sequences of primers were shown in Supplementary Table S1 and S2.

Targeted PCR was performed by using OneTaq Hot Start Quick-Load 2×Master Mix (NEB, Ipswich, MA, USA). Then the amplification products for each single cell were pooled together to construct sequencing libraries using KAPA Hyper Prep Kit (KAPA, Boston, MA, USA) combined with NEBNext Multiplex Oligos for Illumina (NEB, Ipswich, MA, USA) following the manufacturer instructions. The libraries were sequenced by Illumina NovaSeq 2×150bp. The single cell SNVs were called by MuTect2. Genomic sites with mutation frequency  $\geq 5\%$  were defined as mutant, genomic sites with mutation frequency  $< 5\%$  were defined as wild-type, and genomic sites with no reads cover were defined as no coverage.

### **Functional experiments to validate detected concurrent mutations leading to trastuzumab-resistance**

We validated the function of concurrent mutations of AIFM1 P548L and IL1RAPL2 S546C in trastuzumab-resistance in SK-BR-3 cells. Primers were designed at specific sites of plasmids pCMV-FLAG-AIFM1 and pCMV-FLAG-IL1RAPL2 to construct mutant plasmids. The primer sequences were shown in Supplementary Table S3. SK-BR-3 cells were transiently transfected with plasmids of pCMV-FLAG-AIFM1-WT, pCMV-FLAG-AIFM1-Mut, pCMV-FLAG-IL1RAPL2-WT, pCMV-FLAG-IL1RAPL2-Mut, both pCMV-FLAG-AIFM1-WT and pCMV-FLAG-IL1RAPL2-WT, and both pCMV-FLAG-AIFM1-Mut and pCMV-FLAG-IL1RAPL2-Mut using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA), respectively. Trastuzumab was added to the medium to final concentrations of 0, 0.25, 0.5, 1 and 2  $\mu\text{g}/\text{ml}$  for 72 hours. Cell Counting Kit-8 (CCK8) (ZETA, Xi'an, China) was used in accordance with manufacturer guidelines. For AIFM1 and IL1RAPL2 mutations, experiments were repeated three times. And for concurrent mutations of AIFM1 and IL1RAPL2 genes, experiments were repeated twice. Mean values for the three or two experiments were shown.

## **Results**

### **Changes of genomic alterations during the generation of trastuzumab-resistant SK-BR-3 cell line**

SK-BR-3 is a HER2-positive breast cancer cell line with HER2-enriched molecular subtype (ER negative, PR negative, HER2 positive) and is sensitive to trastuzumab. We generated acquired trastuzumab-resistant SK-BR-3 cell line (SK-BR-3 HR) by low dose trastuzumab treatment. By comparing survival rates of SK-BR-3 and SK-BR-3 HR cells under different concentrations of trastuzumab treatment (Supplementary Fig. S2a), we validated the trastuzumab-resistance of SK-BR-3 HR cells ( $\text{IC}_{50}$  for SK-BR-3: 117.4  $\mu\text{g}/\text{ml}$ ;  $\text{IC}_{50}$  for SK-BR-3 HR: 241.3  $\mu\text{g}/\text{ml}$ ). To monitor the changes of genomic alterations during the generation of trastuzumab-resistance, we performed bulk exome sequencing of SK-BR-3 cells after one, two and three months of trastuzumab treatment together with SK-BR-3 cells without trastuzumab treatment.

We used the data from SK-BR-3 cells without trastuzumab treatment as control to call the differential CNVs and SNVs of the three time points. In the aspect of CNVs, for the first two months there were only a few differential CNV regions, but at the third month the CNV regions increased rapidly (Supplementary Fig. S3a). These observations suggested that, in addition to an important role in both carcinogenesis and cancer metastasis [26-28], CNVs may also have a crucial effect on drug resistance. In contrast, SNVs increased gradually across the whole process (Supplementary Fig. S3b).

For exome sequencing data, we could not get CNV information at a high resolution; therefore, we mainly focused on SNV analysis. We first listed all nonsynonymous differential SNVs (a total of 105) and exhibited the mutation frequency changes of them across the three time points, as shown in Fig. 1a. The number of nonsynonymous SNVs appeared at the third month (74 SNVs) was significantly higher than the first two months (first month: 14 SNVs, second month: 17 SNVs), further supporting that dramatic change of trastuzumab sensitivity may occur from the third month.

To find which signaling pathway these nonsynonymous SNVs may affect, we performed GO analysis on KEGG pathways of them. As shown in Fig. 1b, the nonsynonymous SNVs were mainly enriched in MAPK signaling pathway. We marked the positions of these mutated genes in MAPK signaling pathway in Fig. 1c.

Except the enriched genes, mutations of other genes may also play important roles in trastuzumab-resistance. We defined nonsynonymous SNVs that also existed in COSMIC or KEGG pathways in cancer / ErbB signaling pathway / PI3K-Akt signaling pathway as putative trastuzumab-resistance associated SNVs and demonstrated them with protein annotations (Fig. 1d). AIFM1 P548L belonged to COSMIC, with a mutation frequency of 2.47% in breast cancer. This mutation affected the apoptosis-inducing factor mitochondrion associated C-term domain of AIFM1 protein. ERBB2 gene existed in KEGG pathways in cancer and ErbB signaling pathway. ERBB2 M833R affected the protein tyrosine kinase domain of HER2 protein.

### **Concurrent mutations associated with trastuzumab-resistance of SK-BR-3**

To distinguish if two SNVs occurred concurrently in the same cell or one SNV occurred in part of the cells and the other occurred in the other cells, we took advantage of MALBAC for single cell targeted sequencing of SK-BR-3 HR cells. By functional impact prediction of 40 nonsynonymous SNVs with mutation frequency  $\geq 10\%$  (Supplementary Fig. S4a), we selected 21 targeted mutation sites and 94 single cells were targeted sequenced.

The distribution of the 21 SNVs in the 94 single cells were shown in Fig. 2a. Among all of the 1,974 sites, only 28 sites were without coverage, taking account for 1.42%, which confirmed the high genome coverage of MALBAC method. We tried to find two SNVs that more frequently occurred concurrently in the same single cell. The top three pairs were shown in Fig. 2b: AIFM1 P548L and CHD7 G2228W with a concurrent frequency of 29.8%; AIFM1 P548L and UBR5 P2740S with a concurrent frequency of 29.8%; and AIFM1 P548L and IL1RAPL2 S546C with a concurrent frequency of 26.6%. Compared with single

gene mutation, combined mutations of these genes may have more growth advantages under trastuzumab treatment and contribute more to trastuzumab-resistance. Although there was also possibility for more than two mutation combination associated with trastuzumab-resistance, considering the limited single cell sample size and number of targeted mutation sites, we did not perform this analysis.

### **Introduction of the concurrent mutations led to trastuzumab-resistance**

Above we detected pairs of concurrent SNVs in SK-BR-3 HR cells, but whether these detected concurrent mutations lead to trastuzumab-resistance still needed validation. Therefore, we chose one pair of concurrent mutations: AIFM1 P548L and IL1RAPL2 S546C (both of the two SNVs occurred at the first month of trastuzumab addition and the mutation frequencies of them increased over time) for functional experiments.

We transfected SK-BR-3 cells with either wild-type AIFM1 plasmid (AI WT), mutant AIFM1 plasmid (AI Mut), wild-type IL1RAPL2 plasmid (IL WT), mutant IL1RAPL2 plasmid (IL Mut), both wild-type AIFM1 and wild-type IL1RAPL2 plasmids (Double WT) and both mutant AIFM1 and mutant IL1RAPL2 plasmids (Double Mut), respectively. The transfection efficiency was confirmed by western blot in Supplementary Fig. S5. As shown in Fig. 2c, in AI WT and AI Mut group there were no significant differences in trastuzumab sensitivities. In contrast, in IL WT and IL Mut group and Double WT and Double Mut group trastuzumab sensitivity significantly decreased in cells with mutant plasmid compared with cells with wild-type plasmid, especially for Double WT and Double Mut group. In IL WT and IL Mut group the  $IC_{50}$  of cells with mutant plasmid was around 1.61-fold ( $0.6509\mu\text{g/ml}:0.4055\mu\text{g/ml}$ ) of cells with wild-type plasmid. And in Double WT and Double Mut group the  $IC_{50}$  of cells with mutant plasmid was around 2.02-fold ( $0.918\mu\text{g/ml}:0.454\mu\text{g/ml}$ ) of cells with wild-type plasmid. These data indicated that single mutation of AIFM1 gene may give some growth advantages to SK-BR-3 cells under trastuzumab treatment however it did not change their trastuzumab sensitivity significantly. Single mutation of IL1RAPL2 gene can decrease the trastuzumab sensitivity of SK-BR-3 cells, while concurrent mutations of IL1RAPL2 and AIFM1 genes can further decrease their trastuzumab sensitivity, leading to trastuzumab-resistance.

### **Changes of genomic alterations during the generation of trastuzumab-resistant BT-474 cell line**

BT-474 is also a HER2-positive breast cancer cell line, but its molecular subtype is Luminal B (ER positive, PR positive, HER2 positive). Breast cancers with different molecular subtypes exhibited quite distinct biological and clinical phenotypes [29]. To investigate the similarities and dissimilarities of trastuzumab-resistance mechanism between BT-474 and SK-BR-3 cell lines, we also induced acquired trastuzumab-resistant BT-474 cell line (BT-474 HR). The trastuzumab-resistance of BT-474 HR cells was confirmed (Supplementary Fig. S2b) ( $IC_{50}$  for BT-474:  $125.3\mu\text{g/ml}$ ;  $IC_{50}$  for BT-474 HR:  $422\mu\text{g/ml}$ ). Then we performed bulk exome sequencing of BT-474 cells after one, two and three months of trastuzumab treatment together with BT-474 cells without trastuzumab treatment.

Differential CNVs and SNVs of the three time points were called. For CNVs, there were a few differential CNV regions at the first month, and the CNV regions increased rapidly at the second month (Supplementary Fig. S3c). It indicated that the dramatic change of trastuzumab sensitivity for BT-474 cells may occur earlier than SK-BR-3 cells, from the second month of trastuzumab addition. And for SNVs, they also increased gradually across the whole process in BT-474 cells (Supplementary Fig. S3d), as in SK-BR-3 cells.

Then we demonstrated the mutation frequency changes of all nonsynonymous differential SNVs (a total of 197) across the three time points in BT-474 cell line in Fig. 3a. Number of nonsynonymous SNVs occurred at each time point was much higher in BT-474 cells compared with SK-BR-3 cells (the first month: 49 SNVs in BT-474 cells compared with 14 in SK-BR-3 cells; the second month: 54 SNVs in BT-474 cells compared with 17 in SK-BR-3 cells; the third month: 94 SNVs in BT-474 cells compared with 74 in SK-BR-3 cells). This meant that more mutations contributed to trastuzumab-resistance in BT-474 cells compared with SK-BR-3 cells at the same time point, further supporting that the dramatic change of trastuzumab sensitivity may occur earlier in BT-474 cells, maybe from the first or second month of trastuzumab addition.

The GO analysis on KEGG pathways showed distinctions from SK-BR-3 cells, the nonsynonymous SNVs were mainly enriched in mTOR and PI3K-Akt signaling pathways (Fig. 3b), consistent with previous studies [12]. Positions of these mutated genes in mTOR or PI3K-Akt signaling pathway were marked in Fig. 3c.

We also demonstrated the putative trastuzumab-resistance associated SNVs with protein annotations in Fig. 3d. ADAMTS19 V451L and OR5M9 D230N belonged to COSMIC with mutation frequencies of 7.11% and 0.54% in breast cancer, respectively. ADAMTS19 V451L affected the reprolysin (M12B) family zinc metalloprotease domain of ADAMTS19 protein. OR5M9 D230N affected the olfactory receptor domain of OR5M9 protein. COL9A1 and ITGA7 genes existed in KEGG PI3K-Akt signaling pathway. COL9A1 R627T affected the collagen triple helix repeat domain of COL9A1 protein. ITGA7 H911Q affected the integrin alpha domain of ITGA7 protein.

### **Concurrent mutations associated with trastuzumab-resistance of BT-474**

By functional impact prediction of 47 nonsynonymous SNVs with mutation frequency  $\geq 10\%$  in BT-474 cells (Supplementary Fig. S4b), we selected 22 mutation sites for single cell targeted sequencing. Except for one (USP35 E55K, all single cells showed no coverage in this position maybe due to the complex genomic organization making it difficult to be amplified by MALBAC method), the left 21 mutation sites were used and 50 single cells were targeted sequenced.

The distribution of the 21 SNVs in the 50 single cells were shown in Fig. 4a. Among all of the 1,050 sites, only 5 sites were without coverage, taking account for 0.48%. The top three pairs of concurrent SNVs are shown in Fig. 4b: KIAA1107 K771T and MFSD11 L242I with a concurrent frequency of 24%, KIAA1107

K771T and ANAPC4 E16K with a concurrent frequency of 22%; and KIAA1107 K771T and CENPJ E318Q with a concurrent frequency of 22%.

## Discussion

In this study, we investigate the molecular mechanism of acquired trastuzumab-resistance and how genomic alterations accumulate over time in two HER2-positive breast cancer cell lines. The nonsynonymous differential SNV landscapes for trastuzumab-resistant cells in SK-BR-3 and BT-474 cell lines are quite different, sharing only two genes: ERBB2 and BICD1 (with distinct mutation types). However, when we compare the results on the pathway level, the two cell lines show similarity. Although the nonsynonymous SNVs are enriched in different signaling pathways for the two cell lines, all of these signaling pathways are in the downstream of the HER2 kinase. This means that the molecular mechanism of trastuzumab-resistance for both SK-BR-3 and BT-474 cells is alterations of downstream signaling pathways of the HER2 kinase (Fig. 4c). Previous studies also have reported that pathway level alterations rather than mutations in single genes can predict response to HER2-targeted therapies [30], implying the importance of pathway level alterations in trastuzumab-resistance.

The process of genomic alteration accumulation for the two cell lines are also distinctive. The differential CNV regions increase rapidly at the third month in SK-BR-3 cells, but earlier (at the second month) in BT-474 cells, suggesting that BT-474 acquires trastuzumab-resistance earlier than SK-BR-3 cells. We know that although both of SK-BR-3 and BT-474 cell lines are HER2 positive, their molecular subtypes are different (SK-BR-3 with HER2-enriched subtype, BT-474 with Luminal B subtype). Expression of EGFR in the SK-BR-3 cell line is three times of the BT-474 cell line, and BT-474 is more sensitive to trastuzumab compared with SK-BR-3 [31]. The lower trastuzumab sensitivity of SK-BR-3 may be due to its higher expression of EGFR, a bypass signaling of HER2 as compensation. This is consistent with our results that it is more difficult and may take longer time to gain trastuzumab-resistance for SK-BR-3 cells.

We identify some putative trastuzumab-resistance associated SNVs, such as AIFM1 P548L and ERBB2 M833R in SK-BR-3 cells, and OR5M9 D230N, COL9A1 R627T, ITGA7 H911Q and ADAMTS19 V451L in BT-474 cells. What's more, taking advantage of single cell sequencing, we identify concurrent mutations associated with trastuzumab-resistance. Further we validate that concurrent AIFM1 P548L and IL1RAPL2 S546C can indeed lead to trastuzumab-resistance in SK-BR-3 cells, confirming the significance of the detected mutations. These mutation sites have the potential to become drug targets for trastuzumab-resistant breast cancer patients.

In conclusion, we identify that the pathway level mechanism of trastuzumab-resistance is alterations of downstream signaling pathways of the HER2 kinase. We also detect a few putative trastuzumab-resistance mutations, especially concurrent mutations. These mutations are potential biomarkers of trastuzumab-resistance and putative targets for treatment of trastuzumab-resistant breast cancer patients.

# Declarations

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## Conflict of interest

The authors declare that they have no conflict of interest.

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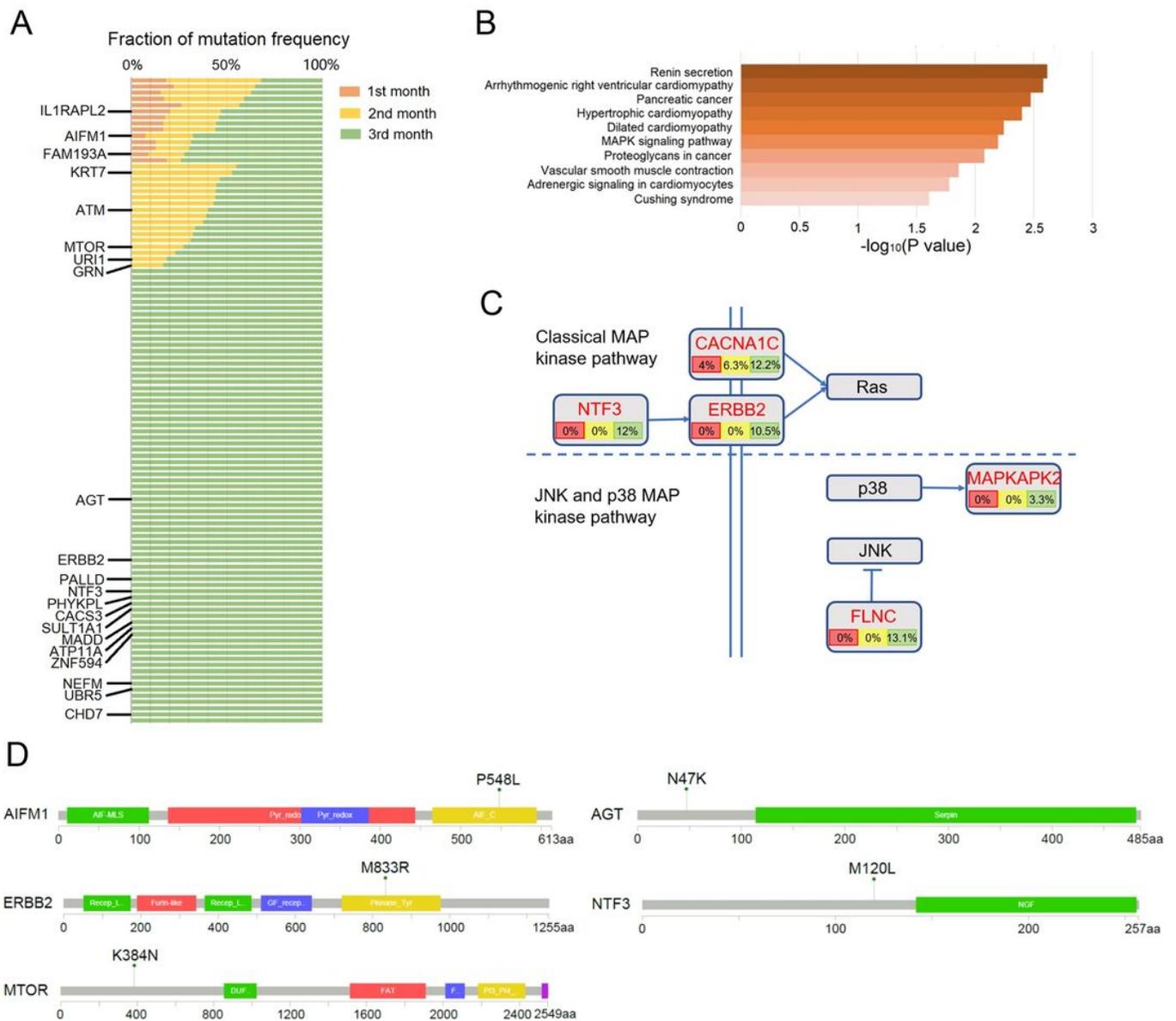
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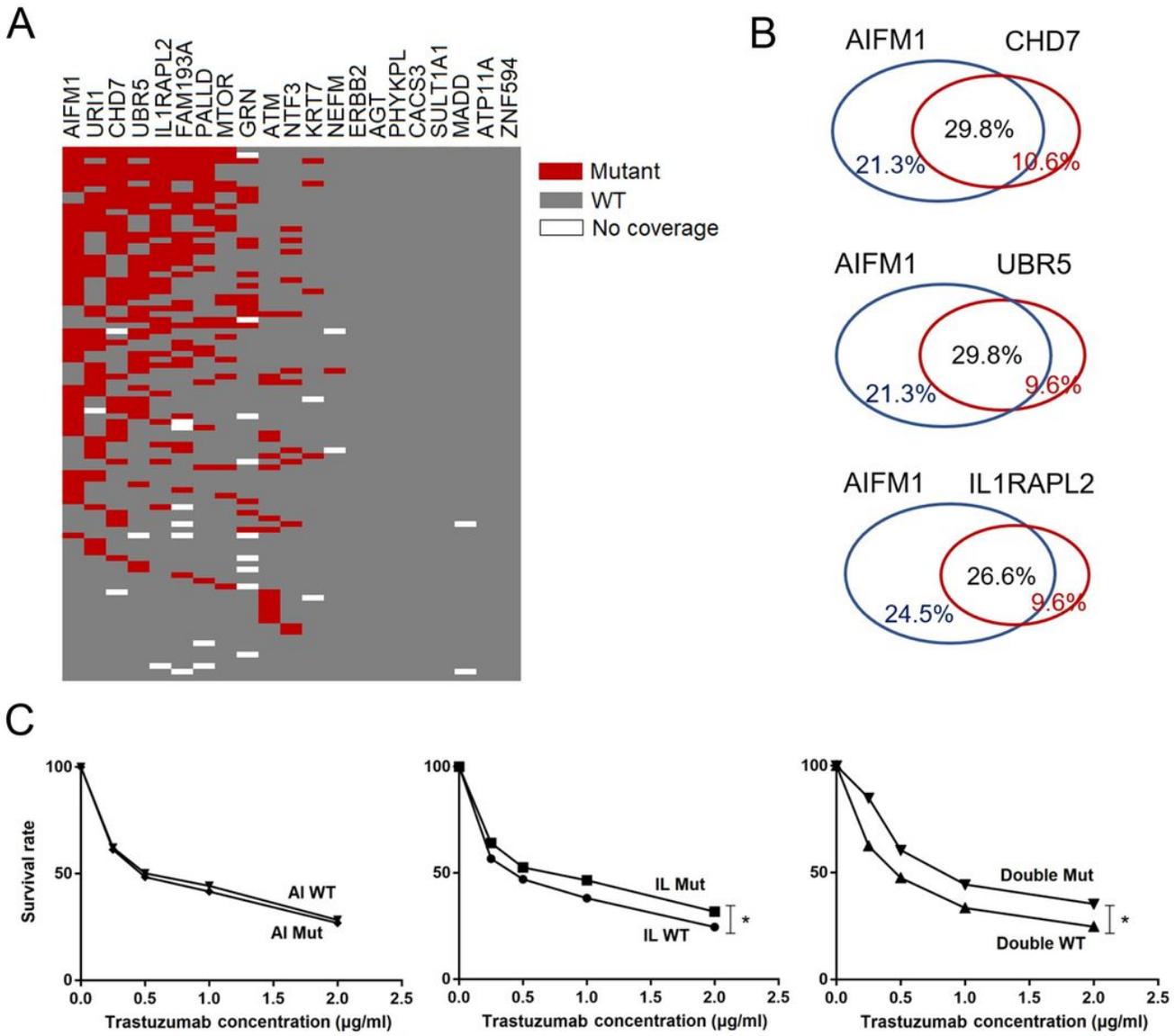
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## Figures



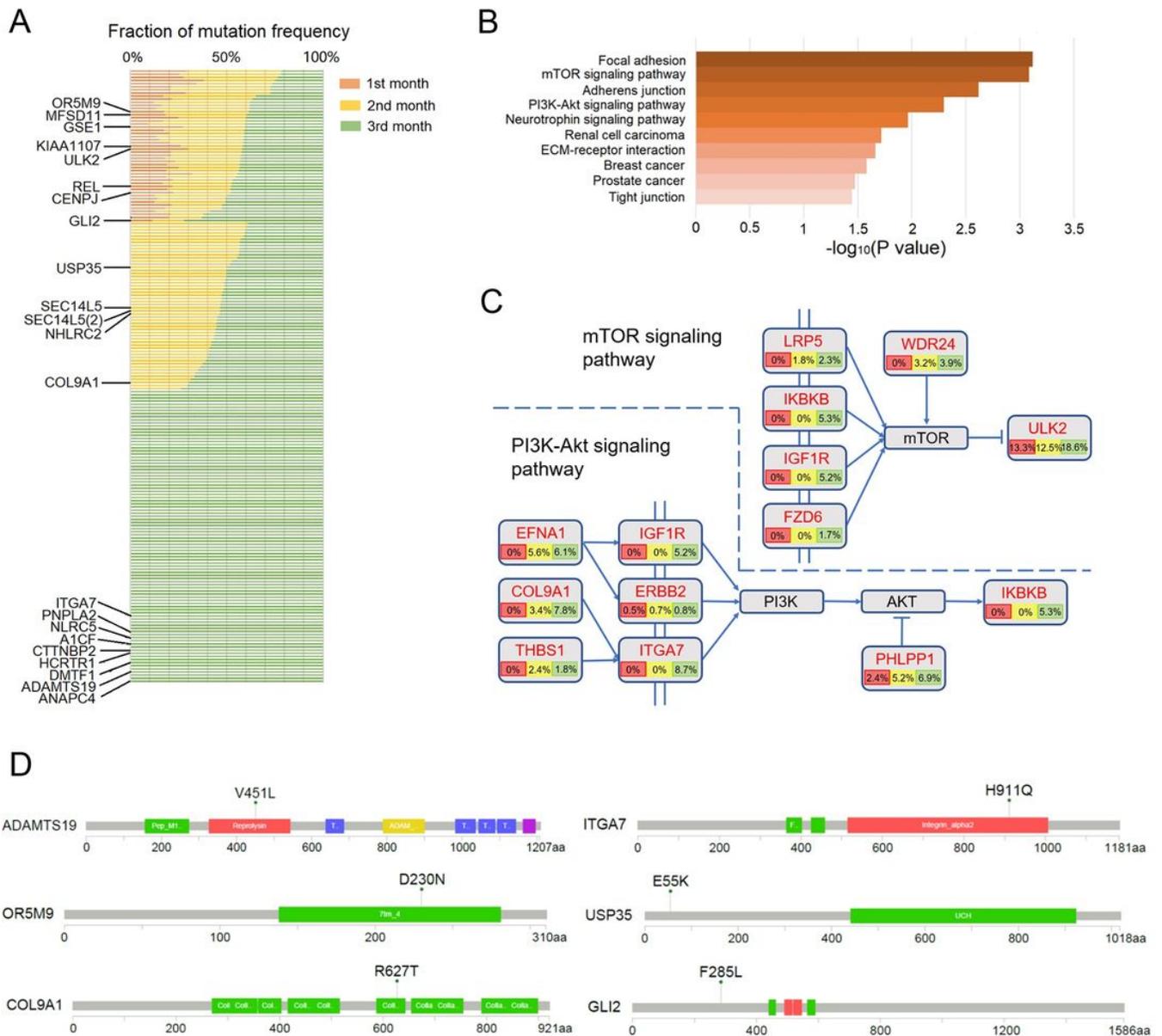
**Figure 1**

Nonsynonymous differential SNVs during the generation of trastuzumab-resistant SK-BR-3 cell line. a Fraction of mutation frequencies for 105 nonsynonymous differential SNVs at different time points (after one, two and three months of trastuzumab addition) in SK-BR-3 cell line. Representative genes are marked on the left. b GO analysis on KEGG pathway of the 105 SNVs shows enrichment in MAPK signaling pathway. c The positions of detected mutated genes (in red) in MAPK signaling pathway with mutation frequencies at different time points (red: first month; yellow: second month; green: third month). d Interpretation of representative putative trastuzumab-resistance associated SNVs for AIFM1, ERBB2, MTOR, AGT and NTF3 genes in SK-BR-3 cell line with protein annotations.



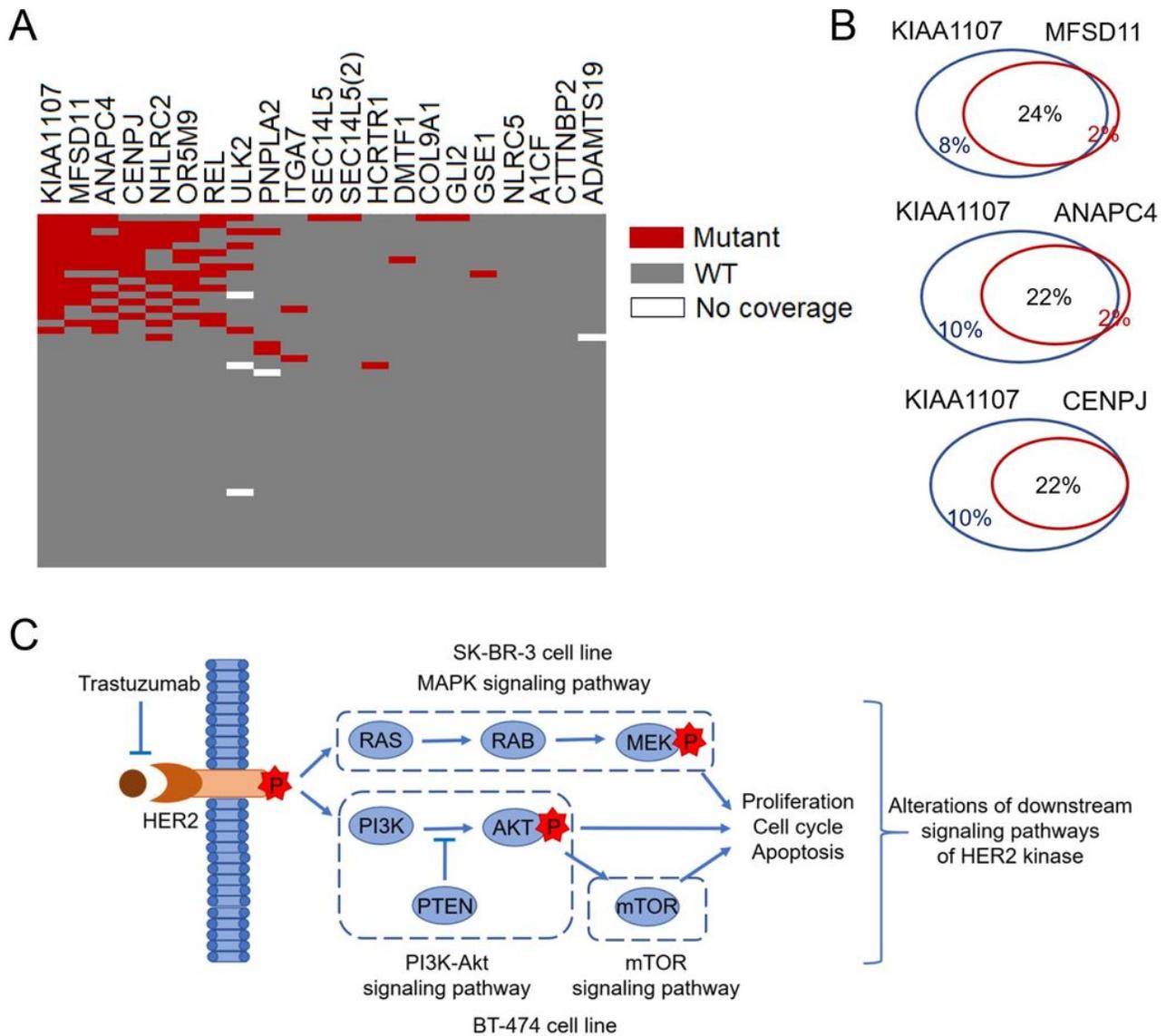
**Figure 2**

Putative trastuzumab-resistance associated SNVs in SK-BR-3 cell line at single cell level. a Distribution of 21 putative trastuzumab-resistance associated SNVs among 94 single cells after three months of trastuzumab addition in SK-BR-3 cell line. b Venn diagrams show the top three pairs of concurrent SNVs with mutant cellular prevalence. c Functional validation of AIFM1, IL1RAPL2 mutations and concurrent mutations of AIFM1 and IL1RAPL2 genes on trastuzumab sensitivities of SK-BR-3 cell line. X axis represents trastuzumab concentration. Y axis represents survival rates. For AIFM1 and IL1RAPL2 mutations, experiments are repeated three times. And for concurrent mutations of AIFM1 and IL1RAPL2 genes, experiments are repeated twice. Mean values for the three or two experiments are shown. \*P value<0.05.



**Figure 3**

Nonsynonymous differential SNVs during the generation of trastuzumab-resistant BT-474 cell line. a Fraction of mutation frequencies for 197 nonsynonymous differential SNVs at different time points (after one, two and three months of trastuzumab addition) in BT-474 cell line. Representative genes are marked on the left. b GO analysis on KEGG pathway of the 197 SNVs shows enrichment in mTOR and PI3K-Akt signaling pathways. c The positions of detected mutated genes (in red) in mTOR or PI3K-Akt signaling pathway with mutation frequencies at different time points (red: first month; yellow: second month; green: third month). d Interpretation of representative putative trastuzumab-resistance associated SNVs for ADAMTS19, COL9A1, ITGA7, USP35 and GLI2 genes in BT-474 cell line with protein annotations.



**Figure 4**

Putative trastuzumab-resistance associated SNVs in BT-474 cell line at single cell level. a Distribution of 21 putative trastuzumab-resistance associated SNVs among 50 single cells after three months of trastuzumab addition in BT-474 cell line. b Venn diagrams show the top three pairs of concurrent SNVs with mutant cellular prevalence. c A diagram shows the common pathway level trastuzumab resistance mechanism for both SK-BR-3 and BT-474 cell lines: alterations of downstream signaling pathways of the HER2 kinase.