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Research

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Title: CDK12 inhibition enhances sensitivity of HER2+ breast cancers to HER2-TKI via suppressing PI3K/AKT

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Abstract

Background: While anti-HER2 tyrosine kinase inhibitors (TKIs) have radically prolonged survival and improved prognosis in HER2-positive breast cancer patients, resistance to these therapies is a constant obstacle leading to TKIs treatment failure and tumor progression. **Methods:** To develop new strategies to enhance TKIs efficiency by combining synergistic gene targets, we performed panel library screening using CRISPR/Cas9 knockout technique based on data mining across TCGA datasets and verified the candidate target in pre-clinical models and breast cancer high-throughput sequencing datasets. **Results:** We identified that CDK12, co-amplified with HER2 in a high frequency, is powerful to sensitize or re-sensitize HER2-positive breast cancer to anti-HER2 TKIs lapatinib, evidenced by patient-derived organoids (PDO) in vitro and cell-derived xenograft (CDX) or patient-derived xenograft (PDX) in vivo. Exploring mechanisms, we found that inhibition of CDK12 attenuated PI3K/AKT signal, which usually serves as an oncogenic driver and is reactivated when HER2-positive breast cancers develop resistance to lapatinib. Combining CDK12 inhibition exerted additional suppression on p-AKT activation induced by anti-HER2 TKIs lapatinib treatment. Clinically, via DNA sequencing data for tumor tissue and peripheral blood ctDNA, we found that HER2-positive breast cancer patients with CDK12 amplification responded more insensitively to anti-HER2 treatment than those without accompanying CDK12 amplification by harboring a markedly shortened progression free survival (PFS) (median PFS: 4.3 months verse 6.9 months; HR 2.26 [95% CI 1.32-3.86]; P=0.0028). **Conclusions:** Dual inhibition of HER2/CDK12 will prominently benefit the outcomes of HER2-positive breast cancer patients by sensitizing or re-sensitizing the tumors to anti-HER2 TKIs treatment.

Keywords

Breast cancer; HER2 positive; Lapatinib resistance; CDK12; Co-amplification

Background

Breast cancer harboring over-expression or gene amplification of human epidermal growth factor receptor 2 (HER2 or ERBB2) but with negative hormone receptors (HR-, estrogen receptor and progesterone receptor) is termed as ERBB2-positive or HER2-positive subtype breast cancer, which accounts for 7.1-21.3% of all breast cancer

patients (1) and represents a more aggressive subtype of breast cancer. HER2-positive breast cancer is addictive to the HER2 oncogene product and signaling pathway. Thus, HER2-targeted therapies, including humanized monoclonal antibody drugs against HER2, such as trastuzumab and pertuzumab, and small molecule TKI, such as lapatinib and pyrotinib, have been developed and, consequently, greatly improved the patient's prognosis (2, 3). However, there are still a large number of HER2-positive breast cancer patients with primary or secondary resistance to lapatinib therapies (4-6). The response rate of HER2-targeted regimens in the first-line setting ranges from 50% to 80%, and from only 20% to 40% in the second-line setting, with most patients eventually resistant to these drugs (7). Clinically, trastuzumab-resistant HER2-positive breast cancer can be treated in combination with lapatinib or treated with lapatinib/capecitabine (8) or lapatinib/pertuzumab (9), while lapatinib-resistant breast cancer does not have reliable alternatives currently. Thus, it is urgent to develop novel oncotherapy strategies for lapatinib-resistant cancers.

Currently, some hypothetical mechanisms on lapatinib resistance have been proposed. Mutations in HER2 gene itself, such as mutations on the HER2 TK domain or those causing loss of the anti-HER2 target, may result in resistance to anti-HER2 therapy (10). Activation of carcinogenic factors, like other receptor tyrosine kinases AXL and MET (11), or signal cross talking between HER2 pathway and ER pathway, are possible mechanisms for the breast cancer cells to develop drug resistance (12-14). However, the mechanisms of lapatinib resistance are still underexplored. Copy number variations (CNVs) of chromosome 17 on which HER2 locates are extremely common in breast cancer. We analyzed the genome sequencing data of 1105 breast cancer patients from TCGA in cBioPortal database and found a significant amplification range on chromosome 17q centering on HER2. The CNVs frequency in the 4 Mbp (17q12-21.2) region flanking HER2 reaches up to 15% in all breast cancer patients. It has been known that CNVs involving multiple genes are frequently found in human tumors and they collaborate to regulate important cellular functions, such as proliferation, angiogenesis and cell movement. However, whether the genes frequently co-amplified with HER2 contribute to the carcinogenic process of the HER2 gene and the response to anti-HER2 treatment efficiency are elusive.

In this study, we constructed a CRISPR/Cas9-based gene knockout library with sgRNAs targeting the genes accompanying HER2 amplification and screened by

lapatinib or pyrotinib pressure in breast cancer cell lines. As a result, cyclin-dependent kinase 12 (CDK12) was identified as a gene critically related to lapatinib therapy resistance. CDK12 is a principal regulator of various cellular biological processes including DNA damage repair and pre-mRNA splicing, participating in tumorigenesis (15, 16). CDK12 globally inhibits intronic polyadenylation (IPA) and regulates DNA repair genes isoforms usage, especially homologous recombination related genes, such as ATM and BRCA2 in prostate adenocarcinoma and ovarian carcinoma(17). Besides, CDK12 also modulates the process of DNA damage repair by regulating the alternative splicing of DNA damage-responsive activator ATM and the last exon of DNAJB6 isoform (ALE), working to promote breast cancer cells' migration and invasion (18). Similarly, being verified in the BRCA-mutated triple-negative breast cancer cells and the PDX model, CDK12 inhibition disrupted homologous recombination and thus reversed the novo resistance to PARP inhibition (19). Although co-amplification of HER2 and CDK12 in patients with HER2-positive breast cancer or gastric cancers has been noticed previously (20, 21), the potential synergistic effects of CDK12/HER2 amplification on biological processes or lapatinib treatment of HER2-positive cancers have never been explored.

Herein, we proposed and verified that the inhibition of CDK12 sensitized HER2-positive breast cancers to lapatinib and markedly suppressed tumor progression by attenuating PI3K/AKT activation.

Materials and Methods

Cell Lines

The human breast cancer cell line SKBR3 and HCC38 were purchased from Cell Culture Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC). MDA-MB-453 cells were cultured in mix medium (L15: DMEM=1:1) with 10% fetal bovine serum (Hyclone) at 37 °C in a 5% CO₂ standard incubator. BT474 cells were cultured by RPMI 1640 with 10% fetal bovine serum (Hyclone) and with 0.1U/ml insulin. The colorectal cancer cell lines HCT15, HCT116, CT26 (mice) as well as 293TN were obtained from the State Key Laboratory of Molecular Oncology, National Cancer Center/Cancer Hospital, CAMS&PUMC. All cell lines, except for HCT15 and 293TN,

were cultured in RPMI 1640 (BIOROC, China) with 10% fetal bovine serum (Hyclone) at 37 °C in a 5% CO₂ standard incubator and HCT15 and 293TN were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone). Copy numbers and mRNA levels of HER2 and CDK12 among 59 breast cancer cell lines were obtained from Cancer Cell Line Encyclopedia (CCLE) database.

***In vivo* xenograft tumor assay**

Female BALB/c immunodeficient mice, 6 weeks old, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). These animals were maintained in the Animal Facilities of National Cancer Center/Cancer Hospital, CAMS&PUMC under pathogen-free conditions. For xenograft experiments, 5×10⁶ HCC38 cells (or 1×10⁶ for CT26 cells) were re-suspended in 100μl of PBS and injected into the right flank of armpit subcutaneously. The tumor size was measured in two dimensions using a caliper, and the volume was expressed in mm³ using the formula: $V = 0.5 \times a \times b^2$ where a and b are the long and short diameters of the tumor, respectively. For the drug treatment experiments, mice were randomized into four groups after tumor formation and treated with either vehicle (DMSO) or Lapatinib (Selleck, S2111, 75mg/kg) by daily stomach injection or/and Dinaciclib (CDK12 inhibitor, Selleck, S2768, 8mg/kg) by intraperitoneal injection. For the lapatinib-resistant HCC38 tumor induction, 5×10⁶ HCC38 cells were injected into nude mice subcutaneously followed by lapatinib treatment (100 mg/kg) once a day upon the tumor volume reached 150mm³. After two weeks, tumor-bearing mice were euthanized and the fresh tumor tissues were transplanted into other tumor-free nude mice followed by lapatinib treatment three days after transplantation. The same procedure was repeated two weeks later. The tumor passage and treatment were repeated up to six generation then the models were prepared for dinaciclib treatment experiments.

Patient-derived organoids (PDO) and patient-derived xenograft (PDX) models

Tumor organoids derived from five HER2-positive breast cancer patients were established by K2 Oncology Co., Ltd. and cultured in BMG Omega, 48-well plate (Thermo 150687) under the conditions of GAS and CellTiter-Lumi™ Plus (Beyotime, Cat.No. C0068L). Primary tumors of HER2-positive/HR-negative patients resistant to lapatinib treatment were obtained from Shanghai LideBiotech CO., LTD (Project No.:

CAH-PDX-PC001) and the Model ID is BRPF211. Briefly, about 50-90 mg tumor tissue blocks were implanted subcutaneously on the right flank of each mouse. The tumor growth was monitored twice weekly using a caliper. The treatment was started when the mean tumor size reached approximately 150 mm³. Experiments were approved by Animal Control Committee of National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College.

Clinical samples

Gene copy numbers of 1105 breast cancer patients were downloaded from the TCGA provisional dataset using the cBioPortal database (<http://www.cbioportal.org>). Peripheral blood ctDNA and tumor tissues were collected from Department of Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, CAMS & PUMC. All ctDNA sequencing were performed before patient undergone indicated oncotherapy and the detailed method was referred to previous report (53). The study was approved by the institutional review boards of the participating centers (Approval No.16-038/1117.), and written informed consents were obtained from all the patients.

Lentiviral CRISPR-Cas9 knockout screening

In the region frequently amplified centralizing HER2 containing 221 known coding genes, 33 gene candidates were selected by their annotation of biological phenotype relevance in GeneCards database to screen their potential involvement in anti-HER2 treatment. Single-guide RNAs (sgRNAs, Table S1) targeting 33 genes were obtained from genome-scale sgRNAs library established by Feng Zhang et al (24). According to the standard CRISPR-Cas9 knockout screening procedure (24), vectors expressing sgRNAs and Cas9 were packaged into lentivirus and then transduced into tumor cells and the puromycin selection (6 mg/ml at first and then 3 mg/ml) was performed after 4-5 days of lentivirus transfection. Then, under the selection pressure of lapatinib or pyrotinib, DNA of remaining alive cells were extracted to perform high-throughput DNA sequencing on Hiseq4000 sequencer. Candidate genes that contribute to the anti-HER2 TKIs resistance were picked up according to the fold change of sgRNA abundance.

Antibodies and Immunoblotting

Whole cell protein lysates were prepared with PMSF and lysis buffer (1:200). Protein concentrations were quantified by Bradford reagent (Beyotime, P0006C-1). Then, the protein samples were load on SDS-PAGE gel at 50 µg protein per lane for separation and then were transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were blocked in 5% non-fat milk powder and probed with antibodies overnight in 4°C: HER2 (Abcam, ab214275), HER2 (Proteintech, 18299-1-AP), CDK12 (Proteintech, 26816-1-AP), mTOR, p-mTOR, Akt, p-Akt (CST, #2972, #2971, #4691, #4060), β-Actin (Sigma, A1978). Incubation of secondary antibodies conjugated to horseradish peroxidase for 1h was followed by enhanced chemiluminescence visualization.

Immunohistochemistry staining

Five µm sections of formalin-fixed, paraffin-embedded tissues were deparaffinized with xylene, rehydrated, and subjected to antigen retrieval with heated antigen-unmasking solution (1.0 mM EDTA, 0.05% Tween 20, pH 8.0). After blocking in goat serum buffer for 1 hr, primary antibodies were applied for overnight at 4 °C. Human antibodies included HER2 (1:100, Abcam, ab214275), CDK12 (1:600, Proteintech, 26816-1-AP), p-Akt (1:100, CST, #4060). For immunohistochemistry, the secondary antibodies were added in ready solutions of PV-9000 Immuno-Bridge Kit for 30 min at room temperature and then DAB staining and counterstaining with hematoxylin QS were followed. Slides were digitally scanned using the Aperio ScanScope CS Slide Scanner with a 20x objective for processing and quantification.

Growth assays and drug or inhibitor sensitivity analyses

xCELLigence RTCA system was adopted to monitor cell growth and sensitivity of breast cancer cells to lapatinib or dinaciclib in a real-time manner. After detecting the background cell index (<0.063), according to the standard instruction manual, CDK12-deficient cells or control cells were transplanted into S16 panel and standing for 30 min before being moved into RTCA station. When cells entered the logarithmic growth phase (0 time-point), lapatinib and/or dinaciclib at desired concentrations were added and the cell growth was monitored for desired time duration.

Total RNA extraction and RNA-sequencing

Total RNA was extracted from control and treated cells using trizol followed by QIAGEN RNAsasy Kit application, and quantified by the NanoDrop ND-2000c. RNA sequencing was performed on HiSeq X Ten sequencer, paired-end 150 bp run. The number of reads aligning to each transcript counted with hisat2, SAM tools and these counts were converted to Fragments Per Kilobase Million (FPKM) for standardization indicating the gene expression signatures. Differentially expressed genes (DEGs) were calculated by edgeR and DEseq2. Unsupervised clustering and Gene Ontology (GO) analyses of DEGs were performed on DAVID (<https://david.ncifcrf.gov>).

Statistical Analysis

Statistical analysis was done by GraphPad Prism 7.0. Data were presented as the means \pm standard deviation (SD). Student *t*-test was applied to assess the statistical significance. Significance of difference in means between experimental groups is represented on the graphs as follow: NS, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

Screening candidate genes related to anti-HER2 TKIs resistance via CRISPR/Cas9-based gene knockout library

To identify genes critical to HER2-targeting TKIs resistance, we analyzed gene CNVs of breast cancer samples from 1105 patients in TCGA provisonal dataset and found that there were 3 amplification hot regions on chromosome 17 among HER2-amplified breast cancers (Fig 1A and S1A). HER2 lies in the second amplification peak and only genes in this region were co-amplified with HER2 in high consistency, covering more than 200 genes upstream and downstream of HER2 locus (Fig S1B). In order to explore the gene candidates potentially contributing to the anti-HER2 TKIs resistance in the HER2-centralized co-amplification region, (22, 23), we retrieved 33 genes with known functions in cell proliferation, apoptosis and movement defined by GeneCards database from the 221 genes covered by this peak from NCBI database. We confirmed their location and co-amplification frequency with HER2 in this region (Fig 1B).

Then, we grew the cancer cells infected by the CRISPR/Cas9 sgRNA library targeting these 33 genes in the presence of lapatinib or pyrotinib. To avoid incomplete knockout of highly amplified target gene copies by CRISPR/Cas9 technique, we selected HCC38 with mildly amplified gene copies (average HER2 copy number 3.2) as the targeted cell model (Fig S1C and S1D). Under the selective pressure of lapatinib or pyrotinib, the remaining drug-resistant cells were collected to perform deep library sequencing and calculated sgRNAs abundance of each targets (24) (Fig 1C). Based on fold change and consistency of sgRNAs, only CDK12-targeted sgRNAs was found to be decreased under either lapatinib or pyrotinib treatment in more than 2/3 of the targeted sgRNAs (Fig 1C). We further analyzed the relationship between these candidate genes and relapse-free survival (RFS) in Kaplan-Meier plotter database and found that level of CDK12, along with other 9 of 33 candidates, is negatively correlated with RFS in HER2-positive subtype breast cancer patients, supporting the liability of the screening processes (Fig 1D and S1E). According to TCGA and CCLE databases, CDK12 and HER2 are strongly correlated to each other at their transcriptional levels (Fig 1E and 1F), and CDK12 also has moderate expression in HCC38 cells (Fig S1F and S1G), assuring that it's an ideal model to investigate the synergistic functions between HER2 and CDK12.

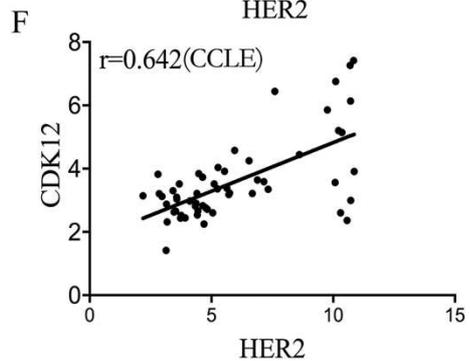
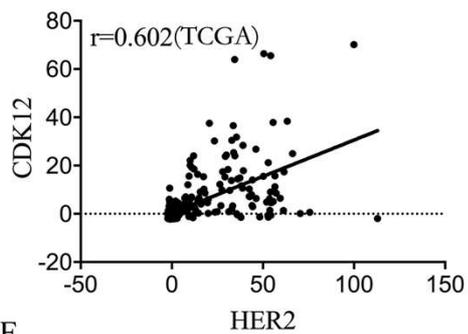
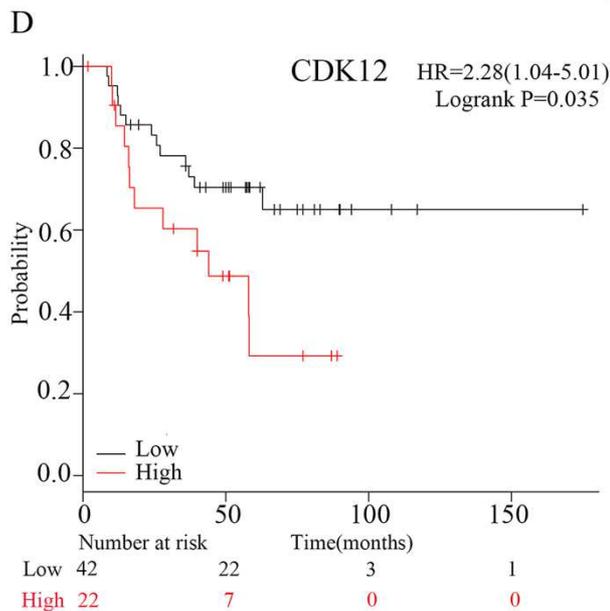
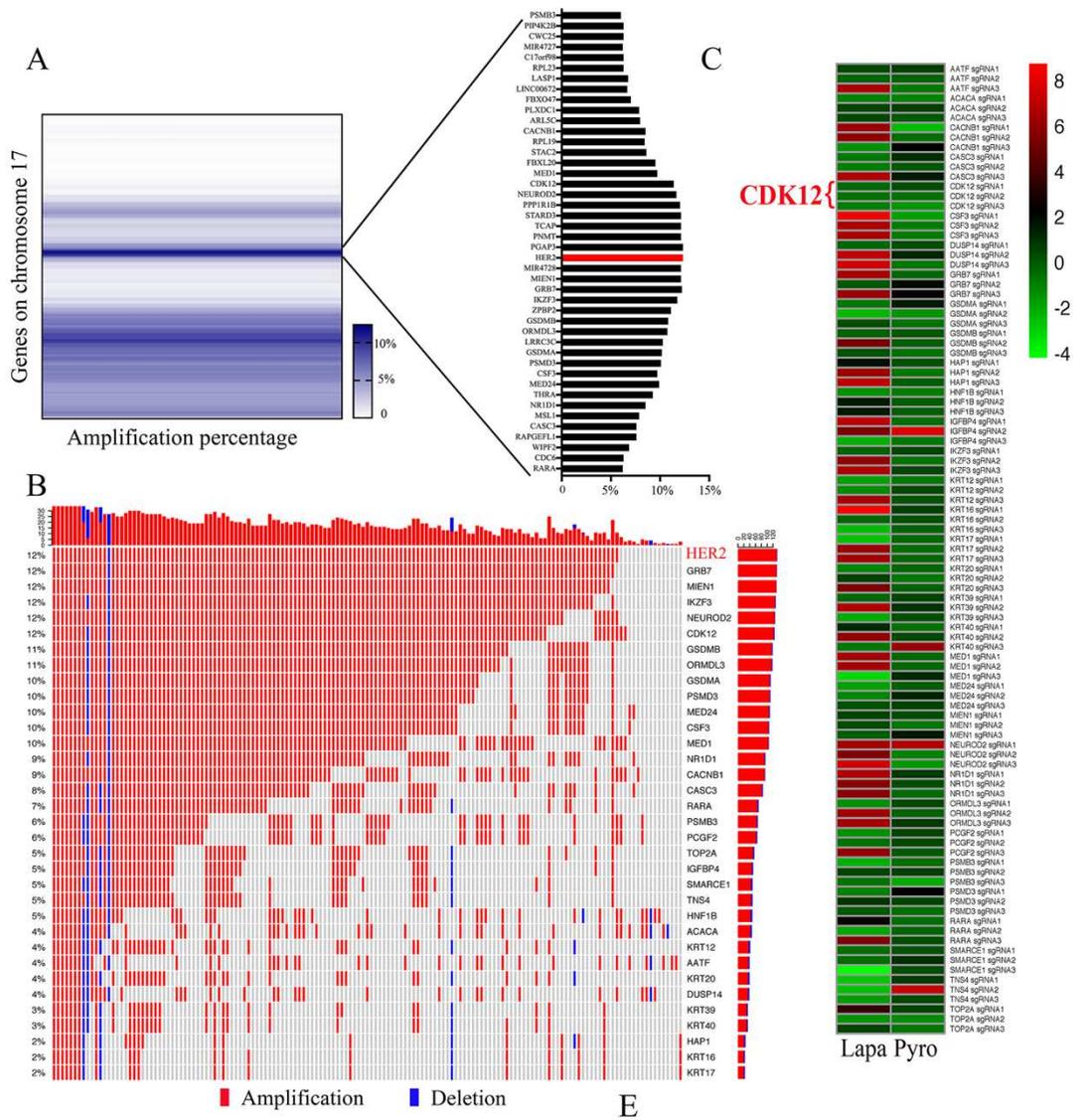


Figure 1. Screening candidate genes associated with anti-HER2 TKIs oncotherapy resistance in HER2-positive breast cancer. **A:** Amplification percentage of genes on chromosome 17. **B:** For any of the 33 candidate genes around HER2 locus, its amplification frequency in the 1105 case set was shown. **C:** Heatmap showing the fold change in sgRNAs abundance targeting 33 candidate genes after lapatinib or pyrotinib treatment compared with control group. **D:** Kaplan-Meier survival curves of HER2-positive breast cancer patient with low or high CDK12 expression. **E and F:** Correlation of HER2 and CDK12 expression at transcriptional levels in breast cancer tissues in TCGA (E) and breast cancer cell lines in CCLE databases (F). See also Fig S1.

CDK12 increased the sensitivity of HER2-positive breast cancer cells to lapatinib *in vitro*

Based on the above screening and database mining results, we hypothesized that CDK12 expression is closely associated with sensitivity of breast cancers to lapatinib therapy. To validate the screening results, we inhibited CDK12 activity by CDK12 inhibitor (Dinaciclib) (25) in two HER2 high amplification cell lines SKBR3 and MDA-MB-453 which are resistant to lapatinib, and found that CDK12 suppression drastically enhanced the sensitivity of these two cells to lapatinib (Fig 2A and 2C) in a dose-dependent manner (Fig 2B). Similarly, CDK12 inhibition also resulted in synergistically suppressed effects on lapatinib-sensitive BT474 cells (Fig 2D). Furthermore, when knocking out CDK12 (Fig S2A), HCC38 cells harboring mildly amplified gene copies (average HER2 copy number 3.2) acquired growth inhibition and increased sensitivity to lapatinib at a concentration that only suppressed SKBR3 and HCC38 cells proliferation subtly (Fig S2D). These revealed that CDK12 is potent to enhance lapatinib efficiency of HER2-positive breast cancer cells, evidenced by a robust growth suppression.

To further support the effect of CDK12 on anti-HER2 TKIs sensitivity, we expanded our conclusion to another cancer type also involving HER2 status, colon cancer. The incidence of HER2 amplification was reported to be about 1% to 6% in colorectal cancer (CRC) (26, 27) and it is predictive of shorter PFS for cetuximab treatment in patients with metastatic colorectal cancer (28). CDK12 and HER2 are also moderately positively correlated to each other across TCGA provisional colorectal cancer datasets in cBioPortal and CCLE platforms, representing their levels in cancer tissues and cell lines (Fig S2B and S2C). We treated human colorectal cancer cell lines HCT15 and HCT116 with lapatinib or/and dinaciclib to assess the cancer cell proliferation ability. In line with our results in breast cancer, we found that CDK12 inhibition dramatically increased lapatinib efficiency both in HCT15 and HCT116 cells at a level of lapatinib otherwise producing limited growth suppression by single usage (Fig 2E and S2E). To comprehensively understand the role of CDK12 in lapatinib sensitivity, we also assayed CDK12 function in mouse colorectal cancer model, CT26 cells, given its robust expression of CDK12 (Fig S2B). Likewise, CDK12 knockdown or inhibition in CT26 cells delivered high sensitivity to lapatinib treatment, reconfirming that CDK12 is a potent modulator of lapatinib antitumor activity (Fig S2F and S2G). Therefore, we

propose that HER2/CDK12 dual inhibition as a potential treatment strategy may warrant further clinical benefits for HER2-positive breast cancer patients.

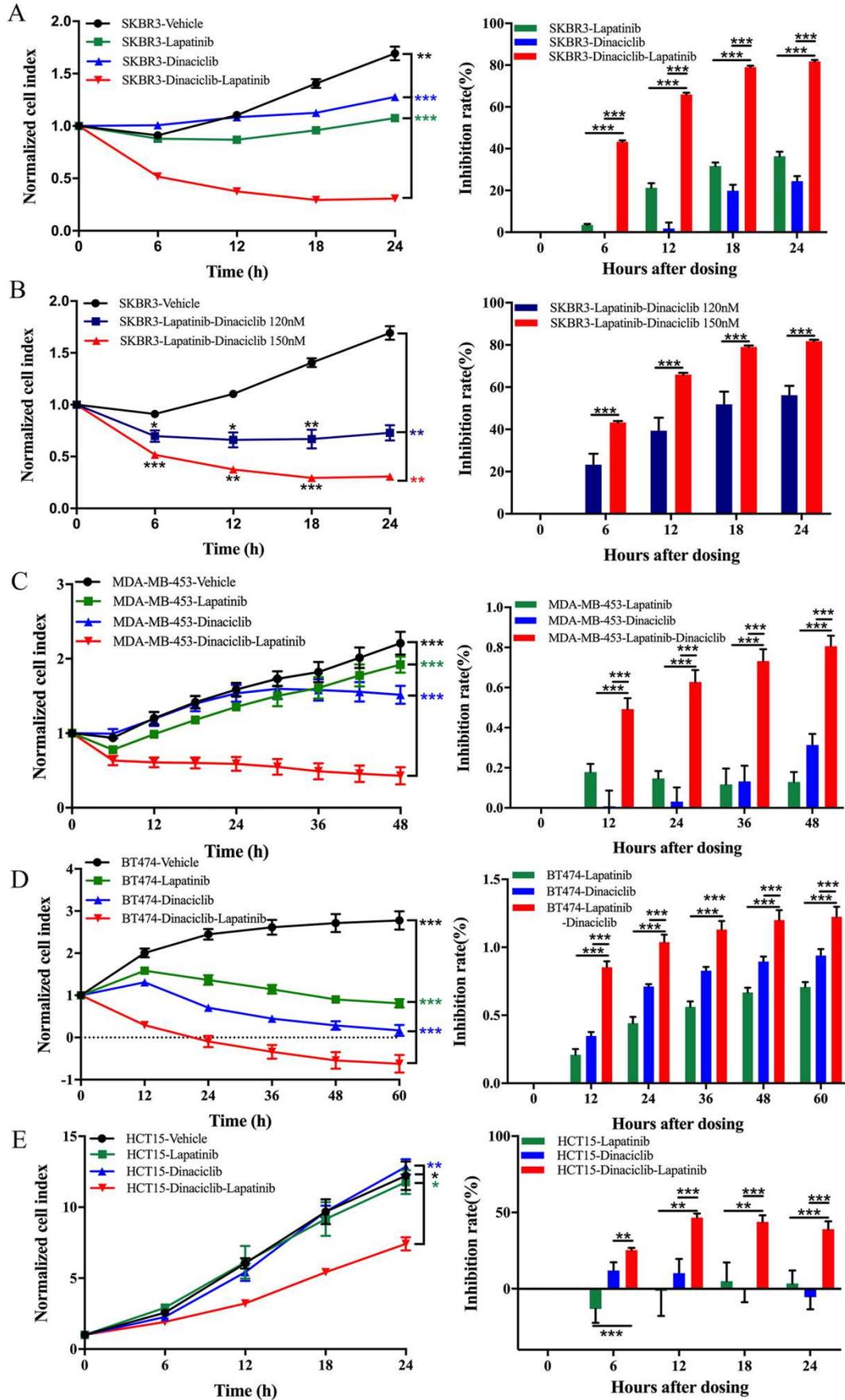


Figure 2. Inhibition of CDK12 sensitized HER2-positive cancer cells to lapatinib in a dose-dependent manner. **A:** Effects of lapatinib (5 μ M) and/or dinaciclib (150 nM) on proliferation of SKBR3 cells. **B:** Lapatinib (5 μ M) combined with dinaciclib (120 nM or 150 nM) significantly suppressed SKBR3 proliferation in a dose-dependent manner. **C and D:** Proliferation of lapatinib-resistant cells (MDA-MB-453) and -sensitive cells (BT474) were monitored by Real-Time Cellular Analysis (RTCA) with lapatinib (5 μ M) and/or dinaciclib (100 nM). **E:** RTCA assays of colorectal cancer cells under pressure of indicated drugs (lapatinib: 5 μ M; dinaciclib: 10nM). Inhibition rate and statistical significance were calculated based on normalized cell index of indicated time points (mean \pm SD, * P < 0.05; ** P < 0.01; *** P < 0.001, two-side Student's t -test). See also Fig S2.

CDK12 inhibition increased the efficacy of anti-HER2 TKIs lapatinib *in vivo*

To provide *in vivo* evidence for the effect of CDK12 inhibition on HER2-positive breast cancer treatment, we subcutaneously implanted HCC38 cells into nude mice to establish the breast cancer xenograft models. Consistent with *in vitro* findings, CDK12 inhibition combined with lapatinib treatment significantly suppressed breast cancer progression, reflected by decreased tumor volume compared with monotherapy or control groups, indicating the enhanced anti-tumor effect of dual treatment by dinaciclib and lapatinib (Fig 3A). Intriguingly, CDK12 repression combined with lapatinib did not markedly affect mice body weight, suggesting that no significant side effect was added by CDK12 inhibition combined with lapatinib (Fig 3B).

Similarly, we constructed CT26 transplanted mouse tumor model followed by dinaciclib or/and lapatinib therapy. In line with results obtained in HCC38 xenograft mice, inhibition of CDK12 markedly rendered CT26 cells highly sensitive to lapatinib treatment compared with control group *in vivo* indicating that CDK12 plays an essential role in HER2-mediated tumor progression, evidenced by interfering lapatinib sensitivity both in breast cancers and colorectal cancers. The tumor volume and tumor weight in combination therapy group were significantly lower than those in other groups (Fig 3C, 3D and 3F), while without significantly increased side effects (Fig 3E). Collectively, we concluded that CDK12 plays an essential role in mediating lapatinib sensitivity in HER2-positive breast cancers, which can even be extended to colorectal cancers.

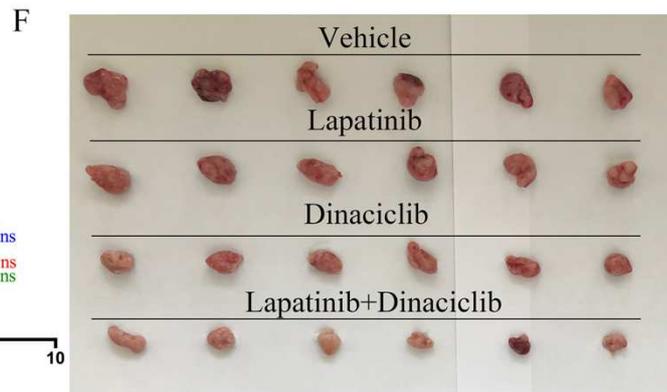
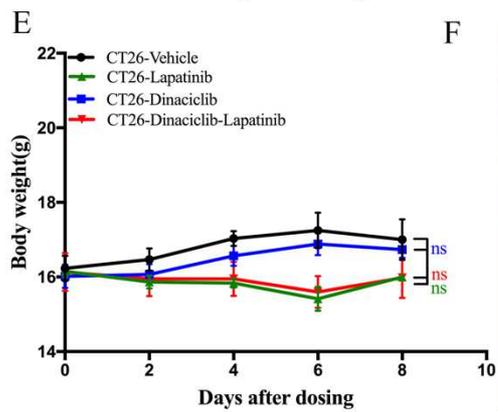
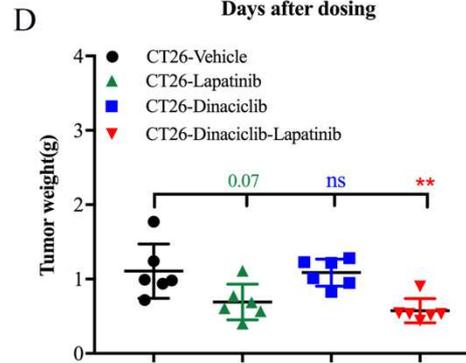
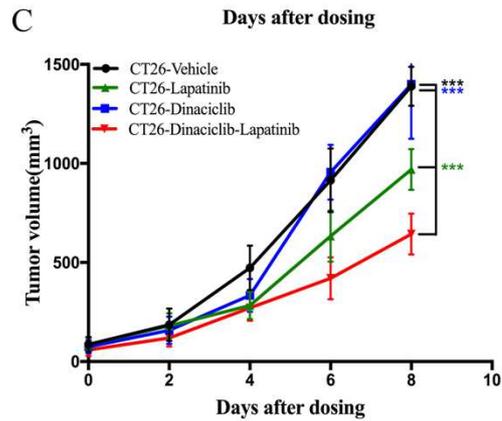
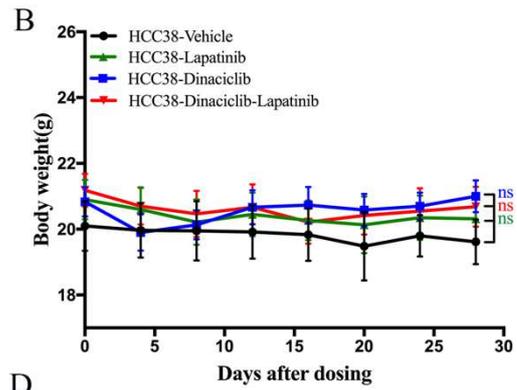
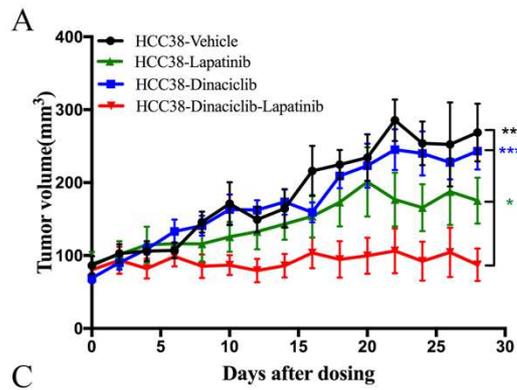


Figure 3. CDK12 inhibition suppressed lapatinib-resistant tumor progression *in vivo*

A and B: Tumor volume of HCC38 cell-derived xenografts treated with lapatinib (75mg/kg), dinaciclib (8mg/kg) or both. Tumor size (**A**) was measured in two dimensions using a caliper, and the volume was expressed in mm³ using the formula: $V = 0.5 a \times b^2$ where a and b are the long and short diameters of the tumor, respectively. (n=6, mean \pm SD, ns: not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, two-side Student's *t*-test). Body weight (**B**) of HCC38 xenografts in each group. **C-F:** Tumor volume (**C**) of CT26-derived xenografts receiving indicated reagents treatment. Tumor weight (**D**) of CT26-derived xenografts. Body weight of CT26-derived xenografts (**E**) and tumor images (**F**) were shown (n=6, mean \pm SD, ns: not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, two-sided Student's *t*-test).

CDK12 suppression increased lapatinib sensitivity via repressing PI3K/AKT activation

To investigate how CDK12 inhibition improves lapatinib efficiency, we performed RNA sequencing in CDK12-deficient HCC38 cells and found that 165 genes were up-regulated and 442 genes were down-regulated significantly (Fig 4A and S3A). Differentially expressed genes were significantly enriched in PI3K/AKT pathway, a key downstream pathway of HER2, by KEGG pathway clustering (Fig 4B). Thus, we hypothesized that CDK12 depletion enhanced efficiency of lapatinib treatment on HER2-positive breast cancers by suppressing PI3K/AKT activity or abrogating the activation of PI3K/AKT pathway upon lapatinib resistance. According to the RNA-seq data we found that the genes enriched in positively regulating PI3K signaling were significantly down-regulated by CDK12 knockout, like NTRK2 (29), UNC5B (30) and KIT (31) (Fig. 4C and S3B). Besides, we downloaded all 534 genes (Pearson correlation coefficient ≥ 0.5 or ≤ -0.5) correlated to CDK12 from cBioPortal database and, consistently, CDK12-associated genes were also clustered on PI3K/AKT pathway (Fig 4D).

Validating the clustering results through Western blot assay revealed that both key players of p-AKT and p-mTOR in PI3K/AKT pathway were significantly inhibited upon CDK12 knockdown or knockout (Fig 4E). These results were also reconfirmed in CT26 cells by silencing CDK12 with shRNA (Fig S3E). What's more, we established lapatinib-resistant CT26 cell line by maintaining the cells in medium with escalating lapatinib concentrations for several generations and found a recovered p-AKT and p-mTOR activity comparing with lapatinib-treated parental cells (Fig S3E). Supporting the notion that CDK12 inhibition suppresses PI3K/AKT to attenuated cell growth, the combination treatment using lapatinib and PI3K inhibitor also markedly repressed proliferation of MDA-MB-453 and CT26 cells in a dose-dependent manner (Fig S3C-H). Above results validated the underlying mechanism of CDK12 inhibition to rescue lapatinib sensitivity by blocking the activity of PI3K/AKT or reactivation of PI3K/AKT upon lapatinib resistance in HER2-positive cancer cells.

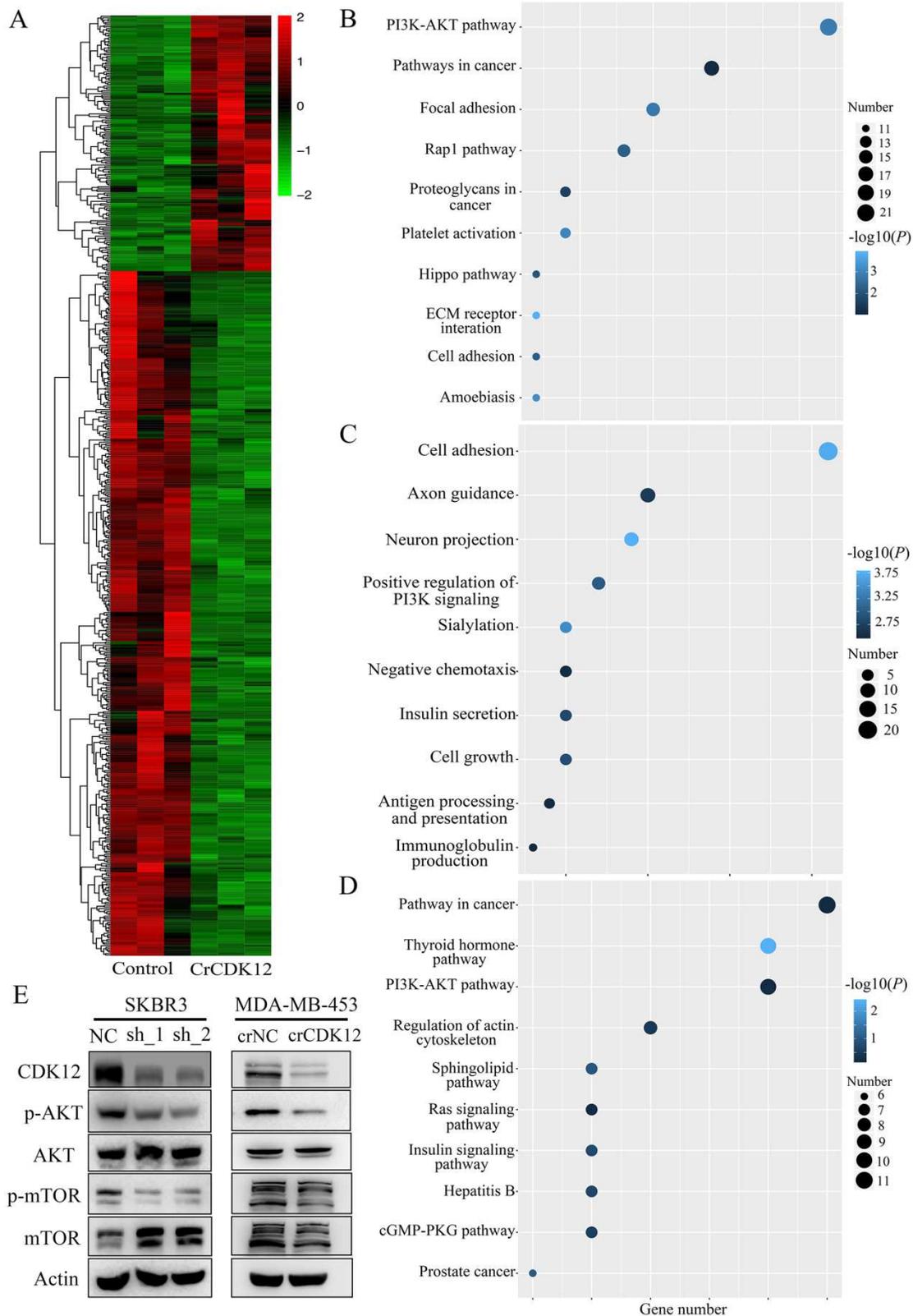


Figure 4. CDK12 suppression reversed lapatinib resistance via repressing PI3K/AKT activation. **A:** Heatmap of differentially expressed genes (DEGs) after CDK12 knockout ($P < 0.05$, $\log_2FC > 1$ or < -1). **B:** Bubble chart of KEGG pathway analysis for all DEGs using DAVID. **C:** Biological process analysis of all down-regulated genes upon CDK12 knockout. Genes positively regulating PI3K signaling were suppressed. **D:** KEGG pathway analysis of CDK12 associated genes from TCGA 1105 breast cancer patient dataset (Pearson correlation coefficient ≥ 0.5 or ≤ -0.5). **E:** Western blotting assay of PI3K/AKT/mTOR pathway after CDK12 knockdown (left) or knockout (right). See also Fig S3.

CDK12 inhibition repressed tumor development of CDX, PDO and PDX tumor models that are resistant to lapatinib

To assess therapeutic benefits, we further constructed HCC38-derived lapatinib-resistant xenograft models (passaged in nude mice with consistent lapatinib treatment for six generations). Compared with parental CDX, lapatinib-resistant CDX harbored higher level of HER2 and CDK12, and the expression of p-AKT also increased after inducing lapatinib resistance (Fig S4A). Then, we treated HCC38-derived lapatinib-resistant xenograft models with dinaciclib or/and lapatinib and observed dramatically decreased tumor volume and tumor weight in the combined treatment group comparing to the control group (Fig 5A, S4B and S4C). Accordingly, we also saw a significant repression of p-AKT and CDK12 in the combined treatment group (Fig 5B and S4D).

Trying to evaluate the effects in models closer to naïve tumor microenvironment, we established patients-derived organoids (PDO) in 3D culture models harboring resistance to lapatinib from five HER2-positive breast patients. The PDO models were treated with single or combinatory drugs (Fig 5C and S4E). CDK12 repression combined with lapatinib produced consistent and inspiring anti-tumor effect among all five cases of organoids. Similarly, we also found that there was no marked difference between PI3K inhibitor (PI103) monotherapy group and control group, while the PDOs growth were significantly inhibited in PI103 and lapatinib combination treatment group reconfirming PI3K/AKT serves as a downstream signaling pathway to enhance lapatinib therapeutic effects mediated by CDK12 inhibition (Fig 5C and S4E).

To further demonstrate the clinical relevance of our findings *in vivo*, we established PDX models, with resistance to lapatinib, derived from HER2-positive breast cancer patients, in which lapatinib single treatment led to no significant efficacy comparing to control group. CDK12 inhibition prominently sensitized lapatinib-resistant PDX tumors to lapatinib, demonstrated by the significant tumor growth suppression in dinaciclib and lapatinib combination treatment group (Fig 5D and 5E). We carried out HE and IHC staining for tumors tissues from PDX mouse and found that there were more tumor necrosis and a significantly lower p-AKT level in combined treatment group compared with vehicle or monotherapy groups, symboling enhanced anti-tumor effects and attenuated PI3K/AKT signaling (Fig 5F).

Collectively, our results suggested that CDK12 inhibition enhances lapatinib therapeutic efficacy by down regulating PI3K/AKT pathway in patient-derived

preclinical tumor models of HER2-positive breast cancer. Collectively, our results suggested that CDK12 inhibition enhances lapatinib therapeutic efficacy by down regulating PI3K/AKT pathway in patient-derived preclinical tumor models of HER2-positive breast cancer.

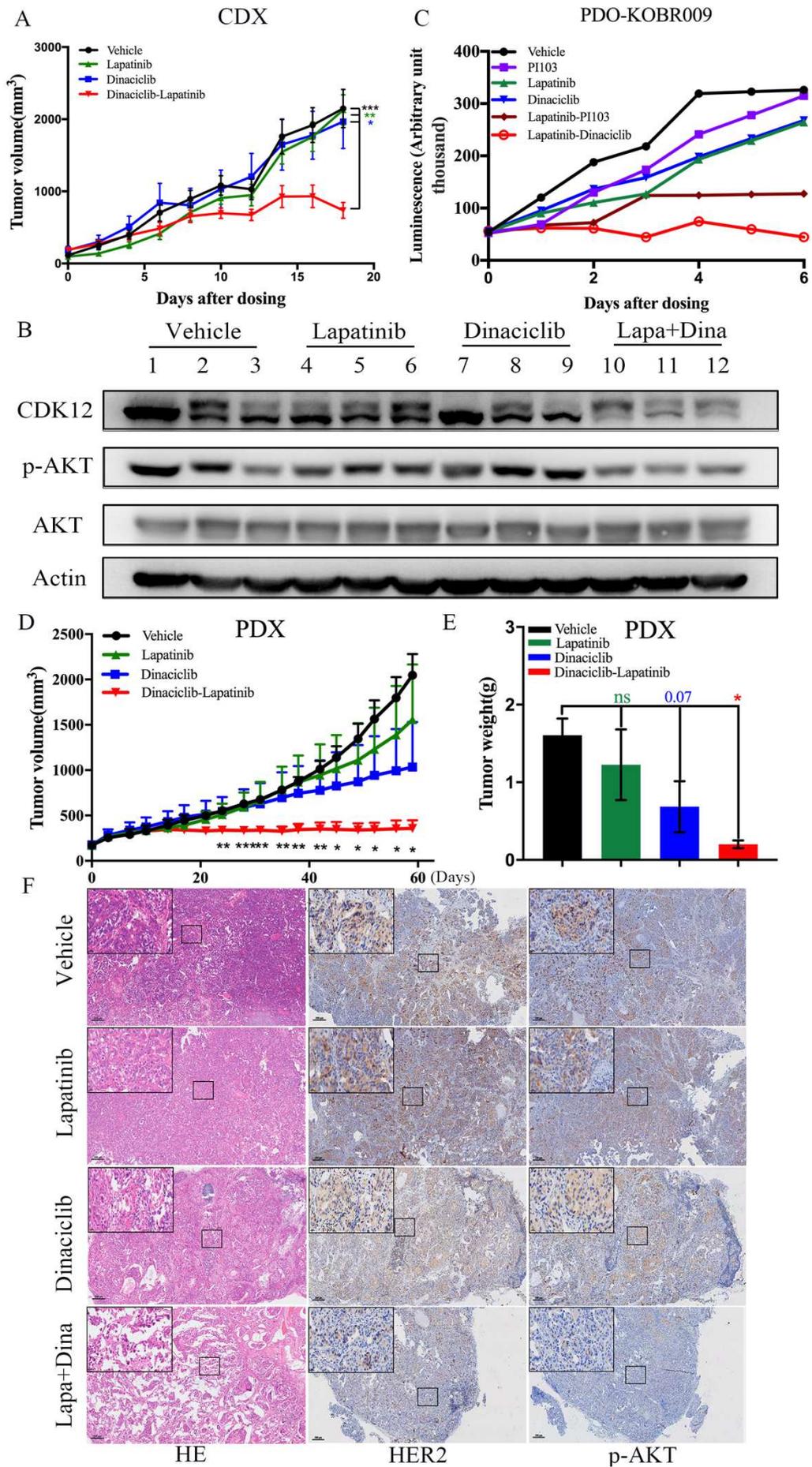


Figure 5. CDK12 inhibition repressed tumor progression of CDX and PDX models resistant to lapatinib. **A:** Tumor progression of HCC38 CDX with induced lapatinib resistance (n=6, mean \pm SD, * P < 0.05; ** P < 0.01; *** P < 0.001, two-sided Student's t -test). **B:** Representative images show western blotting for p-AKT in variously treated HCC38 CDX mice. **C:** HER2-positive breast cancer patient-derived organoids (PDO) were treated with different drug combinations (lapatinib, 5 μ M; dinaciclib, 50 nM; PI103, 5 μ M). **D** and **E:** Tumor volume (**D**) and tumor weight (**E**) of PDX models that resistant to lapatinib treatment (n=3, lapatinib, 50 mg/kg; dinaciclib, 8 mg/kg; * P < 0.05; ** P < 0.01, *** P < 0.001, two-sided Student's t -test). **F:** Representative images show HE staining and IHC staining for HER2 and p-AKT in PDX models. Scale bar, 20 μ m and 200 μ m. See also Fig S4.

HER2-positive breast cancers with CDK12-amplification are resistant to HER2-targeted treatment.

To further clinically confirm that CDK12 amplification or high expression is associated with the response of HER2-positive subtype breast cancers to anti-HER2 treatment in a clinical cohort, we retrieved and assayed clinical treatments and genomic sequencing data of 1918 breast cancer patients in Memorial Sloan Kettering Cancer Center (MSKCC) dataset. To rule out the effects of endocrine therapy on HR positive patients, we excluded Luminal B type (HER2+/HR+) patients and focused on HER2-positive (HER2+/HR-) subtype breast cancer patients who undergone anti-HER2 therapy containing 77 patients with CDK12 amplification (CDK12-Gain) and 132 patients without CDK12 amplification (Non CDK12-Gain). Based on treatment regimens and lines of therapy in metastatic settings, we matched 77 pairs of patients for CDK12-amplified and non-CDK12 amplified group, respectively, for their PFS duration analysis. Kaplan-Meier survival analysis showed that HER2-positive subtype breast cancer patients harboring CDK12 amplification are poorly responsive to HER2-targeted treatment (Fig 6A). The median PFS are 4.4 months in CDK12-amplified group and 7.1 months in non-CDK12-amplified group (HR 2.39 [95% CI 1.64–3.50]; $P < 0.0001$). By establishing Cox-regression proportional hazards model and performing multivariable analysis, we found that CDK12 is an independent factor linked to anti-HER2 therapy efficiency in HER2-positive subtype patients (Fig 6B and Table S2, HR 3.44 [95% CI 2.20–5.40]; $P < 0.001$). Above all, our results indicated that CDK12 is a potential driver for HER2-targeted treatment resistance and dual inhibition of HER2/CDK12 will prominently benefit the outcomes of HER2-positive breast cancer patients by sensitizing or re-sensitizing the tumors to anti-HER2 treatment.

Circulating tumor DNA (ctDNA) is a promising blood-based biomarker to monitor disease status of patients with advanced cancers. ctDNA detection showed profound clinical benefits as an alternative method for screening clinically targetable mutations for the assessment of response to oncotherapy. We recruited and comprehensively analyzed clinical treatments and CNV data of 417 breast cancer patients who undergone ctDNA sequencing in our institute. Excluding HER2-negative patients and patients treated with non-HER2 targeted oncotherapy, we recruited 107 HER2-positive breast cancer patients who received anti-HER2 therapy after ctDNA detection (Fig S5A). According to the ctDNA sequencing results, there are 28 and 79 patients with or without

CDK12 amplification, respectively. As expected, we found a shortened PFS and poorer prognosis in CDK12-gaining patients compared with patients without CDK12 amplification (Fig 6C). The median PFS (mPFS) are 4.3 months in CDK12-amplified subset and 6.9 months in non-CDK12-amplified group (HR 2.26 [95% CI 1.32-3.86]; $P=0.0028$). Besides, there are 12 patients with CDK12 amplification and 32 patients without CDK12 amplification in our HER2-positive subtype group. Similarly, we found a shortened mPFS in CDK12-gaining group (Fig 6D, 4.0 months versus 5.3 months, HR 2.64 [95% CI 1.12-6.23]; $P=0.027$). Analysis combining CDK12 status, HR status and treatment regimens and lines, showed that CDK12 is an independent factor significantly related to HER2 targeted treatment (Table S3, HR 1.89 [95% CI 1.203-2.970]; $P=0.006$). However, the trend was not significant for synergistic effects of CDK12 to anti-HER2-targeted treatment in Luminal B type (HER2+/HR+) breast cancer patients and more complexed hormone signaling as mixed factors may be responsible for this discrepancy (Fig S5B). Larger cohorts and more mechanism investigations are needed to clarify this inconsistency. Additionally, immunohistochemical staining showed that HER2-positive breast cancers resistant to anti-HER2 treatment (PFS \leq 5 months) expressed higher CDK12 protein than those sensitive to anti-HER2 treatment (PFS \geq 12 months) (Fig 6E). Therefore, in HER2-positive breast cancer subtype, higher CDK12 level generally connected to poorer anti-HER2 treatment response and shorter PFS. We displayed as an example the CT scan images of a HER2-positive case with CDK12 overexpression, showing the disease progression during Trastuzumab or Pyrotinib treatment (Fig 6F, S5C and S5D). Therefore, the CDK12-linked anti-HER2 treatment sensitivity also applied to blood-based ctDNA sequencing, as a marker to predict treatment sensitivity.

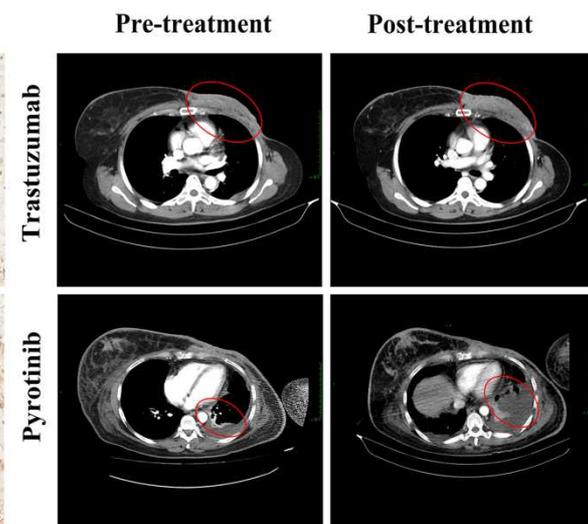
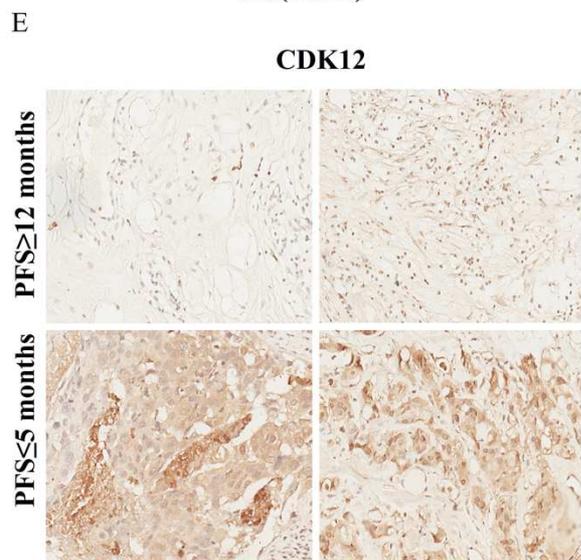
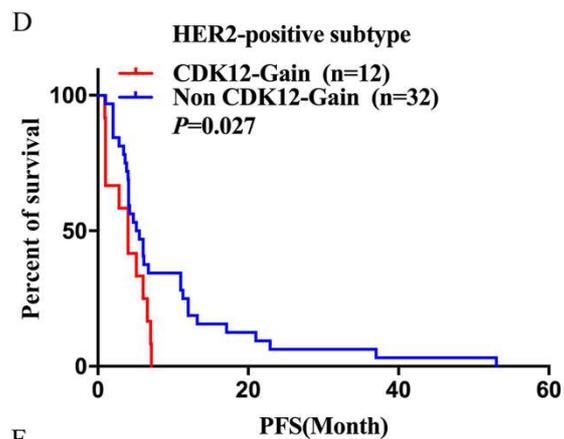
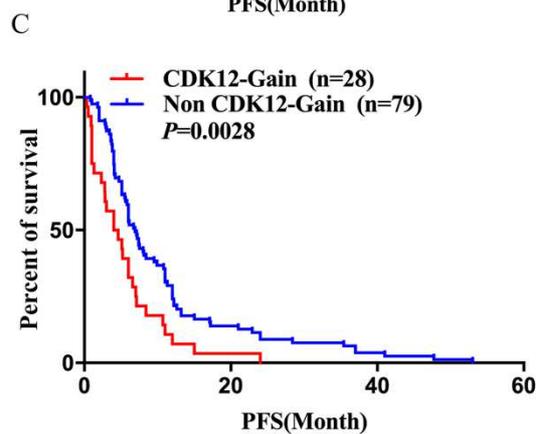
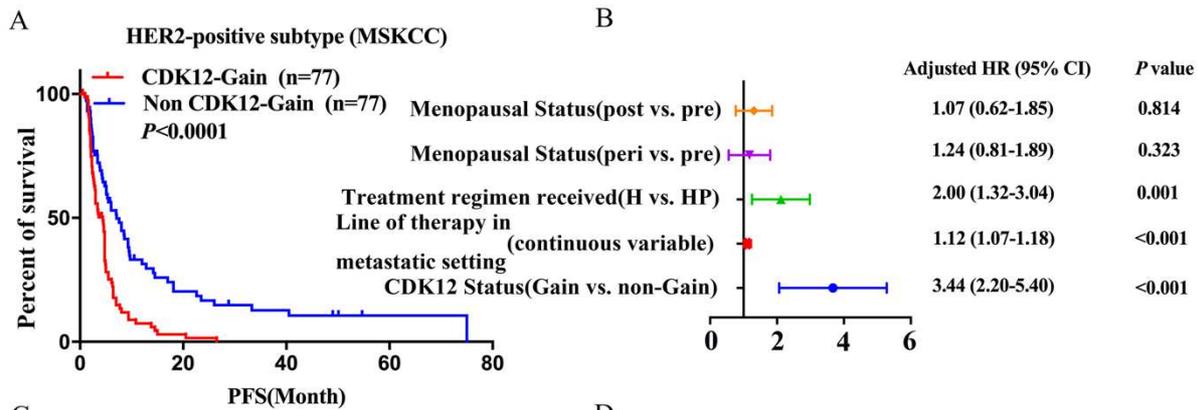


Figure 6. CDK12-amplified HER2-positive breast cancer patients are resistant to HER2-targeted treatment. **A:** Kaplan-Meier survival analysis of HER2-positive subtype breast cancer patients (HER+/HR-) in Memorial Sloan Kettering Cancer Center (MSKCC). **B:** Weighted multivariable Cox proportional hazards regression analyses of MSKCC data are shown for PFS. The hazard ratio (HR) compares the CDK12-gain vs the CDK12 non-gain status, and adjusted by menopausal status, treatment regimen received and line of therapy in metastatic setting (H, trastuzumab; HP, trastuzumab-pertuzumab). **C:** Kaplan-Meier survival analysis of all 107 HER2 positive patients, including HR- or HR+, who received anti-HER2 therapy after ctDNA detection. Patients were dichotomized by CDK12 gene status. **D:** Kaplan-Meier survival analysis of 44 HER2-positive subtype patients (HER2+/HR-). **E:** IHC assays for CDK12 expression in HER2-positive sensitive (PFS \geq 12 months) and drug resistant (PFS \leq 3 months) groups, respectively. **F:** Representative images from breast cancer patients with HER2/CDK12 co-amplification detected by peripheral blood ctDNA sequencing whose cancers were resistant to anti-HER2 oncotherapy. CT scan shows the thickness of chest wall with metastasis to the soft tissue before and after HER2-targeted treatment. See also Fig S5.

Discussion

TKIs oncotherapy, lapatinib as a typical drug, is an important advance for HER2-positive breast cancer treatment, however, intrinsic and acquired drug resistance is still the intractable clinical challenge. Multiple mechanisms are involved in the occurrence of lapatinib resistance, including RTKs or other intracellular kinases recoveries which are usually acquired during TKIs treatment. Although there are clinical strategies in development to overcome lapatinib resistance (32), no study has been focused on the genomic or epigenetic alterations surrounding HER2 locus, which much more frequently come together with HER2 amplification or activation than abnormalities scattering on other locations. Thus, elucidating the mechanism of lapatinib resistance, based on the intrinsic genome aberrations like mutations or co-amplifications frequently accompanying HER2 abnormality, is a quite important and very applicable strategy to improve the efficacy or discover useful biomarkers predicting the prognosis of anti-HER2 treatment. (33).

CNV alteration is a very common biological event during cancer development and progression as well as in treatment processes. CNV usually involves more than one gene, while previous studies mainly focused on one typical oncogene or tumor suppressor gene in one region. There are potential complex interactions between the co-amplified or co-deleted genes affected by a CNV event, which synergistically interfere with cancer treatment efficacy. Like *ACTL6A*, it frequently co-amplified with *p63* in squamous cell carcinoma and physical interaction between them controlled a transcriptional program which drives YAP activation, regenerative proliferation and poor prognosis (34). Reportedly, HER2 is one of the genes most commonly affected by copy number amplification event (35, 36). Bioinformatics analysis of copy number abnormalities in HER2-positive patients from TCGA dataset indicates that HER2 is co-amplified at high frequency with 221 genes covering the upstream and downstream of its locus. Previous reports have mentioned that the co-amplification of HER2 with EGFR (37), FGFR1 (38) or uPAR (39) promoted tumor development and predicted poor clinical outcome, but they were not on the same amplicon with HER2 and only co-amplified with HER2 at a very low chance. In order to identify genes related to anti-HER2 TKIs resistance with high occurrence, we retrieved the 221 genes covering the amplicon around HER2 from NCBI database, and chose 33 genes related to cellular

proliferation, apoptosis, invasion or metastasis referring to the GeneCards database for further screening. By performing CRISPR/Cas9 knockout library screening under lapatinib or pyrotinib pressure, we found CDK12-depleted cells displayed enhanced sensitivity to anti-HER2 reagents on HER2-positive background.

CDK12 is a transcriptional cyclin-dependent kinase (CDK) with known roles in transcriptional elongation, mRNA processing, proliferation and development. It composes a complex with cyclin K to regulate cellular responses to DNA damage, heat shock and stress (40). Analysis of CDK12 and HER2 across TCGA provisional datasets in cBioPortal revealed a positive correlation between each other regardless of copy number or transcription level. It has been previously discovered that CDK12 was co-amplified with HER2 in breast cancer and lung cancer (20, 41), while there was no further functional verification and mechanism illustration. Until recently, Rusan et al reported that CDK7/12 inhibition in combination with erlotinib may serve as a therapeutic paradigm for enhancing the effectiveness of targeted therapies in bladder cancer RT112, NSCLC PC9 cells (42).

In our study, both depletion and inhibition of CDK12 significantly suppressed the growth of HER2 positive cancer cells as well as tumor progression of breast cancer cells *in vitro* and *in vivo* assays. Given the incidence of HER2 amplification and positive correlation between CDK12 and HER2 in colorectal cancer (26, 27), we performed confirmative assays on colorectal cancer cells and achieved the concordant conclusion that CDK12 inhibition enhanced the sensitivity of colorectal cancer cells to lapatinib. In order to accurately recapitulate tumor tissue architecture and function, we developed PDO, and PDX models, which are promising tumor models not only for understanding the biology but also for testing drug efficacy *in vitro* and *in vivo*, respectively (42, 43). Compared with traditional 2-dimensional (2D) cultures lacking real cell-matrix interaction episode as *in vivo*, organ-like microenvironment 3D cell culture conformations have been granted as a promising model to mimic, in a micro-scale, the cellular functions and interactions presented in whole tumor *in vivo* [29, 30]. Consistently, CDK12 inhibition increased the sensitivity of HER2-positive breast cancer PDO and PDX mouse models to lapatinib. Further, we collected 417 breast cancer cases undergone ctDNA analyzing. Given the reports that acquired resistance to lapatinib can be resulted from overexpression of estrogen receptor (ER) and lapatinib promotes the transcription of ER-regulated genes (44), we screened out a total of 44

HER2-positive subtype (HER2+/HR-) breast cancer patients undergone HER2-targeted oncotherapy. We found HER2-positive breast cancer patients harboring CDK12 amplification by ctDNA detection are poorly sensitive to anti-HER2 treatment, indicating CDK12 is a potential driver of lapatinib resistance and promising target to settle this clinical challenge. Similarly, after controlling the impact of treatment options and lines, we draw the same conclusion for CDK12 amplification and the PFS relationship in 77 pairs of HER2-positive breast cancer patient cohort in MSKCC. Besides, HER2-positive breast cancer patients with lower CDK12 expression tend to benefit more from anti-HER2 treatment than those with higher CDK12 level. Interestingly and importantly, the conclusion for the role of CDK12 in anti-HER2 TKIs treatment is not consistent when it comes to Luminal B type (HER2+/HR+) patients, indicating that CDK12 is an essential factor, besides HR status, which should be taken into account when applying anti-HER2 targeted therapy and it may interactively work with HR. Overall, these findings validate and expand the therapeutic potential of CDK12 inhibition in the treatment of breast cancer patients with resistance to anti-HER2 oncotherapy.

To dissecting the molecular mechanisms underlying the anti-tumor effects by CDK12 down-regulation, we performed transcriptome sequencing and GO/KEGG analysis and revealed that CDK12 depletion markedly suppressed the activation of PI3K/AKT signaling pathway. PI3K/AKT pathway is well studied and clearly implicated in the tumor proliferation, metastasis and drug resistance of breast cancer, and is a key candidate to be targeted during cancer therapy (43, 44). As reported, oncogenic hyperactivation of PI3K partly resulted from HER2 amplification and phosphorylated-AKT are often detected in many cancer types and especially at high frequencies in breast cancer patients (45-47). Upon activation, AKT serves as a tumorigenesis driver to phosphorylate downstream substrates such as mTOR/Raptor complex 1 (mTORC1) and further to promote tumor progression and resistance to apoptosis (48-50). Moreover, it has been reported aberrant activation of PI3K-AKT signaling is one of the mechanisms for the resistance to anti-HER2 oncotherapy in HER2-positive breast cancer patients (51, 52). From our RNA-seq data, the positive regulators (*NTRK2* and *UNC5B*) of PI3K activity and PI3K downstream gene (*KIT*), largely decreased upon CDK12 depletion. KIT is a type III RTK operating in cell signal transduction, which can be overexpressed by PI3K activation and lead to imatinib resistance (31). Herein,

knockdown or inhibition of CDK12 significantly decreased p-AKT and p-mTOR level, and consistent results were found in breast cancer tissue-derived mice xenografts. This indicates CDK12 inhibition could be an effective strategy to overcome lapatinib resistance to anti-HER2 therapies via suppressing PI3K/AKT activity, but how CDK12 ablation inhibited PI3K/AKT activation remains to be further studied in the future.

Conclusion

In conclusion, by CRISPR/Cas9 knockout library screening, we identified genes related to anti-HER2 treatment resistance. Among the identified candidates, CDK12 depletion negatively regulates PI3K/AKT signaling activity. Knocking down CDK12 expression suppresses the progression of HER2-positive breast cancers that were resistant to lapatinib oncotherapy or further increases treatment efficiency, suggesting that dual targeting of HER2 and CDK12 could benefit HER2-positive breast cancer patients. Further clinical trials are warranted to confirm and optimize dose and treatment conditions for the combinational oncotherapy.

Abbreviations

TKIs: Tyrosine kinase inhibitors

PDO: patient-derived organoids

CDX: cell-derived xenograft

PDX: patient-derived xenograft

PFS: progression-free survival

mPFS: median PFS

RFS: relapse-free survival

HER2: human epidermal growth factor receptor 2

HR: hormone receptors

CDK12: cyclin-dependent kinase 12

IPA: intronic polyadenylation

sgRNAs: Single-guide RNAs

PVDF: polyvinylidene difluoride

FPKM: Fragments Per Kilobase Million

DEGs: Differentially expressed genes

Declarations

Ethics approval and consent to participate

The study was approved by the institutional review boards of the participating centers (Approval No.16-038/1117.), and written informed consents were obtained from all the patients. Experiments were approved by Animal Control Committee of National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

HL.Q., F.M., HJ.W. and QM.Z. designed and supervised the study. H.L. performed most in vitro and in vivo experiments with the significant assistance of CX.L., JY.Z., T.W., P.N. and F.L. HJ.W., H.L. and JS.W. prepared the writing and organization of manuscript. F.M. DK.X. and ZB.Y. collected and inspected human patient samples, and analyzed ctDNA data and MSK database. All authors read and contributed to the

manuscript polish and approved the submission.

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Supplementary Materials

Fig S1. Candidate genes screening and analysis for their expression and survival relevance.

Fig S2. CDK12 effected on lapatinib sensitivity in HER2-positive colorectal cancer cell lines.

Fig S3. CDK12 suppression inhibited PI3K/AKT activation and proliferation in colorectal cancer cells, facilitating lapatinib efficacy.

Fig S4. CDK12 inhibition repressed tumor development of CDX and PDX that are resistant to lapatinib.

Fig S5. HER2-positive subtype breast cancer patients with CDK12 amplification are insensitive to HER2-targeted oncotherapy.

Table S1. Single-guide RNAs targeting 33 candidate genes.

Table S2. Cox regression analyses of the associations between the PFS and CDK12 status and clinical characteristics in HER2+/HR- breast cancer patients of MSKCC.

Table S3. Cox regression analyses of the associations between the PFS and CDK12 status and clinical characteristics in our HER2+/HR- breast cancer patients' cohort.

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Figures

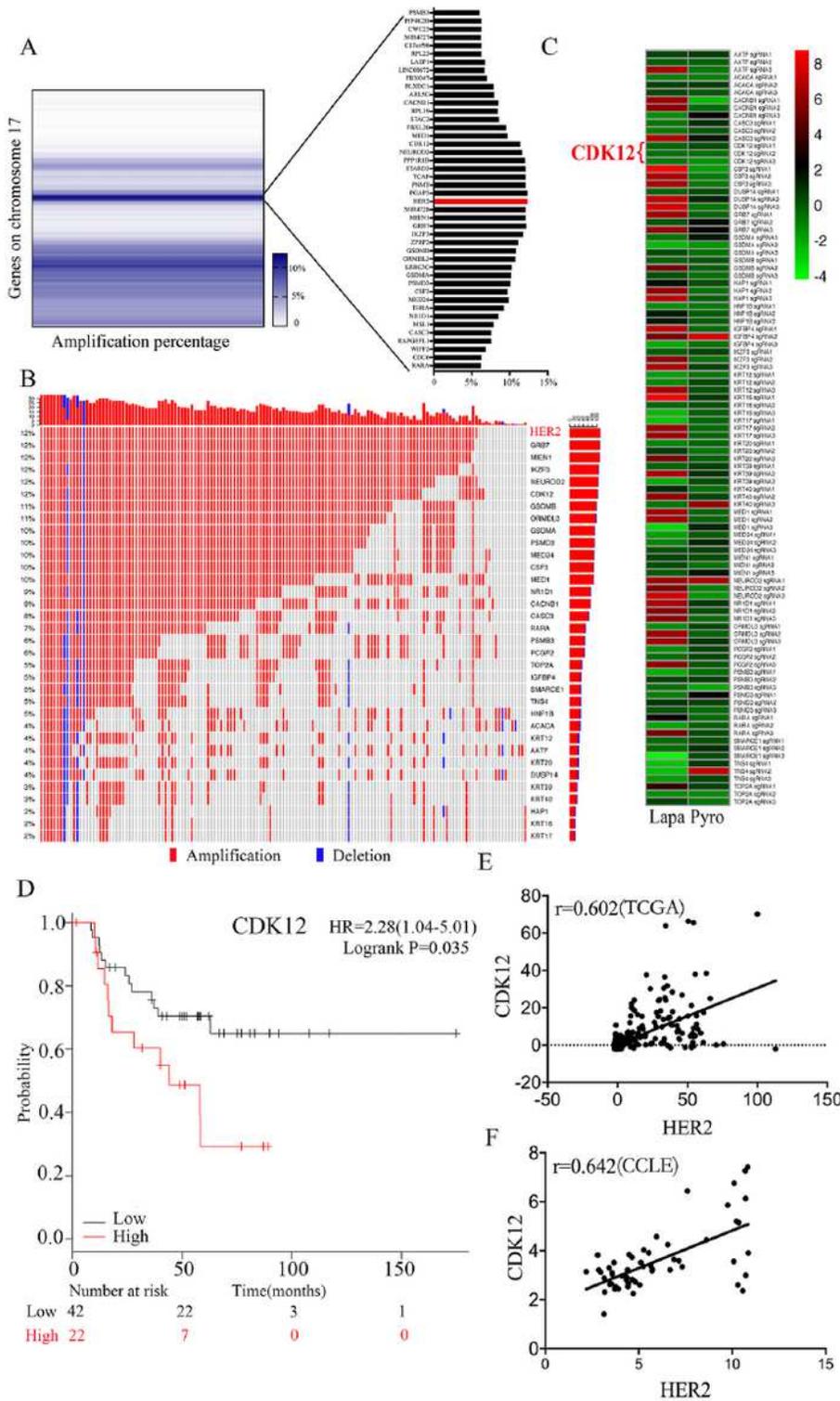


Figure 1

Screening candidate genes associated with anti-HER2 TKIs oncotherapy resistance in HER2-positive breast cancer. A: Amplification percentage of genes on chromosome 17. B: For any of the 33 candidate genes around HER2 locus, its amplification frequency in the 1105 case set was shown. C: Heatmap

showing the fold change in sgRNAs abundance targeting 33 candidate genes after lapatinib or pyrotinib treatment compared with control group. D: Kaplan-Meier survival curves of HER2-positive breast cancer patient with low or high CDK12 expression. E and F: Correlation of HER2 and CDK12 expression at transcriptional levels in breast cancer tissues in TCGA (E) and breast cancer cell lines in CCLE databases (F). See also Fig S1.

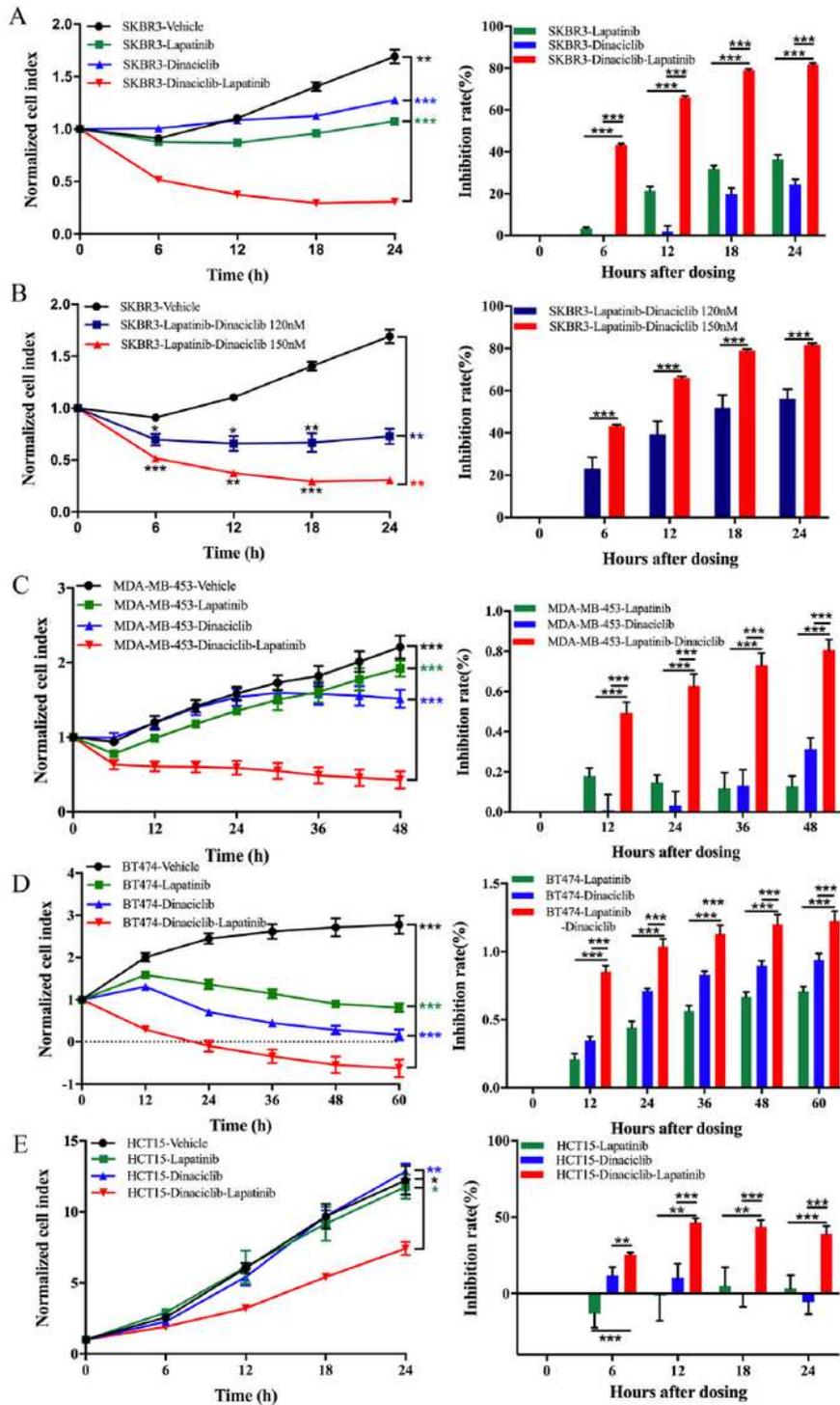


Figure 2

Inhibition of CDK12 sensitized HER2-positive cancer cells to lapatinib in a dose-dependent manner. A: Effects of lapatinib (5 μ M) and/or dinaciclib (150 nM) on proliferation of SKBR3 cells. B: Lapatinib (5 μ M) combined with dinaciclib (120 nM or 150 nM) significantly suppressed SKBR3 proliferation in a dose-dependent manner. C and D: Proliferation of lapatinib-resistant cells (MDA-MB-453) and - sensitive cells (BT474) were monitored by Real-Time Cellular Analysis (RTCA) with lapatinib (5 μ M) and/or dinaciclib (100 nM). E: RTCA assays of colorectal cancer cells under pressure of indicated drugs (lapatinib: 5 μ M; dinaciclib: 10nM)). Inhibition rate and statistical significance were calculated based on normalized cell index of indicated time points (mean \pm SD, *P < 0.05; **P < 0.01; ***P < 0.001, two-side Student's t-test). See also Fig S2.

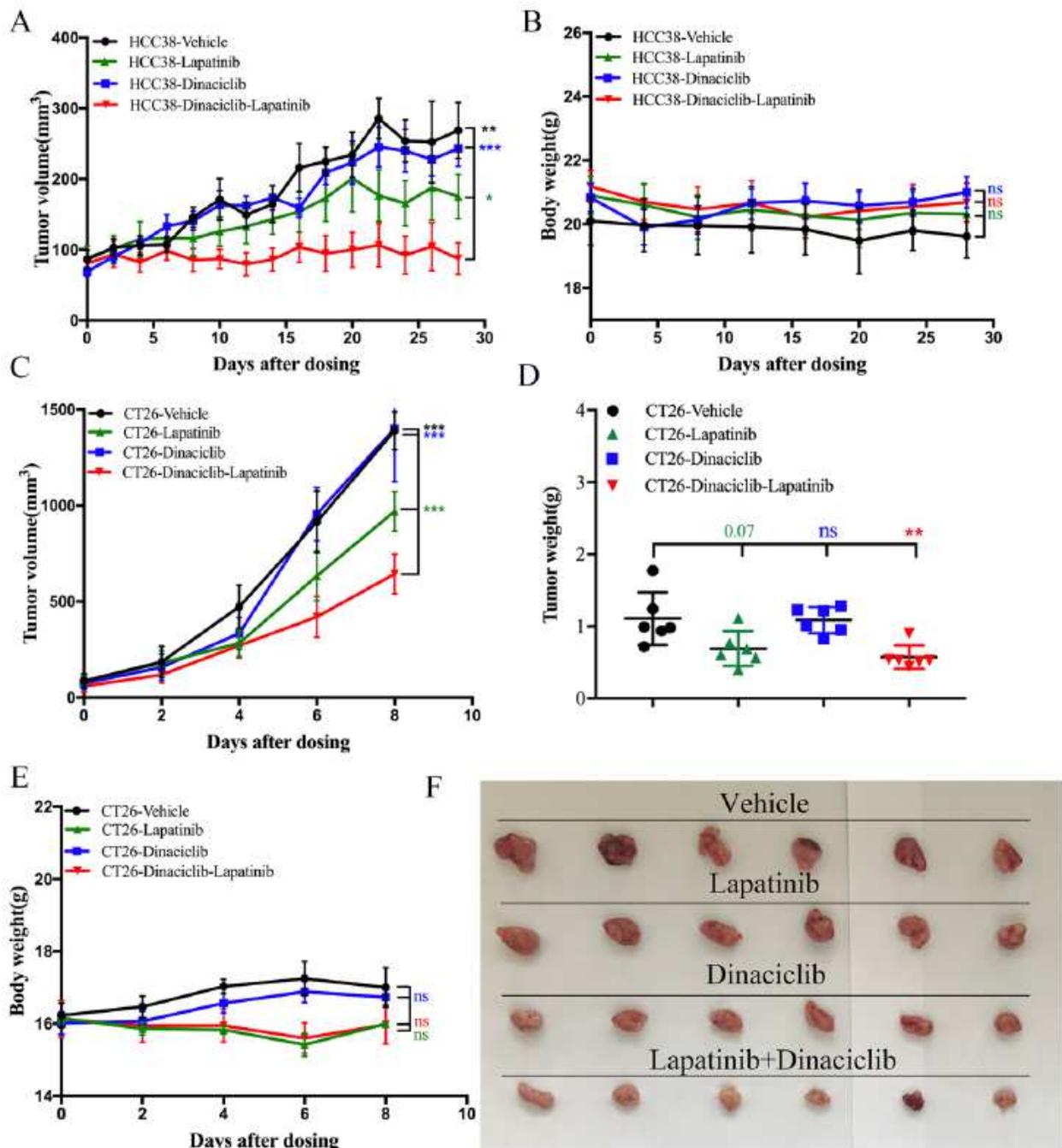


Figure 3

CDK12 inhibition suppressed lapatinib-resistant tumor progression in vivo A and B: Tumor volume of HCC38 cell-derived xenografts treated with lapatinib (75mg/kg), dinaciclilb (8mg/kg) or both. Tumor size (A) was measured in two dimensions using a caliper, and the volume was expressed in mm³ using the formula: $V = 0.5 a \times b^2$ where a and b are the long and short diameters of the tumor, respectively. (n=6, mean \pm SD, ns: not significant, * P < 0.05; ** P < 0.01; *** P < 0.001, two-side Student's t-test). Body weight

(B) of HCC38 xenografts in each group. C-F: Tumor volume (C) of CT26-derived xenografts receiving indicated reagents treatment. Tumor weight (D) of CT26-derived xenografts. Body weight of CT26 derived xenografts (E) and tumor images (F) were shown (n=6, mean \pm SD, ns: not significant, *P < 0.05; **P < 0.01; *** P < 0.001, two-sided Student's t-test).

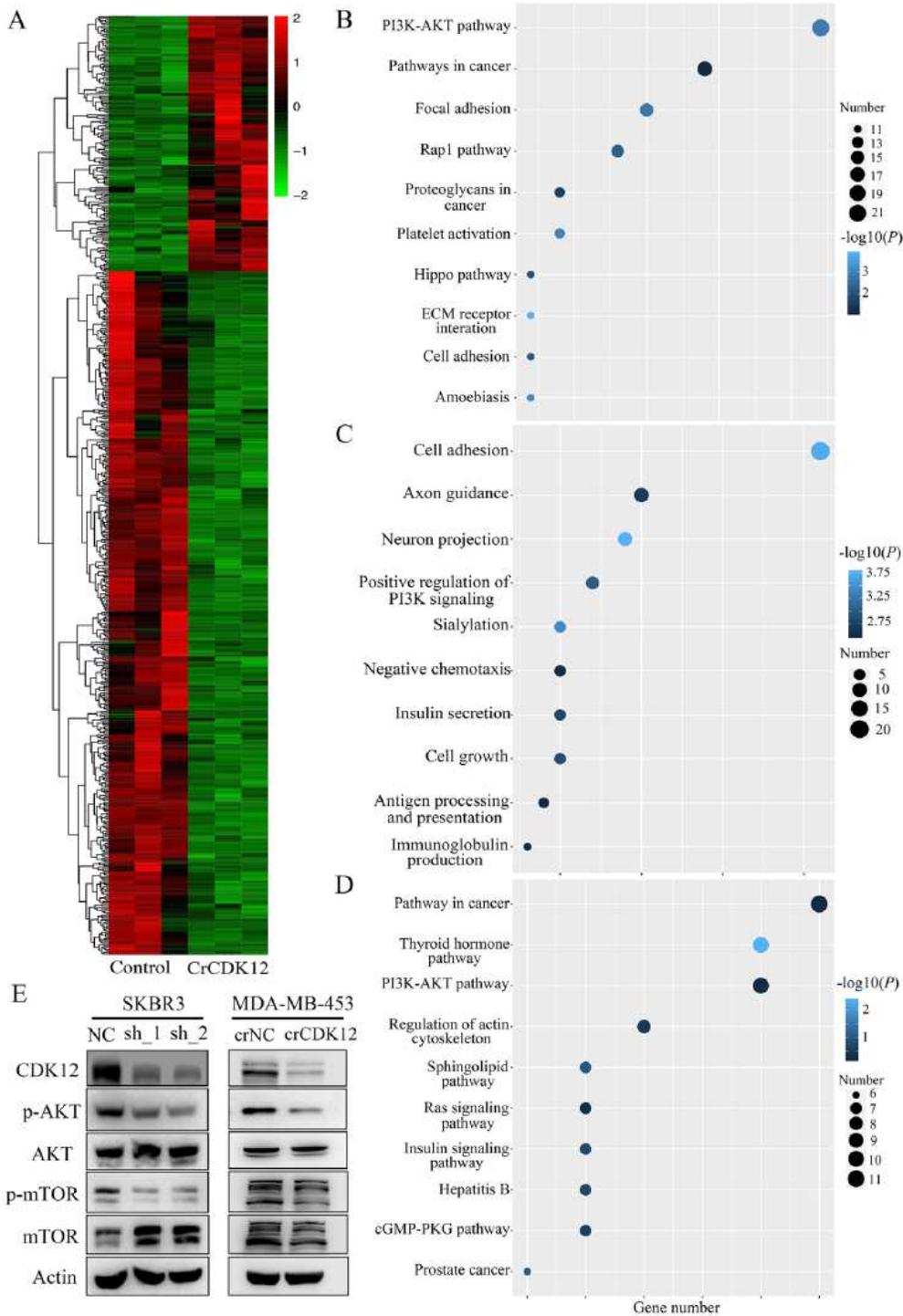


Figure 4

CDK12 suppression reversed lapatinib resistance via repressing PI3K/AKT activation. A: Heatmap of differentially expressed genes (DEGs) after CDK12 knockout ($P < 0.05$, $\log_2FC > 1$ or < -1). B: Bubble chart of KEGG pathway analysis for all DEGs using DAVID. C: Biological process analysis of all downregulated genes upon CDK12 knockout. Genes positively regulating PI3K signaling were suppressed. D: KEGG pathway analysis of CDK12 associated genes from TCGA 1105 breast cancer patient dataset (Pearson correlation coefficient ≥ 0.5 or ≤ -0.5). E: Western blotting assay of PI3K/AKT/mTOR pathway after CDK12 knockdown (left) or knockout (right). See also Fig S3.

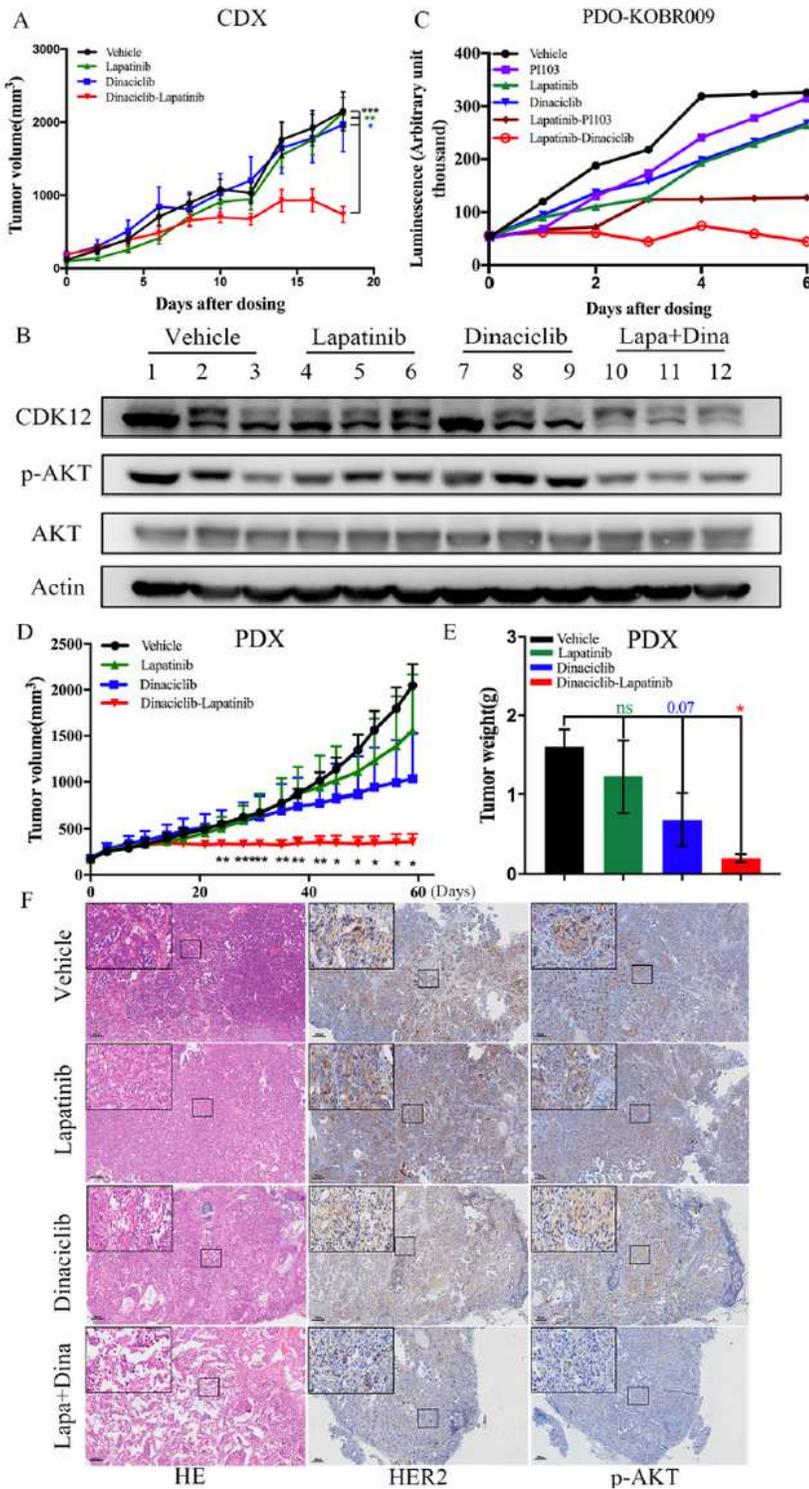


Figure 5

CDK12 inhibition repressed tumor progression of CDX and PDX models resistant to lapatinib. A: Tumor progression of HCC38 CDX with induced lapatinib resistance (n=6, mean \pm SD, *P < 0.05; **P < 0.01; ***P < 0.001, two-sided Student's t-test). B: Representative images show western blotting for p-AKT in variously treated HCC38 CDX mice. C: HER2-positive breast cancer patient-derived organoids (PDO) were treated with different drug combinations (lapatinib, 5 μ M; dinaciclib, 50 nM; PI103, 5 μ M). D and E: Tumor volume (D) and tumor weight (E) of PDX models that resistant to lapatinib treatment (n=3, lapatinib, 50 mg/kg; dinaciclib, 8 mg/kg; *P < 0.05; **P < 0.01, ***P < 0.001, two-sided Student's t-test). F: Representative images show HE staining and IHC staining for HER2 and p-AKT in PDX models. Scale bar, 20 μ m and 200 μ m. See also Fig S4.

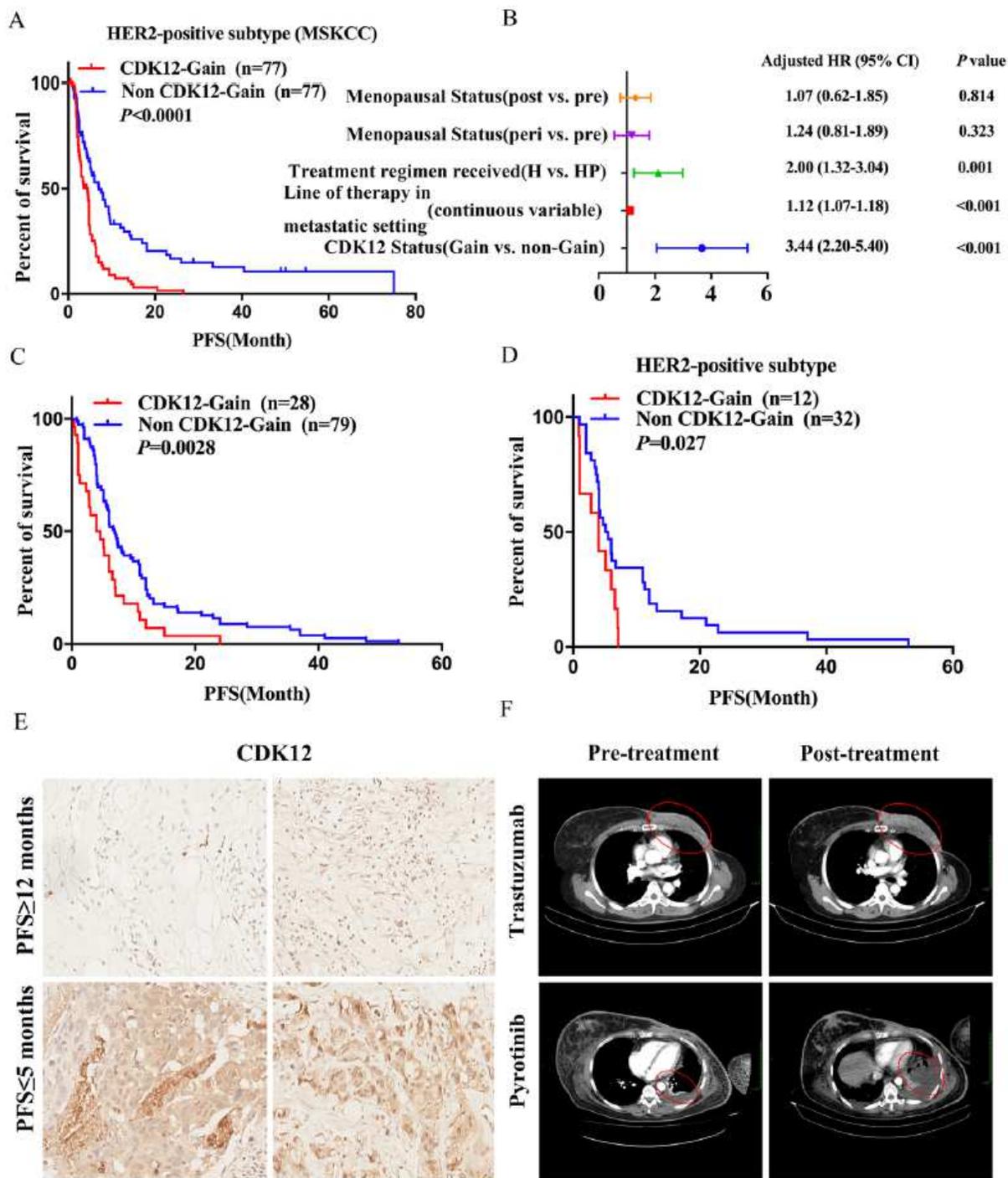


Figure 6

CDK12-amplified HER2-positive breast cancer patients are resistant to HER2-targeted treatment. A: Kaplan-Meier survival analysis of HER2-positive subtype breast cancer patients (HER+/HR-) in Memorial Sloan Kettering Cancer Center (MSKCC). B: Weighted multivariable Cox proportional hazards regression analyses of MSKCC data are shown for PFS. The hazard ratio (HR) compares the CDK12-gain vs the CDK12 non-gain status, and adjusted by menopausal status, treatment regimen received and line of

therapy in metastatic setting (H, trastuzumab; HP, trastuzumab-pertuzumab). C: Kaplan-Meier survival analysis of all 107 HER2 positive patients, including HR- or HR+, who received anti-HER2 therapy after ctDNA detection. Patients were dichotomized by CDK12 gene status. D: Kaplan-Meier survival analysis of 44 HER2-positive subtype patients (HER2+/HR-). E: IHC assays for CDK12 expression in HER2-positive sensitive (PFS \geq 12 months) and drug resistant (PFS \leq 3 months) groups, respectively. F: Representative images from breast cancer patients with HER2/CDK12 co-amplification detected by peripheral blood ctDNA sequencing whose cancers were resistant to anti-HER2 oncotherapy. CT scan shows the thickness of chest wall with metastasis to the soft tissue before and after HER2-targeted treatment. See also Fig S5.

Supplementary Files

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