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Original Article

PIK3CA mutations contribute to fulvestrant resistance in ER-positive breast cancer

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Abstract: p110α is an important subunit of phosphatidylinositol-3-kinases (PI3Ks) encoded by *PIK3CA*. Though PI3Ks are known as crucial regulators of cellular growth and proliferation, the function of *PIK3CA* mutations in fulvestrant resistance remains elusive. Thus, this study aimed to investigate the roles of *PIK3CA* mutations in fulvestrant resistance and tumor progression. Using circulating tumor DNA (ctDNA) from four fulvestrant-resistant patients, we found three of them were *PIK3CA* mutated, one with a novel *PIK3CA* mutation (p.R115P). *In vitro* experiments further evaluated the functions of those mutations and underlying mechanisms. We identified *PIK3CA* mutations could not only confer to fulvestrant resistance, but also promote cell proliferation and migration. Inhibition of corresponding activated pathways could effectively suppress cell growth.

Keywords: Fulvestrant resistance, estrogen receptor-positive breast cancer, *PIK3CA* mutation

Introduction

Currently, the mortality of breast cancer has still remained relatively high compared with others, despite of early diagnosis and comprehensive treatment [1]. Highly heterogeneous, different subtypes of breast cancer have different biological behaviors and clinical prognosis, among which estrogen receptor (ER)-positive breast cancer is the most common type and accounts for 65%-75% of breast cancer [2-4]. ER positivity is also the rationale that antiestrogen therapeutics were developed. Binding to ER, estradiol forms estradiol/ER complex, which mediates gene transcription via receptor dimerization and nuclear translocation. What is more, through non-genomic pathway, the complex can also activate mitogen-activated-protein-kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT so as to promote cell growth [5, 6].

Targeting ER, endocrine therapy includes selective ER modulators (SERMs, e.g., Tamoxifen), aromatase inhibitors (AIs, e.g., Letrozole) and selective ER downregulators (SERDs, e.g., Fulvestrant) [7-9]. However, along with benefits, the resistance in ER-positive breast cancer to these agents is inevitable, which drives tumor progression [10]. Whereas mechanisms concerning SERMs and AIs resistance have been widely studied, those of fulvestrant resistance are still waited to be elucidated [11, 12].

Based on the recent progress on circulating DNA (ctDNA) testing, we had four fulvestrant-resistant patients sequenced and found three of them carrying *PIK3CA* mutations [13]. As previous studies have shown, PI3K pathway might be implicated in fulvestrant resistance. Subsequent to growth factor binding and activation of receptor tyrosine kinases (RTKs), phosphatidylinositol 4,5-bisphosphate (PIP2) is phosphorylated by PI3K to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3), thus to recruit pleckstrin homology (PH) domain-containing proteins, such as phosphoinositide-dependent kinase 1 (PDK1) and AKT, so as to activate multiple downstream targets. P110α, encoded by *PIK3CA*, is a key component of PI3K pathway [14, 15]. Interestingly, *PIK3CA* mutation occurs frequently in tumors and is found

closely associated with tumor progression [16]. Nevertheless, the relation between fulvestrant resistance and *PIK3CA* is still not clear.

Thus, in this study, we explored the functions of *PIK3CA* mutations and their roles in generating resistance to fulvestrant. Furthermore, this study also sought to identify the strategy to treat fulvestrant-resistant breast cancer with mutant *PIK3CA*.

Patients and methods

Patients and samples collection

In total, four blood samples were collected from patients treated in Nanjing Jinling Hospital from 2015 to 2016. Regardless of previous treatment, ER-positive breast cancer patients who progressed during the treatment of fulvestrant were eligible. The study protocol was approved by the Ethics Committee of Jinling Hospital. All patients gave their informed consent prior to inclusion in the study. Specimens and all experimental procedures were handled and performed in accordance with the approved guidelines. Blood samples (5-10 mL) were collected from each patient when disease progressed.

Sample processing

The whole blood samples were collected in EDTA tubes and then centrifuged at 1000 g for 15 minutes within 1 hour after collection. Plasma and blood cells were collected into separate tubes and stored at -80°C. After DNA extraction and quantification, samples were further sequenced for genes associated with endocrine resistance.

Cell lines, reagents and lentivirus transfection

Breast cancer cell line MCF-7 was purchased from the Chinese Academy of Science Committee type culture collection cell bank (Shanghai, China) in 2017. Cells were cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin. Then, cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C. Fulvestrant, BKM120 and Palbociclib were purchased from MedChem Express. To identify the function of *PIK3CA* mutations p.R115P, p.N345K and p.E542K, we transfected MCF-7 cells with recombinant lentivirus of wild-type *PIK3CA or mutant PIK3CA* (p.R115P, p.N345K or p.E542K), which were purchased from Applied Biological Materials (ABM) (Zhenjiang, China) and confirmed by DNA sequencing. A nonspecific control was also purchased from ABM. Cells were harvested for further study after 72 hours of transfection.

Cell survival assay

Cell viability was measured using Cell Counting Kit-8 (CCK-8) (MedChem Express, China). Briefly, cells were seeded into a 96-well plate at a density of 5×10^3 cells/well with 6 repeats for each condition. After 24 hours, the cells were treated with fulvestrant or BKM120 with or without Palbociclib for another 72 or 24 hours. Then, the supernatants were removed and 100 μl medium with 10 μl CCK-8 was added into each well of the plate and incubated at 37°C. After 2 hours, the absorbance value (OD) of each well was measured at 450 nm using an ELX-800 spectrometer reader (Bio-Tek Instruments, Winooski, USA).

Colony formation assay

Cells transfected with wild-type or mutant *PIK3CA* and/or treated with medication were diluted and seeded into six-well plates at a density of 500 cells per well. After being incubated in a CO_2 incubator at 37°C for 14 days, cells were fixed with 100% methanol and stained with 0.5% crystal violet. Colonies larger than 1 mm were manually counted. These experiments were performed at least three times.

Apoptosis and cell cycle assays

Cells transfected with wild-type or mutant *PIK3CA* and/or treated with medication were incubated for 24 or 72 hours, then harvested by trypsinization (no EDTA) and washed three times with phosphate-buffered saline (PBS). For apoptosis analysis, the cells were resuspended in 500 μl of 1 × binding buffer and stained with 5 μl of Annexin V-APC and 5 μl of 7-AAD for 15 minutes at room temperature in the dark. For cell cycle analysis, cells were washed with PBS and fixed in 70% ethanol overnight at -20°C, then fixed cells were resuspended in PBS and stained by PI/RNase for 30 minutes in the dark. A flow cytometer (Becton-Dickinson) was used to evaluate the apoptotic

rates and cell cycle distribution in each sample. Each sample was tested in triplicate.

Wound healing assay

Cells were seeded in six-well plates and incubated to generate confluent cultures. Using 200 μl sterile pipette tips, wounds were scratched in the cell monolayer and rinsed with PBS. Subsequently, the cells were cultured in serum-free medium for 48 hours. The migration of the cells was photographed at time 0 and 48 hours.

Western blotting

The whole protein was extracted by RIPA buffer supplemented with protease and phosphatase inhibitors. 20 μg cell lysates were loaded per lane and resolved by sodium dodecylsulfate-polyacrylamide (SDS-PAGE) electrophoresis and blotted onto polyvinylidene fluoride (PVDF) membranes. Following 2-hour blockade with 5% skim milk in tris-buffered saline/0.1% tween-20, the membranes were incubated with the primary antibodies overnight at 4°C and with a horseradish peroxidase-conjugated secondary antibody (1:10000) for 2 hours the next day. Antibodies for detecting p110α (#4249), Akt (#4685), phospho-Ser473 Akt (#4060), Cyclin D1 (#2978), β-Actin (#4967) were from Cell Signaling Technology. Antibodies for detecting mTOR (ab32028), phospho-Ser2481 mTOR (ab137133), Rb (ab181616) and phospho-Ser780 Rb (ab47763) were from Abcam. Results from at least two separate experiments were analyzed.

Statistical analysis

All the *in vitro* experiments were performed in triplicate. One-way ANOVA and t-test were performed using GraphPad Prism version 7 (GraphPad Prism, San Diego). A P value of < 0.05 was considered statistically significant.

Results

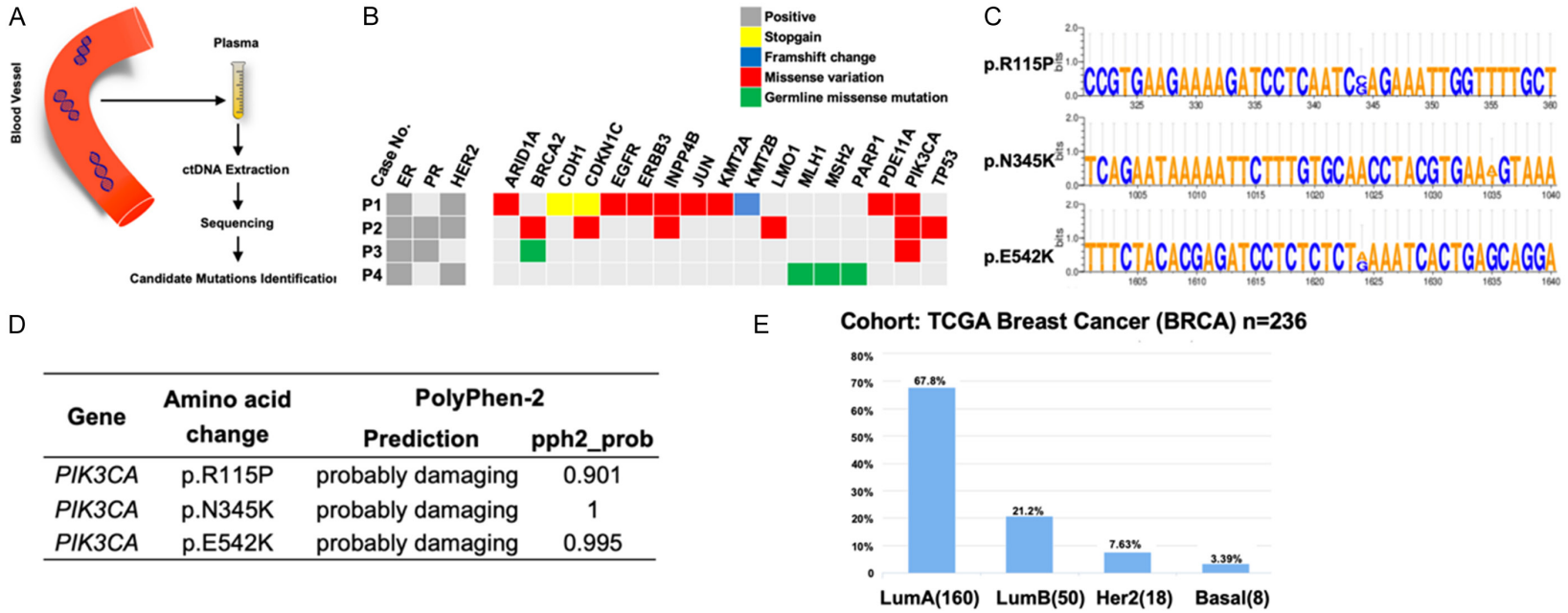
Investigation of PIK3CA mutations in circulating tumor DNA

We collected the blood samples from four patients with fulvestrant-resistant ER-positive breast cancer to sequence common mutations in a panel of genes involved in endocrine therapy resistance (**Figure 1A**, **1B**). As a result, it was identified that *PIK3CA* mutations were the most common mutations found in circulating tumor DNA (ctDNA) with three in four being *PIK3CA* mutation carriers (**Figure 1B**). While two of them carried previously described mutations p.N345K and p.E542K, one was found to carry a novel mutation which has not been reported in the breast cancer dataset of the COSMIC database (p.R115P). The corresponding altered nucleobases were shown in **Figure 1C**. By using Polyphen-2, we predicted the potential functional impacts, brought by the mutations, on protein. It showed that all three mutations were "probably damaging", indicating a further analysis was needed (**Figure 1D**).

Subsequently, we analyzed the distribution of *PIK3CA* mutations in different breast cancer subtypes and found that *PIK3CA* were mostly mutated in ER-positive breast cancer and clustered in exons 9 and 20, with the most frequently mutations being E545K and H1047R (**Figure 1E**).

Functional analysis of the PIK3CA mutations in vitro

In order to investigate the effects of *PIK3CA* mutations found in patients, MCF-7 cells were transfected with wild-type *PIK3CA, PIK3CA* mutations p.R115P, p.N345K and p.E542K respectively. Subsequently, we performed cell viability to explore the effects of such mutations in cell sensitivity to fulvestrant. By using CCK-8 assay, we found that cells with transfection of *PIK3CA* mutations exhibited a significant resistance to fulvestrant compared with these with wild-type *PIK3CA* (**Figure 2A**). What is more, the results of colony formation showed that the proliferation of wild-type *PIK3CA* transfected cells were markedly lower than that of cells transfected with *PIK3CA* mutations (**Figure 2B**). As for the results of cell apoptosis, compared with control, cells with mutant *PIK3CA* displayed only a slightly decreased apoptosis rate (**Figure 2C**). Next, the influence of *PIK3CA* mutations on cell cycle was analyzed. Interestingly, whereas cells transfected with *PIK3CA* mutations p.R115P and p.N345K showed a similar distribution of cells in different phases, cells with *PIK3CA* mutations p.E542K displayed a decreased percentage in G1 phase and a slightly increased percentage of S phase and G2 phase (**Figure 2D**), implying a G1-to-S phase transition. Furthermore, the wound healing



Gene	Amino acid change	PolyPhen-2	
		Prediction	pph2_prob
PIK3CA	p.R115P	probably damaging	0.901
PIK3CA	p.N345K	probably damaging	1
PIK3CA	p.E542K	probably damaging	0.995

Figure 1. Screening and functional analysis of endocrine resistance related mutations. A. The chart of mutations identification in ctDNA. ctDNA, circulating tumor DNA. B. Genetic mutations identified in the ctDNA of patients resistant to fulvestrant. C. Specific mutation sites of detected mutant *PIK3CA*. D. Potential effects brought by identified candidate mutations on protein function, as predicted by PolyPhen-2. E. Distribution of *PIK3CA* mutations among different subtypes of breast cancer in database.

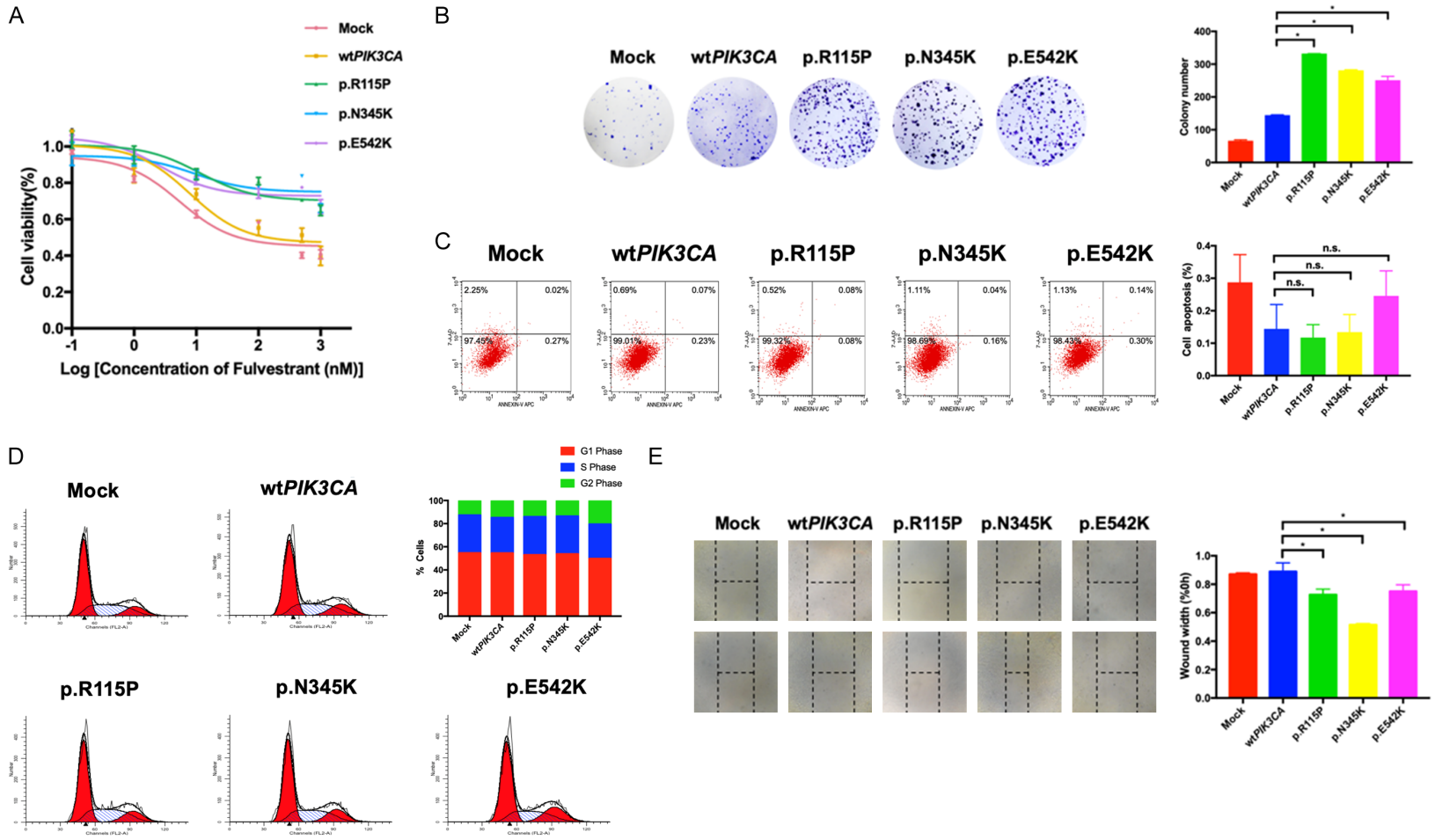


Figure 2. Mutant *PIK3CA* promotes fulvestrant resistance, cell proliferation and migration. A. The effect of fulvestrant on cell growth of ER-positive breast cancer cells with wild-type or mutant *PIK3CA* was measured by CCK-8. B. Colony formation of cells transfected with wild-type or mutant *PIK3CA*. C. The representative images of cell apoptosis analyzed by flow cytometry in cells transfected with wild-type or mutant *PIK3CA*. D. The representative images of cell cycle analyzed by flow cytometry in cells transfected with wild-type or mutant *PIK3CA*. E. Wound-healing assay in transfected cells were performed. *P < 0.05. n.s., nonsignificant.

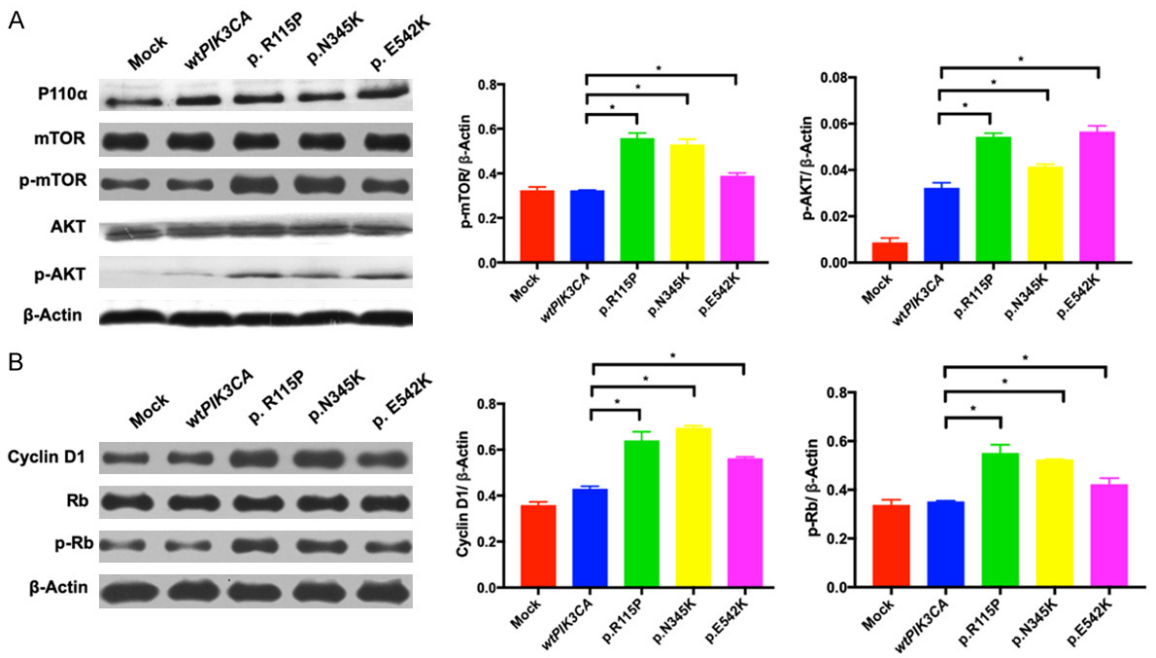


Figure 3. PI3K pathway and downstream cell cycle proteins are activated. A. Western blotting of PI3K pathway proteins p110α, mTOR, p-mTOR, AKT and p-AKT were performed in wild-type or mutant *PIK3CA* ER-positive breast cancer cells. B. Western blotting analysis of cell cycle proteins Cyclin D1, Rb and p-Rb in wild-type or mutant *PIK3CA* cells. β-Actin expression was used as the loading control. *P < 0.05.

assay illustrated that the *PIK3CA* mutations might have the potential to promote cell migration, which finally contributed to the metastasis of fulvestrant-resistant breast cancer (**Figure 2E**).

Taken together, our results showed that the *PIK3CA* mutations p.R115P, p.N345K and p.E542K could not only induce an insensitivity to fulvestrant treatment, but also contribute to the promotion of cell growth and cell migration.

Activation of PI3K pathway by PIK3CA mutations

It has been widely recognized that the mutations of *PIK3CA* could activate downstream PI3K pathway to promote the cell growth. To confirm the activating ability of *PIK3CA* mutations, we performed western blotting assays for p-mTOR (phosphorylated mTOR) and p-AKT (phosphorylated AKT) in cells transfected with these mutations. In comparison with phosphorylation levels of mTOR and AKT in control group, the expressions of these in cells with *PIK3CA* mutations p.R115P, p.N345K and p.E542K were remarkably elevated, suggesting an activation of PI3K pathway (**Figure 3A**). As shown above (**Figure 2D**), the G1-to-S phase transition was promoted in cells with *PIK3CA* mutations p.E542K. Thus, we further explored the expression of cell cycle-related key proteins Cyclin D1 and p-Rb (phosphorylated Rb). The results showed that in cells with the mutation, Cyclin D1 and p-Rb were upregulated compared with these of control (**Figure 3B**). However, although *PIK3CA* mutations p.R115P and p.N345K seemed not to have strong impacts on cell cycle promotion (**Figure 2D**), the levels of Cyclin D1 and p-Rb were also increased (**Figure 3B**). In view of the complexity of cell cycle and mutations, it was speculated that different mutations might involve different proteins in the regulation of cell cycle.

Briefly, the confirmed *PIK3CA* mutations could further promote cell proliferation by activating downstream signaling pathway such as PI3K pathway and altering cell cycle proteins.

PI3K inhibitor plus CDK4/6 inhibitor inhibits growth of cells transfected with mutant PIK3CA

Given the activation of both PI3K pathway and its downstream cell cycle proteins in *PIK3CA*-mutant cells, as well as previous studies which

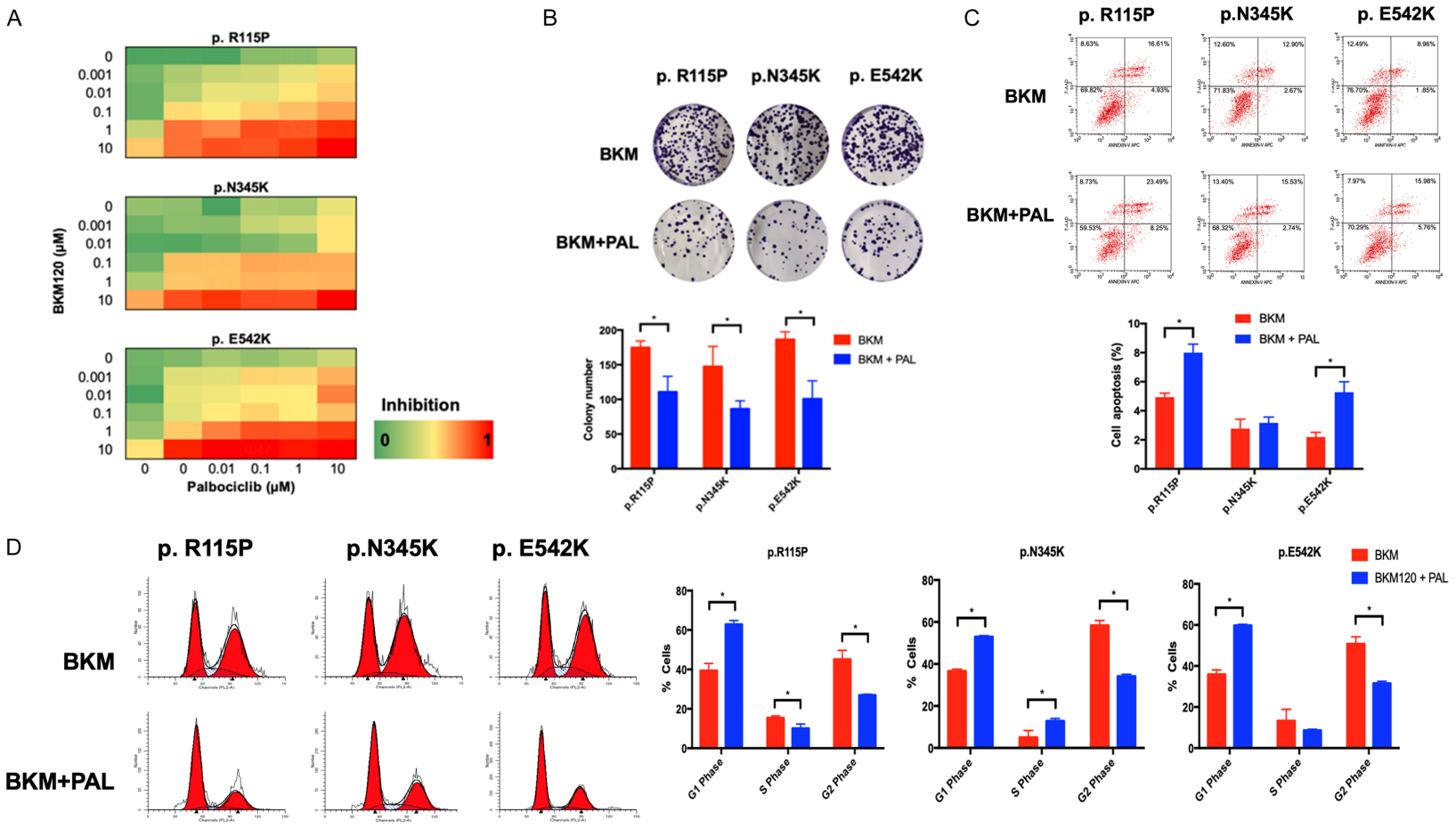


Figure 4. The impacts of combined inhibitors of PI3K and CDK4/6 on *PIK3CA* mutated cells. A. A dose matrix of PI3K inhibitor BKM120 and CDK 4/6 inhibitor Palbociclib was performed in cell lines transfected with mutant *PIK3CA*. Viability was assessed one day after treatment. The concentrations and percentages of inhibition at each dose of drug were presented. B. Colony formation of cells transfected with mutant *PIK3CA* were carried out after treatments of single BKM120 alone or with Palbociclib. C. The representative images of cell apoptosis were analyzed by flow cytometry in cells transfected with wild-type or mutant *PIK3CA* following medication. D. The representative images of cell cycle were analyzed by flow cytometry in cells transfected with mutant *PIK3CA* after exposure to BKM120 alone or with Palbociclib for 24 hours. Percentages of cells in G1, S, and G2 phases were quantified. *P < 0.05.

demonstrated that PI3K blockade alone was not as potent as initially assumed [17, 18], we hypothesized that the combination of PI3K inhibitor with CDK4/6 inhibitor might exhibit a stronger inhibition on cell proliferation than PI3K inhibitor alone. Therefore, we subsequently performed a dose matrix of PI3K inhibitor BKM120 and CDK4/6 inhibitor Palbociclib in all three mutant *PIK3CA*-transfected cells to confirm the synergistic activity. It was observed that single PI3K inhibition was relatively ineffective in mutant cells, while the combination of PI3K inhibitor and CDK4/6 inhibitor successfully suppressed cell growth in a low concentration (**Figure 4A**). What is more, following treatment of BKM120 alone or with Palbociclib, the results of colony formation showed that the combination could significantly inhibit cell proliferation (**Figure 4B**). To explore the role the combined agents played in cell growth inhibition, cell cycle and apoptosis assays were carried out. As the results showed, we assumed that the combination effect might depend on both apoptosis and cell cycle arrest, ultimately leading to decreased cell viability (**Figure 4C**, **4D**).

Together, compared with PI3K inhibitor alone, combination of PI3K inhibitor with CDK4/6 inhibitor was more potent in cell proliferation inhibition, apoptosis induction and cell cycle arrest.

Discussion

Potent as endocrine therapy is in treating ER-positive breast cancer, however, the resistance to antiestrogen therapeutics has gradually developed. To further improve the life span and quality for those patients, it is urgent to clarify mechanisms underlying such resistance so as to identify and develop new diagnostic methods and agents. PI3K pathway is a crucial signaling hub for both normal cells and cancer cells, and its implication in fulvestrant resistance has been demonstrated. In spite of this, roles of PI3K alterations, such as mutations of *PIK3CA*, in conferring resistance to fulvestrant are still unsettled [19, 20].

Limited by current conditions, we only found four patients who were resistant to fulvestrant after treatment with fulvestrant. We sequenced endocrine therapy resistance-related genes to identify mutations that might contribute to the insensitivity. As the most commonly mutated genes in four patients, *PIK3CA* was selected for further research. A retrospective study of 19,784 patients showed that compared with expressions of ER and progesterone receptor (PR) in samples with wild-type *PIK3CA*, expressions of ER and PR were higher among *PIK3CA*-mutant patients [21]. What is more, it was demonstrated that alterations of *PIK3CA* was correlated with persistent ER expression, which might result in the irresponsiveness to antiestrogen agents. According to previous studies, most mutations in *PIK3CA* could constitutively activate the corresponding kinase and subsequently induce cellular transformation and tumor proliferation [16, 22]. Thus, we speculated that it might be via activated PI3K pathway that the detected mutations led to fulvestrant resistance.

In this study, we discovered one novel mutations in *PIK3CA* exon 1 and two reported mutations in *PIK3CA* exons 4 and 9. Protein functional assay predicted that these three mutations would impact the functions of protein and deserve a further analysis. By a series of *in vitro* studies, we confirmed that the mutations were responsible for the insensitivity to fulvestrant and could promote cell growth, colony formation and cell migration. These findings were in agreement with results shown in other tumors and verified the gain of functions of *PIK3CA* in breast cancer cells. Besides, we also demonstrated that it was due to the activated PI3K/AKT pathway and downstream altered Cyclin D1 and Rb that mutant *PIK3CA* conferred a resistance to fulvestrant.

Since the activated PI3K pathway was observed in cells transfected with mutant *PIK3CA*, we conjectured PI3K inhibitors might have a preferential inhibition of these cells. Although some laboratory and clinical studies showed tumors with *PIK3CA* mutations would benefit more from PI3K inhibitors, some studies showed opposite results [23-25]. What is more, it was revealed that a combination inhibition of PI3K with other inhibitors (e.g., mTORC inhibitor) could strengthen tumor suppression [26]. Considering the increased p-Rb in cells with mutant *PIK3CA* and efficacy of CDK4/6 inhibitors shown in breast cancer, we then sought to explore the effectiveness of PI3K inhibitor BKM120 with CDK4/6 inhibitor Palbociclib. As expected, CDK4/6 inhibition not only sensitized *PIK3CA*-mutant cells to PI3K inhibitor, but also

exhibited an additional benefit. Interestingly, as shown, the combinatorial effect depended on both cell apoptosis and cell cycle arrest in cells with mutations p.R115P and p.E542K. Different from the previous two types of cells, cells with *PIK3CA* mutations p.N345K did not show a higher apoptosis rate after being exposed to the combination treatment. These results suggested that though addition of CDK4/6 inhibitor to PI3K inhibition would be more effective than single PI3K inhibitor, different mutations might respond in different mechanisms.

Despite the potency of inhibition of both cell cycle proteins and PI3K, the side effects and toxicities should also be taken into consideration. Fortunately, the toxicity profiles of these two drugs do not cross over much. While the toxicity profiles of PI3K inhibitors are mainly rash, hyperglycemia and diarrhea [27, 28], CDK4/6 inhibitor differs, with the most common toxicities being hematologic neutropenia [29-31].

Briefly, this study demonstrated that the mutations of *PIK3CA* contributed to fulvestrant resistance through the activation of PI3K pathway and alteration of cell cycle proteins. Further, it was also found that combined inhibitors of PI3K and CDK4/6 could successfully suppress *PIK3CA* mutated cells. While further studies of combined agents would be needed with no doubt, such combination might shed light on the future development direction of combination therapeutics.

Acknowledgements

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Disclosure of conflict of interest

None.

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