


## ORIGINAL ARTICLE

# miR-221 confers lapatinib resistance by negatively regulating p27<sup>kip1</sup> in HER2-positive breast cancer

Thanh Kieu Huynh<sup>1,2</sup> | Chih-Hao Huang<sup>1,3</sup> | Jhen-Yu Chen<sup>1</sup> | Jin-Han Yao<sup>4</sup> |  
Yi-Shiang Yang<sup>2</sup> | Ya-Ling Wei<sup>2</sup> | Hsiao-Fan Chen<sup>2</sup> | Chia-Hung Chen<sup>4,5</sup> |  
Chih-Yen Tu<sup>4,5</sup> | Yuan-Man Hsu<sup>6,7</sup> | Liang-Chih Liu<sup>3,4</sup> | Wei-Chien Huang<sup>1,2,8,9</sup> 

<sup>1</sup>Graduate Institute of Biomedical Sciences, Drug Development Center, China Medical University, Taichung, Taiwan

<sup>2</sup>Center for Molecular Medicine, China Medical University Hospital, Taichung, Taiwan

<sup>3</sup>Division of Breast Surgery, China Medical University Hospital, Taichung, Taiwan

<sup>4</sup>School of Medicine, China Medical University, Taichung, Taiwan

<sup>5</sup>Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, China Medical University Hospital, Taichung, Taiwan

<sup>6</sup>Department of Biological Science and Technology, China Medical University, Taichung, Taiwan

<sup>7</sup>Department of Animal Science and Technology, Agriculture College, Tunghai University, Taichung, Taiwan

<sup>8</sup>The Ph.D. program for Cancer Biology and Drug Discovery, China Medical University and Academia Sinica, Taichung, Taiwan

<sup>9</sup>Department of Biotechnology, Asia University, Taichung, Taiwan

## Correspondence

Wei-Chien Huang, Graduate Institute of Biomedical Sciences, China Medical University, Taichung, Taiwan.  
Email: whuang@mail.cmu.edu.tw (WCH)

Liang-Chih Liu, School of Medicine, China Medical University, Taichung, Taiwan  
Email: d7495@mail.cmu.edu.tw (LCL)

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## Abstract

Development of acquired resistance to lapatinib, a dual epidermal growth factor receptor (EGFR)/human epidermal growth factor receptor 2 (HER2) tyrosine kinase inhibitor, severely limits the duration of clinical response in advanced HER2-driven breast cancer patients. Although the compensatory activation of the PI3K/Akt survival signal has been proposed to cause acquired lapatinib resistance, comprehensive molecular mechanisms remain required to develop more efficient strategies to circumvent this therapeutic difficulty. In this study, we found that suppression of HER2 by lapatinib still led to Akt inactivation and elevation of FOX3a protein levels, but failed to induce the expression of their downstream pro-apoptotic effector p27<sup>kip1</sup>, a cyclin-dependent kinase inhibitor. Elevation of miR-221 was found to contribute to the development of acquired lapatinib resistance by targeting p27<sup>kip1</sup> expression. Furthermore, upregulation of miR-221 was mediated by the lapatinib-induced Src family tyrosine kinase and subsequent NF- $\kappa$ B activation. The reversal of miR-221 upregulation and p27<sup>kip1</sup> downregulation by a Src inhibitor, dasatinib, can overcome lapatinib resistance. Our study not only identified miRNA-221 as a pivotal factor conferring the acquired resistance of HER2-positive breast cancer cells to lapatinib through negatively regulating p27<sup>kip1</sup> expression, but also suggested Src inhibition as a potential strategy to overcome lapatinib resistance.

Thanh Kieu Huynh and Chih-Hao Huang contributed equally to this work.

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## KEYWORDS

lapatinib resistance, miR-221, NF- $\kappa$ B, p27<sup>kip1</sup>, Src inhibition

## 1 | INTRODUCTION

The aberrant activation of members of the HER (ErbB) family, including EGFR, HER2, HER3, and HER4, has been implicated in tumor growth and progression. Lapatinib (Tykerb®, GW-572016), a dual inhibitor of EGFR and HER2 tyrosine kinase, in combination with Capecitabine, has been approved for advanced HER2-positive breast cancer patients who have failed to respond to chemotherapy or Trastuzumab therapies.<sup>1</sup> Although lapatinib inhibits both HER2 and EGFR tyrosine kinases by competing for the Mg<sup>2+</sup>-ATP binding site of their cytoplasmic tyrosine kinase pocket, its anti-tumor activity in breast cancer seems to be more dependent on HER2 overexpression compared with EGFR.<sup>2,3</sup> Lapatinib inhibits HER2 tyrosine phosphorylation and attenuates downstream signaling pathways to reduce tumor cell growth, survival, and metastasis.<sup>4,5</sup>

The PI3K/Akt axis is a major downstream pathway of HER2 and contributes to tumor progression of HER2-positive breast cancer cells through the regulation of a variety of cellular events, including phosphorylation and inactivation of transcription factor FOXO3a.<sup>6,7</sup> In response to lapatinib, inhibition of the HER2/PI3K/Akt axis relieves FOXO3a suppression<sup>8</sup> and upregulates the expression of FOXO3a-dependent genes such as cell cycle inhibitor p27<sup>kip1</sup>,<sup>9,10</sup> Bim, Puma,<sup>11</sup> and CDK6,<sup>12</sup> involving cell cycle arrest and pro-apoptosis. However, the anti-cancer effects and survival benefits from lapatinib treatment are limited due to the development of acquired resistance that typically occurs within 12 mo of initial treatment.<sup>13</sup>

The compensatory activation of the PI3K/AKT/mTOR signaling pathway and the substantial increase of c-MET receptor tyrosine-protein kinase were proposed to mediate the resistance to HER2-targeted therapies.<sup>14,15</sup> The perturbation of Akt activation and FOXO3a expression was found in lapatinib-resistant cells, where the levels of phosphorylated Akt are aberrantly maintained, while FOXO3a expression is suppressed.<sup>16</sup> In preclinical data, growth inhibition was restored by targeting overexpressed Akt and c-MET,<sup>17,18</sup> but this effect was not found in some lapatinib-resistant cells, in which Akt remains inactivated by lapatinib.<sup>19</sup> The addition of an oral selective allosteric inhibitor of Akt (MK-2206) to lapatinib-recurrent breast cancer patients only showed stable disease for 28 wk in a phase I study (NCT01281163). The results from a phase I/II clinical trial also did not show superior benefits from the addition of c-Met/VEGF receptor kinase inhibitor foretinib in lapatinib-recurrent HER2-positive metastatic breast cancer patients.<sup>20</sup> Therefore, other factors conferring the acquired resistance to lapatinib await to be explored for the development of new therapeutic strategies.

MicroRNAs (miRNAs) with ~22 nucleotides belong to small non-coding RNAs and play an essential role in the post-transcriptional

regulation of messenger RNA (mRNA). miRNAs have been found to be dramatically deregulated in many cancer types, highlighting their essential roles in tumorigenesis, tumor growth, and cancer metastasis.<sup>21</sup> The expression of miRNAs is tissue-specific, and their functions in targeting different mRNAs involved in various effects depend on cancer types. The expression profile of miRNA showed marked differences in sensitive and resistant cells in response to various anti-cancer therapies, indicating miRNAs as major regulatory factors in the control of abnormal gene expression involved in cell cycle arrest, cell proliferation, and apoptosis in drug-resistant cells.<sup>22,23</sup> In this study, we discovered that upregulation of miR-221 by the Src/NF- $\kappa$ B pathway contributed to the development of lapatinib acquired resistance by targeting p27 expression. Our results indicated inhibition of miR-221 by Src inhibitors as the potential therapeutic strategies to overcome drug resistance.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture and culture conditions

Human SkBr3 (HTB-30) and BT474 (HTB-20) breast cancer cell lines were obtained from the American Type Culture Collection (ATCC). Lapatinib-resistant (LR) clones were generated from long-term treatment with lapatinib (GlaxoSmithKline).<sup>24,25</sup> All cell lines were cultured in Dulbecco's modified Eagle medium/F12 (SH30004.04, HyClone) supplemented with 10% fetal bovine serum (10437-028, Gibco), 1% penicillin-streptomycin (SH40003.01, HyClone) at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. LR clones were maintained in the presence of 1  $\mu$ mol/L lapatinib.

### 2.2 | RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated using TRIzol™ reagent (15596026, Invitrogen) following the manufacturer's instructions. Reverse transcription of p27<sup>kip1</sup> mRNA was performed using Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (28025013, Invitrogen) followed by qPCR using the KAPA PROBE FAST qPCR Master Mix (2 $\times$ ) Universal (KM4701, Kapa Biosystems) with a p27<sup>kip1</sup>-specific primer pair: p27-F (5'-ATAAGGAAGCG ACCTGCAACCG-3') and p27-R (5'-TTCTTGGGCGTCTGCTCCACAG-3'). RT-qPCR of miRNAs was conducted using the TaqMan microRNA Reverse Transcription Kit (4366596, Applied Biosystems, Foster City, CA, USA) and TaqMan probes (hsa-miR-221, 000524; hsa-miR-222, 002276) as described previously.<sup>26</sup> qPCR was performed on a LightCycler 480/384 system (Roche Diagnostics).

### 2.3 | Protein extraction and western blot analysis

Total protein was harvested using RIPA lysis buffer (50 mmol/L pH7.4 Tris-HCl, 1% NP-40, 0.15% Na-DOC, 150 mmol/L NaCl, and 1 mmol/L EDTA) plus EDTA-free Protease Inhibitor Cocktail (4693132001, Roche Diagnostics) as described previously.<sup>27,28</sup> The extracted proteins were separated by 8%-12% SDS-PAGE and transferred to PVDF (10600023)/NC(10600001) membrane (GE Healthcare). Antibodies used in this study included anti-p27 antibody (sc-528, Santa Cruz), anti-phospho-HER2 (Tyr1221/1222) antibody (2243, Cell Signaling Technology), anti-HER2 antibody (sc-393712, Santa Cruz Biotechnology, Inc.), anti-Akt antibody (9272, Cell Signaling Technology), anti-phospho-Akt(Ser473) antibody (sc-7985, Santa Cruz Biotechnology, Inc.), anti-FOXO3a antibody (sc-11351, Santa Cruz Biotechnology, Inc.), anti-Bim antibody (2933, Cell Signaling Technology), anti-PARP antibody (9542, Cell Signaling Technology), anti-p65 antibody (sc-372, Santa Cruz Biotechnology, Inc.), anti-phospho-p65 (Ser536) antibody (sc-101752, Santa Cruz Biotechnology, Inc.), anti-IκBα antibody (9242, Cell Signaling Technology), anti-phospho-IκBα (Ser32/36) antibody (2859, Cell Signaling Technology), anti-Rb antibody (9309, Cell Signaling Technology), anti-phospho-Rb (Ser807/811) antibody (9308, Cell Signaling Technology), anti-caspase-3 antibody (9662, Cell Signaling Technology), anti-Src kinase family antibody (9320, Cell Signaling Technology), and anti-phospho-Src (Tyr416) antibody (6943, Cell Signaling Technology).

### 2.4 | Luciferase reporter constructs and luciferase assay

The 3'UTR of p27 cDNA was amplified from the reverse-transcript product of SkBr3 cells by PCR with specific primers, p27-3'UTR-F (5'-GCGGTACCACAGCTCGAATTAAGAATATGT-3') and p27-3'UTR-R (5'-GAGCTCAATAGCTATGGAAGTTTT-3') and was subcloned into the pGL4.22[luc2CP/Puro] vector (9PIE677, Promega) between the *KpnI*/*SacI* sites. pGL4.22-p27 3'UTR was co-transfected with pSV-β-galactosidase control vector (E1081, Promega Corporation) into the indicated cell lines using the TransIT-X2 transfection reagent (MIR6000, Mirus Bio) for 48 h. Luciferase activity was performed using a luciferase reporter assay system (E1501, Promega Corporation) following the manufacturer's instructions and detected by GloMax Discover Microplate Reader (Promega) as described previously.<sup>29</sup> The relative luciferase activity was normalized to β-galactosidase activity.

### 2.5 | Lentivirus production and infection

The p27-lentivirus shRNA plasmid, pMD.G envelope-expressing plasmid, and pCMV-Δ8.91 packaging plasmid were co-transfected into 293T cells using Lipofectamine 2000 Transfection Reagent (11668019, Invitrogen) in accordance with the manufacturer's instructions. Virus-containing media were collected after 24 h of post-transfection. Cells were infected with shRNA lentivirus at MOI 125 and selected with 2 μg/mL puromycin for 3 d before

being subjected to functional assays. p27-lentiviral shRNA-1 (5'-GAGCAATGCGCAGGAATAAGG-3') and p27-lentiviral shRNA-2 (5'-GAATGGTGATCACTCCAGGTA-3').

### 2.6 | Cell viability assay

As described previously,<sup>30</sup> cells were seeded into 96-well plates at  $3 \times 10^3$  cells/well and were cultured for the indicated time followed by MTT assay or IncuCyte zoom live-cell analysis (Essen Bioscience). For MTT assay, the culture medium was replaced by 100 μL DME/F12 serum-free medium containing 5 mg/mL MTT (M5655, Sigma Aldrich) and was incubated at 37°C for 3 h. The solution was discarded and then 100 μL DMSO was added to dissolve the precipitate. OD<sub>570</sub> was detected by ELISA reader (BioTek Instruments).

### 2.7 | Clonogenic assay

Cells were seeded into 6-well plates at  $1 \times 10^3$  to  $1 \times 10^4$  cells/well followed by treatments with the indicated drugs for 7 d. Cells were dyed with 1% crystal violet solution prepared in 30% ethanol.

### 2.8 | Cell cycle assay

After treatment, cells were trypsinized, washed with PBS, and fixed with 75% ethanol overnight at -20°C. The fixed cells were stained for 30 min at 37°C with propidium iodide (PI) solution containing 1% Triton X-100, 0.1 mg/mL RNase A (12091021, Invitrogen), and 20 μg/mL PI. In total, 10 000 nuclei were examined in a FACSVerse flow cytometer (BD Biosciences).

### 2.9 | Prediction of miRNA targets

The miRNA targets predicted by computer-aided algorithms were obtained from PicTar ([http://pictar.bio.nyu.edu/cgi-bin/PicTar\\_vertebrate.cgi](http://pictar.bio.nyu.edu/cgi-bin/PicTar_vertebrate.cgi)), TargetScan Release 6.1 (<http://www.targetscan.org>), and PITA ([https://tools4mirs.org/software/target\\_prediction/pita/](https://tools4mirs.org/software/target_prediction/pita/)).

### 2.10 | Mouse xenograft model

Female BALB/c (nu/nu) nude mice (6-8 wk old) were subcutaneously injected with  $1 \times 10^7$  BT/LR1 lapatinib-resistant cells. Before injection, mice were implanted with a 17β-estradiol pellet (0.72 mg, 60-d release, Innovative Research of America) on the nape. After 4 d, a 150 μL mixture (1:1) of cells preparing in PBS and Matrigel (354230, Corning Life Sciences) was injected into the mammary fat pad. After an additional 2 wk, mice were randomly divided into 4 groups receiving the following treatments, corn oil (control), lapatinib (20 mg/kg), dasatinib (10 mg/kg), and the combination. Drugs were prepared in 10% DMSO, 90% corn oil.

Tumors were measured every 3 d and the following formula used to calculate tumor volume: large diameter × small diameter × small diameter/2.

### 2.11 | Data analysis

All experiments were repeated for at least 3 independent assays. Data are summarized as mean ± SD. Statistical analysis of the results was determined by GraphPad Prism 8.0 with a two-tailed Student *t* test. *P* < .05 was considered statistically significant.

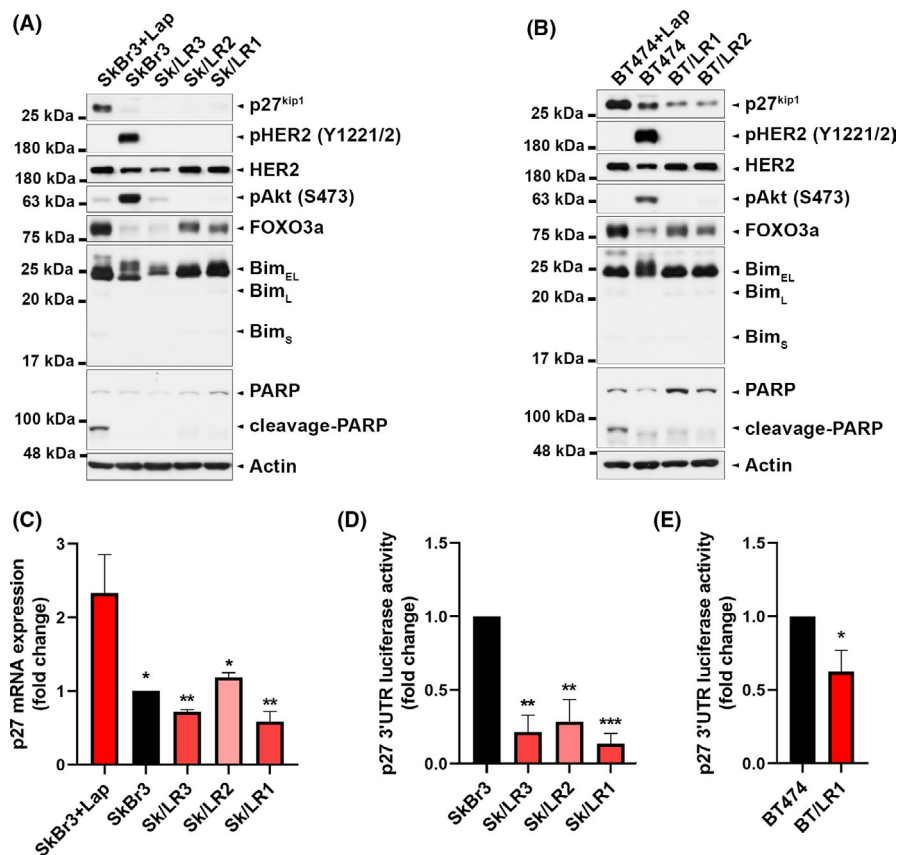
## 3 | RESULTS

### 3.1 | Failure of p27<sup>kip1</sup> induction contributes to lapatinib resistance

Elevated FOXO3a expression by lapatinib treatment has been shown to induce p27<sup>kip1</sup> upregulation, leading to G1 phase cell cycle arrest and cell growth inhibition.<sup>10</sup> We first addressed whether the

dysregulation of the FOXO3a/p27<sup>kip1</sup> axis is involved in lapatinib resistance. Transient treatment of lapatinib in 2 HER2-positive breast cancer cell lines, SkBr3 (Figure 1A) and BT474 (Figure 1B), induced FOXO3a and its downstream p27<sup>kip1</sup> protein levels, pro-apoptotic Bim expression, and PARP cleavage. Interestingly, the induction of p27<sup>kip1</sup> was not found in LR clones and even FOXO3a remained upregulated in response to HER2/Akt inhibition by lapatinib (Figure 1A,B). Because of Skp2-mediated p27<sup>kip1</sup> ubiquitination and degradation,<sup>31,32</sup> we sought to verify the involvement of protein degradation in the p27<sup>kip1</sup> reduction in different LR clones of SkBr3 cells (Sk/LR). Sk/LR1 and Sk/LR2 cells were treated with the proteasome inhibitor MG132, and TNF-α-induced IκBα degradation was used as the positive control for the efficacy of proteasome inhibition by MG132. However, p27<sup>kip1</sup> protein expression only slightly increased in LR clones upon proteasome inhibitor treatment (Figure S1), demonstrating that loss of p27<sup>kip1</sup> expression in lapatinib-resistant clones was not due to post-translational regulation.

We next examined the involvement of transcriptional and post-transcriptional regulation in the dysregulation of p27<sup>kip1</sup> expression in response to lapatinib resistance, and quantitative RT-PCR was



**FIGURE 1** p27<sup>kip1</sup> is not induced by lapatinib in lapatinib-resistant cells. A, B, Total lysates harvested from HER2-positive SkBr3 and BT474 breast cancer cell lines with or without lapatinib treatment (1 μmol/L, 24 h) and their lapatinib-resistant clones in the presence of lapatinib were prepared to detect the expressions of the indicated proteins in western blot analysis. C, p27<sup>kip1</sup> mRNA expression in SkBr3 cells with or without lapatinib treatment (1 μmol/L, 24 h) and in its lapatinib-resistant clones was measured by RT-qPCR. The bar graph showed the relative levels of p27<sup>kip1</sup> mRNA under various conditions in comparison with that in parental SkBr3 cells. (Student *t* test, \**P* < .05, and \*\**P* < .01). D, E, SkBr3 cells were transfected with pGL4.22 luciferase reporter gene containing p27<sup>kip1</sup> 3'UTR in indicated cell lines followed by treatment with 1 μmol/L lapatinib for 24 h. The bar graph represented the fold change of p27<sup>kip1</sup>-3'UTR luciferase activity with normalization to β-galactosidase activity. Data are presented as mean ± SD (Student *t* test, \**P* < .05, \*\**P* < .01 and \*\*\**P* < .001)

performed to measure mRNA expression of  $p27^{kip1}$ . Consistently,  $p27^{kip1}$  mRNA expression was suppressed in LR clones while it was induced by lapatinib transiently treatment (Figure 1C). Notably, the 3'-untranslated region (3'-UTR) activity of  $p27^{kip1}$  was decreased in LR clones in the luciferase reporter assays (Figure 1D,E). Collectively, these data suggested that post-transcriptional downregulation of  $p27^{kip1}$  may contribute to lapatinib resistance.

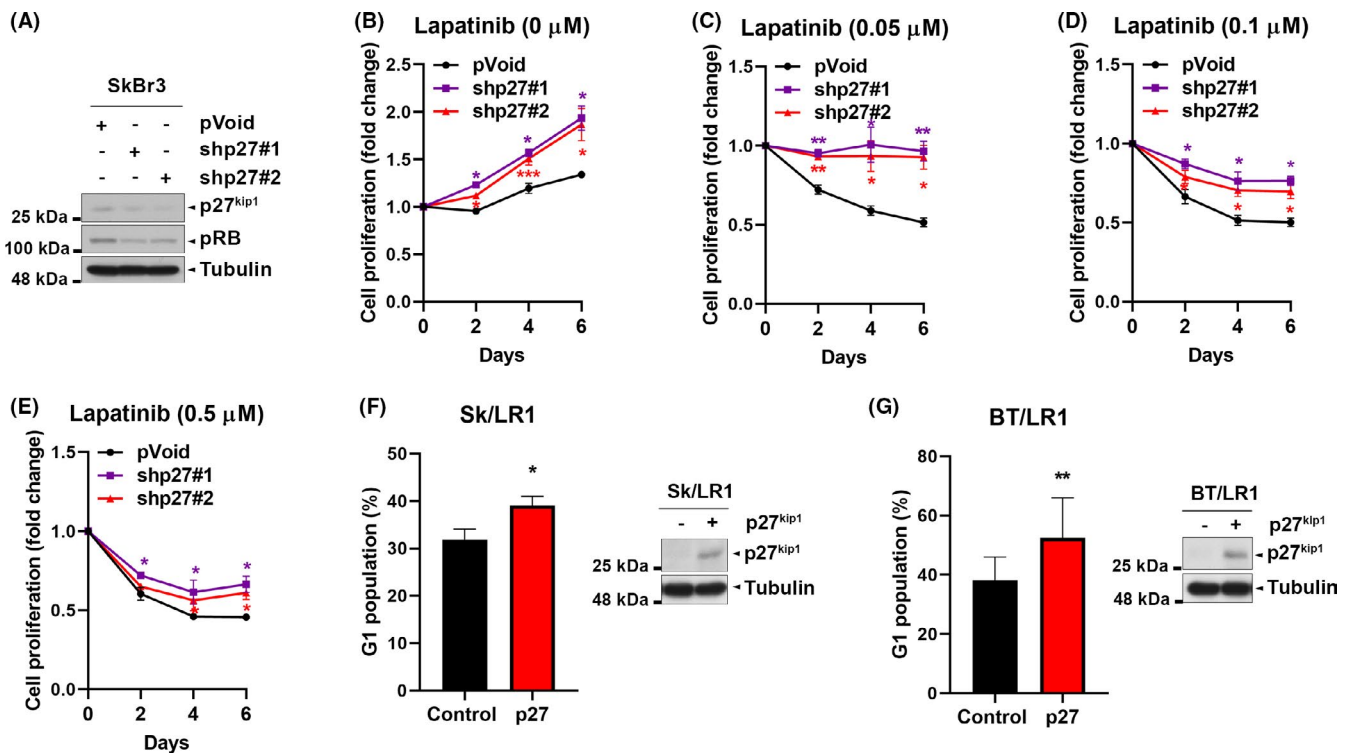
### 3.2 | Loss of $p27^{kip1}$ expression reduced susceptibility to lapatinib

As  $p27^{kip1}$  functions as a cell cycle inhibitor to slow down cell division and progression, we then silenced  $p27^{kip1}$  expression in SkBr3 cells with 2 individual shRNA clones (Figure 2A) to check their effect on cell proliferation by MTT assay (Figure 2B-E). In line with previous findings, we also observed that  $p27^{kip1}$  knockdown increased cell growth (Figure 2B), which was consistent with the reduction of the negative control of the cell cycle pRB (Figure 2A), and susceptibility of SkBr3 cells to lapatinib was decreased when  $p27^{kip1}$  was silenced (Figure 2C-E). To further dissect the regulatory role of  $p27^{kip1}$  in the cell cycle of LR clones, we overexpressed  $p27^{kip1}$  in LR cell lines and evaluated the cell cycle by flow cytometry analysis. Overexpression of  $p27^{kip1}$  induced the G1 population of Sk/LR1 (Figure 2F) and BT/

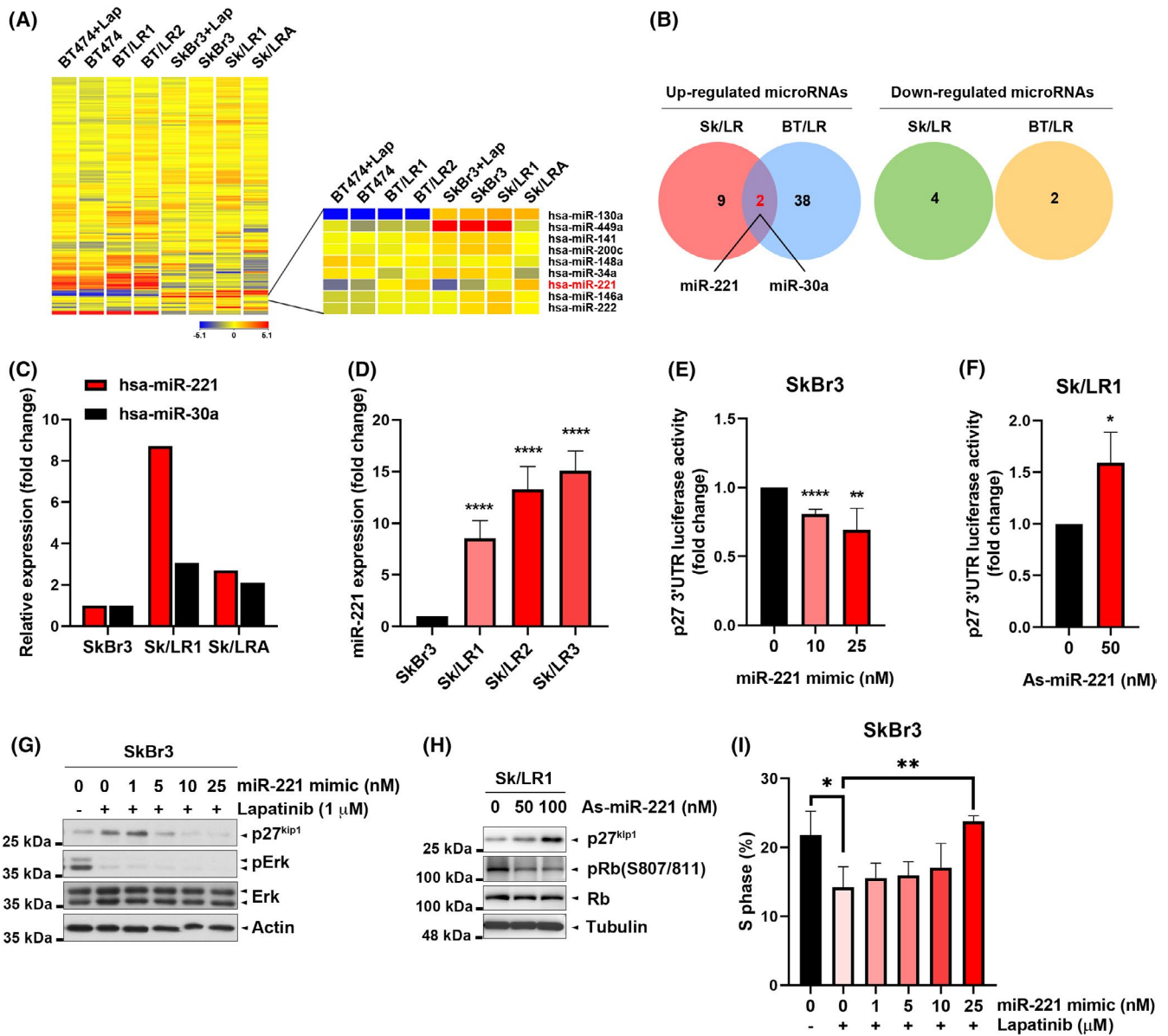
LR1 clones (Figure 2G). These results portrayed that no induction of  $p27^{kip1}$  effectively reduced lapatinib sensitivity and suppressed G1 cell cycle arrest to maintain the cell growth of LR clones.

### 3.3 | Upregulation of miR-221 suppresses $p27^{kip1}$ expression in LR cells

It is known that microRNAs dysregulation contributes to drug resistance in various cancer types, including breast and lung cancers.<sup>33,34</sup> To identify the role of miRNAs dysregulation in contributing to lapatinib resistance, we performed a microRNA array to compare the differential microRNA expression between parental cell lines (SkBr3 and BT474) and their lapatinib-resistant clones (SkBr3/LR1, SkBr3/LRA, BT474/LR1, and BT474/LR2; Figure 3A). There were 4 and 2 down-regulated microRNAs, while 11 and 40 upregulated microRNAs with an at least 2-fold change in SkBr3/LR and BT474/LR clones, respectively, compared with that in their parental cells (Figure 3B). Of these candidate miRNAs, the data showed that only miR-221 and miR-30a were commonly upregulated in these 2 populations, and miR-221 showed higher expression in these LR clones (Figures 3C and S2A). We further confirmed the expression of miR-221 in LR clones by RT-qPCR analysis. As a result, miR-221 expression was upregulated in LR clones of SkBr3 (Figure 3D) and BT474 (Figure S2B) cells.



**FIGURE 2** Inhibition of  $p27^{kip1}$  reduced the susceptibility of HER2-positive breast cancer cells to lapatinib. A, Total lysates harvested from SkBr3 cells infected with shp27 lentivirus were subjected to detect  $p27^{kip1}$  and pRB expression in western blot analysis. B-E, MTT assay was performed to identify cell proliferation upon  $p27^{kip1}$  silencing in SkBr3 cells treated with different concentrations of lapatinib. The line graph represented fold changes in cell growth. F, G, Sk/LR1 cells (F) and BT/LR1 cells (G) with overexpression of  $p27^{kip1}$  were subjected to cell cycle analysis with PI staining (left) and western blot analysis for  $p27^{kip1}$  expression (right). The bar graph presented the percentage of the G1 population as presented as mean  $\pm$  SD (Student *t* test, \**P* < .05, \*\**P* < .01 and \*\*\**P* < .001)



**FIGURE 3** Upregulation of miR-221 suppresses p27<sup>kip1</sup> expression in lapatinib-resistant cells. A, The heatmap from microRNA microarray analysis illustrates miRNA expression profiling in HER2-positive BT474 or SkBr3 breast cancer cell lines and in their LR clones. B, Venn diagram showing the number of miRNAs upregulated (right) or downregulated (left) in LR clones compared with their parental. Fold change >2 ( $P < .05$ ). C, Bar graph depicting the relative fold changes of hsa-miR-221 and hsa-miR-30a expression from microRNAs analysis between SkBr3 and its LR clones, Sk/LR1 and Sk/LRA. D, The relative fold changes in miR-221 expression in Sk/LR clones compared with parental SkBr3 cells were measured by RT-qPCR, and data are presented as mean  $\pm$  SD (Student  $t$  test, \*\*\*\* $P < .0001$ ). E, F, SkBr3 cells were co-transfected with pGL4.22 luciferase reporter gene containing p27 3'UTR and different concentrations of miR-221 mimic (E) or 50 nmol/L as-miR-221 (F) for 3 d. Bar graph shows the fold changes of p27<sup>kip1</sup> 3'UTR luciferase activity normalized to  $\beta$ -galactosidase activity (Student  $t$  test, \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$  and \*\*\*\* $P < .0001$ ). G, H, Total lysates harvested from SkBr3 (G) or Sk/LR1 (H) cells transfected with different concentrations of a miR-221 mimic or as-miR-221, respectively, followed by lapatinib treatment (1  $\mu$ M/L, 24 h) were used to detect the indicated protein expression in western blot analysis. I, SkBr3 cells transfected with different concentrations of miR-221 mimic followed by lapatinib treatment (1  $\mu$ M/L, 24 h) and subjected to cell cycle analysis with PI staining. Bar graph presents the percentage of the S population as mean  $\pm$  SD (Student  $t$  test, \* $P < .05$ , and \*\* $P < .01$ )

Previous studies have shown that miR-221 suppresses p27<sup>kip1</sup> expression by targeting its 3'UTR.<sup>35,36</sup> Consistent with these findings, the 3'UTR luciferase activity of p27<sup>kip1</sup> revealed a suppression when the synthetic oligo mimics of miR-221 (miR-221 mimic) were transiently expressed in SkBr3 (Figure 3E) and BT474 (Figure S2C) cells. Conversely, inhibition of miR-221 expression by

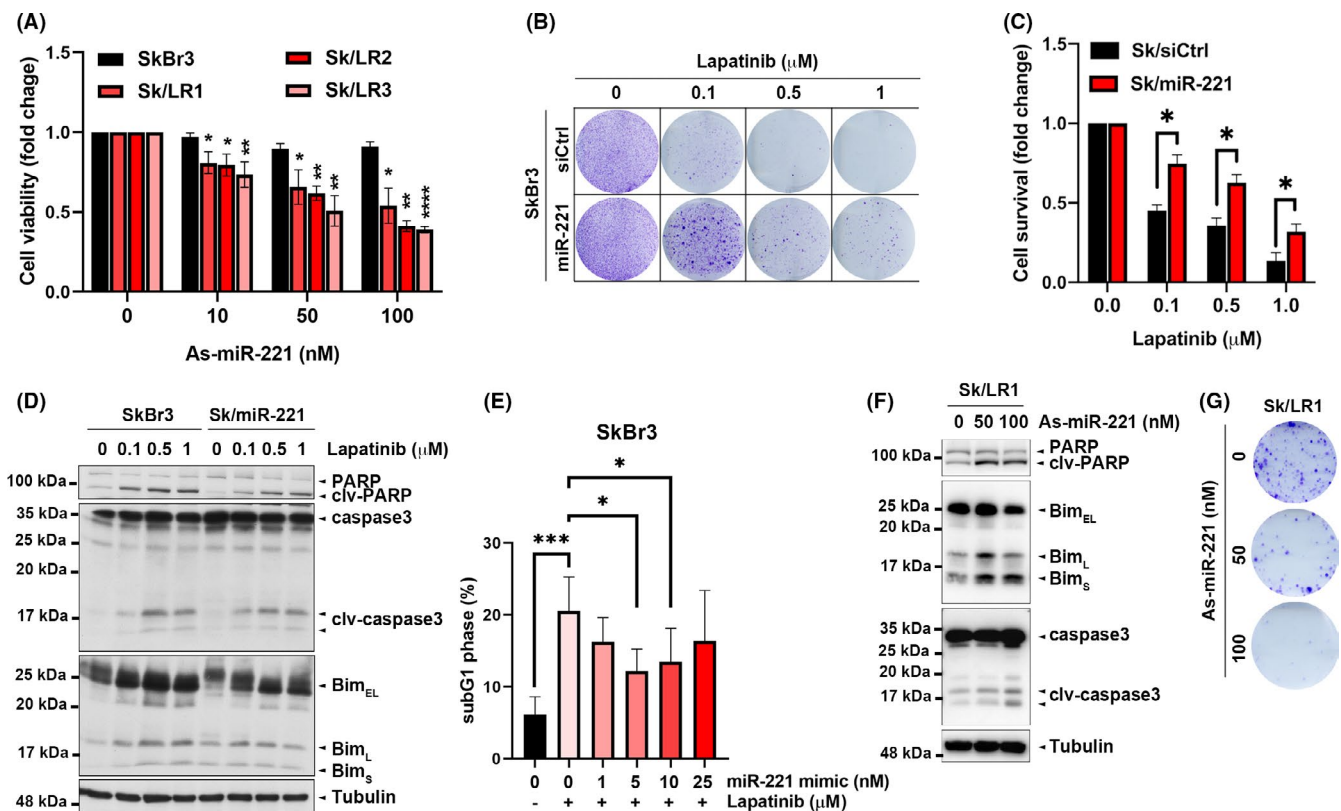
the anti-sense oligonucleotides of miR-221 (as-miR-221) reversed p27<sup>kip1</sup> 3'UTR luciferase activity in LR cell clones (Figures 3F and S2D). Next, we examined the involvement of miR-221 in p27<sup>kip1</sup> repression in LR clones. miR-221 mimic was transfected into parental cells, followed by lapatinib treatment. It was found that the expression of p27<sup>kip1</sup> protein was induced after treatment with

lapatinib, but this effect was reduced by miR-221 mimics in a dose-dependent manner (Figures 3G and S2E). Conversely, silencing miR-221 using as-miR-221 reversed the suppression of p27<sup>kip1</sup> expression in Sk/LR1 clone (Figure 3H). In parallel, phosphorylation of Rb at Ser<sup>807</sup>/Ser<sup>811</sup> by CDK2, which promotes cell cycle progression from G1 phase to S phase and is inhibited by p27<sup>kip1</sup>, was also suppressed by as-miR-221 (Figure 3I). Consistent with this finding, miR-221 mimics also rescued the suppression of the S phase population of SkBr3 (Figure 3I) and BT474 (Figure S2F) cells by lapatinib. Although miR-221 and miR-222 share highly homologous sequences and are expressed as a gene cluster,<sup>36,37</sup> as-miR-221 showed a definite inhibitory effect in both parental and LR clones (Figure S3). Therefore, these data coherently suggested that miR-221 was upregulated to suppress p27<sup>kip1</sup> expression by targeting its 3'UTR and disturbed cell cycle progression in LR clones.

### 3.4 | miR-221 contributes to lapatinib resistance in breast cancer cells

To further confirm the essential role of miR-221 in conferring lapatinib resistance, the effect of as-miR-221 on the cell viability of both parental and LR cell lines was examined in an MTT assay. Inhibition of miR-221 expression by its anti-sense oligonucleotides suppressed the viability only in LR clones, but not in their parental cells (Figures 4A and S4A). These results suggested that miR-221 was the critical regulator in conferring cell survival in LR cell lines.

Next, miR-221-overexpressing stable clones were established from SkBr3 and BT474 cells to demonstrate the involvement of miR-221 in the acquired resistance to lapatinib. The expression of miR-221 was significantly higher compared with miR-222 in these clones (Figure S4B). As predicted, treatment



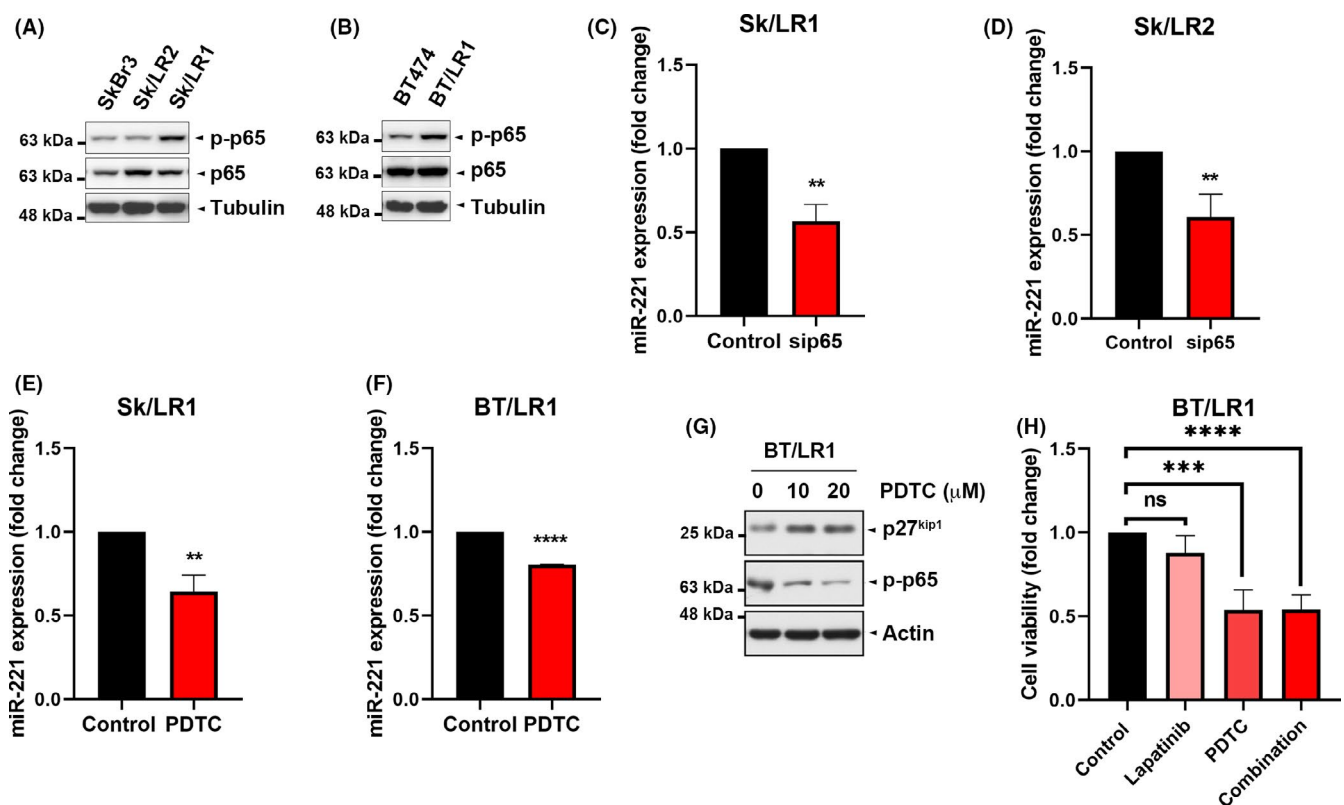
**FIGURE 4** miR-221 contributes to lapatinib resistance. A, Cell viability of SkBr3 cells and their LR clones transfected with different concentrations of as-miR-221 for 5 d and determined by MTT assay. Bar graph presents the fold change (mean  $\pm$  SD) of cell viability (Student *t* test, \**P* < .05, \*\**P* < .01, and \*\*\*\**P* < .0001). B, Colony formation of stable miR-221-overexpressing SkBr3 cells in response to lapatinib treatment for 7 d was determined in clonogenic assays. C, Cell viability of stable miR-221-expressing SkBr3 cells in response to lapatinib treatment for 7 d and determined by MTT assay. Bar graph presents the fold change of cell viability as mean  $\pm$  SD (Student *t* test, \**P* < .05). D, Inhibitory effects of miR-221 overexpression on lapatinib-induced apoptotic protein levels were determined in western blot analysis. E, SkBr3 cells transfected with different concentrations of miR-221 mimic followed by lapatinib treatment (1  $\mu$ mol/L, 24 h) were subjected to cell cycle analysis with PI staining. Bar graph shows the changes in the sub-G1 phase population (%) as mean  $\pm$  SD (Student *t* test, \**P* < .05, and \*\*\**P* < .001). F, G, Sk/LR1 cells transfected with different concentrations of as-miR-221 for 72 h were subjected to analyses for the indicated protein expression (F) and colony formation (G)

with lapatinib induced cell death of parental SkBr3 and BT474 cells, and this effect was rescued by overexpression of miR-221 in the clonogenic assay (Figures 4B and S4C) and MTT assay (Figures 4C and S4D). Consistently, overexpression of miR-221 also repressed the lapatinib-induced expression of proapoptotic protein Bim and the cleavage of PARP and caspase-3 (Figure 4D). To exclude the clonal effect of these results, miR-221 was transiently overexpressed in parental SkBr3 and BT474 cells and these cell apoptosis markers determined. Similarly, lapatinib-induced expression of Bim and cleavage of caspase-3 were suppressed by miR-221 in a dose-dependent manner (Figure S4E,F). Flow cytometric analysis further demonstrated that the lapatinib-induced sub-G1 population was reduced by transient overexpression of miR-221 (Figure 4E). Conversely, overexpression of as-miR-221 induced Bim expression and cleavage of caspase-3 and PARP (Figure 4F) and suppressed the colony formation of Sk/LR1 cells (Figure 4G). These data demonstrated that miR-221 contributes to the development of acquired resistance to lapatinib in breast cancer by attenuating cell cycle arrest and apoptosis.

### 3.5 | miR-221 is upregulated by Src/NF- $\kappa$ B signaling axis in response to lapatinib treatment

Transcription factor NF- $\kappa$ B has previously been reported to upregulate miR-221 in prostate carcinoma and glioblastoma cells.<sup>38,39</sup> The phosphorylation of p65, a subunit of NF- $\kappa$ B, was commonly increased in most LR clones compared with parental cells (Figure 5A,B). To further confirm the involvement of NF- $\kappa$ B activation in miR-221 upregulation in LR clones, p65/NF- $\kappa$ B expression was silenced by specific siRNA. As shown in Figure 5C,D, miR-221 expression was decreased by p65 siRNA compared with the control group. Moreover, inhibition of p65/NF- $\kappa$ B activity by pharmacological inhibitor pyrrolidine dithiocarbamate (PDTC) also suppressed miR-221 expression in RT-qPCR analysis (Figure 5E,F), further reversed p27 expression in western blot analysis (Figure 5G), and thereby inhibited the viability of LR cells (Figure 5H). These results supported the finding that the p65/NF- $\kappa$ B pathway is a critical regulator in lapatinib-induced miR-221 expression.

It has been recognized that Src induces gene transcription and cell survival through activation of NF- $\kappa$ B.<sup>40-42</sup> Our previous findings

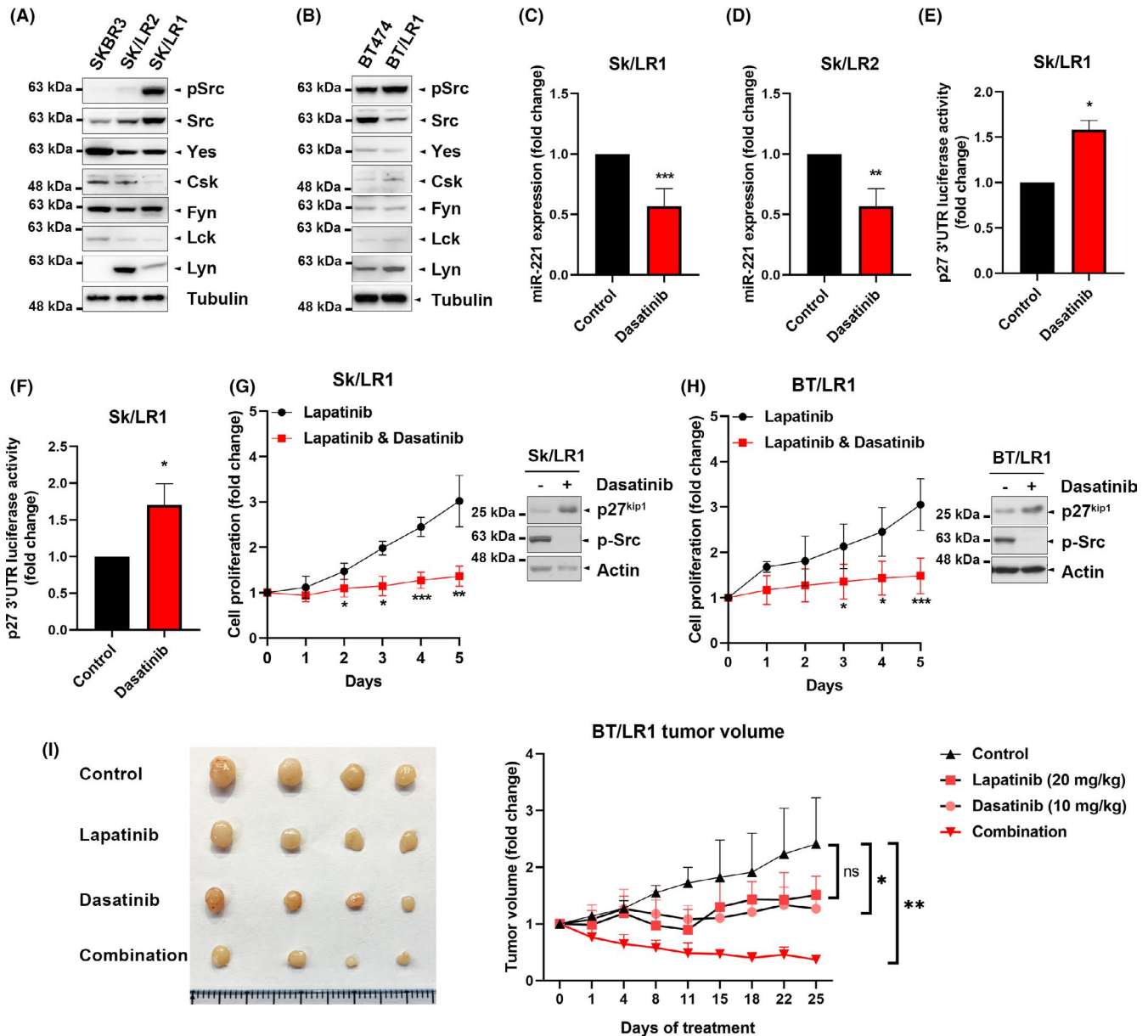


**FIGURE 5** NF- $\kappa$ B pathway involved in miR-221 upregulation in lapatinib-resistant clones. A, B, Levels of p65/p-p65 in SkBr3 or BT474 cells and their LR clones were determined in western blot analysis. C, D, Inhibitory effect of p65 siRNA on miR-221 expression in Sk/LR1 (c) and Sk/LR2 (d) cells was measured by RT-qPCR. Bar graph shows the fold changes of miR-221 level as mean  $\pm$  SD (Student t test, \*\* $P$  < .01). E, F, Inhibitory effect of NF- $\kappa$ B inhibitor PDTC (72 h, 10  $\mu$ mol/L) on miR-221 level in Sk/LR1 (E) and BT/LR1 (F) cells was measured by RT-qPCR. Bar graph shows the fold changes of miR-221 levels as mean  $\pm$  SD (Student t test, \*\* $P$  < .01, \*\*\*\* $P$  < .0001). G, Inhibitory effect of NF- $\kappa$ B inhibitor PDTC on the expression of p27<sup>kip1</sup> and p-p65 level in BT/LR1 cells as determined by western blot analysis. H, MTT assay determined the cell viability of BT/LR1 cells with lapatinib 1  $\mu$ mol/L and/or PDTC 10  $\mu$ mol/L for 24 h. Bar graph presents the fold change (mean  $\pm$  SD) of cell viability (Student t test, ns, not significant, \*\* $P$  < .01, and \*\*\*\* $P$  < .0001)



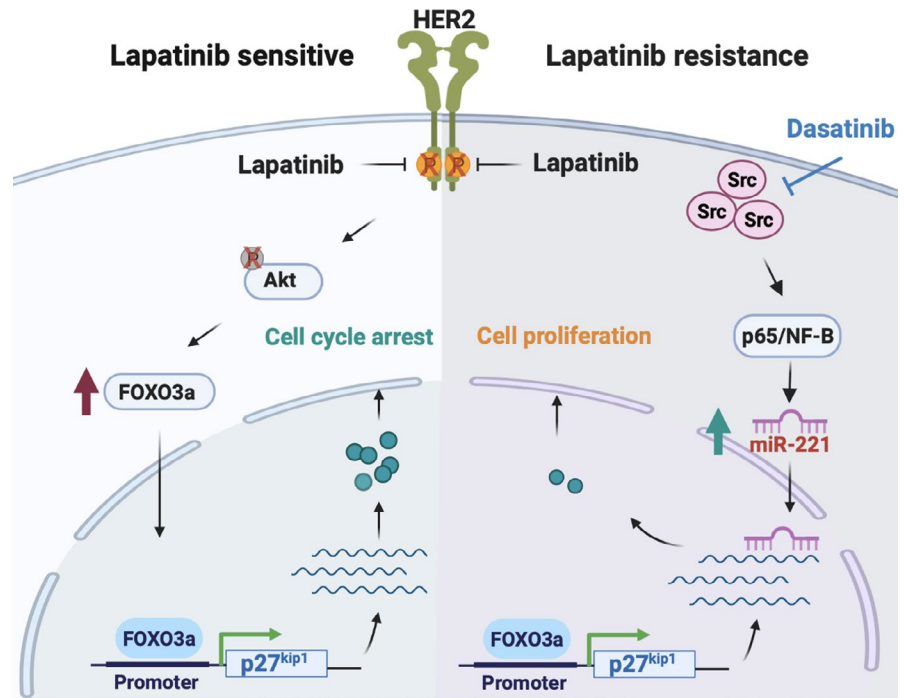
also demonstrated that Src activity was enhanced in lapatinib-resistant clones.<sup>24</sup> To study whether Src activation upregulated miR-221 expression in LR clones, we compared the expression of Src kinase family, including Src, Yes, Csk, Fyn, Lck, and Lyn in parental and LR clones. Consistent with the previous study,<sup>24</sup> Src phosphorylation was higher in LR clones compared with its parental

cells (Figure 6A,B). Inhibition of Src by dasatinib suppressed miR-221 expression in LR clones (Figures 6C,D and S5A) but not in parental cells (Figure S5B,C). In contrast, p27<sup>kip1</sup> 3'UTR luciferase activity was increased by dasatinib in these LR clones (Figure 6E,F). Therefore, Src inhibitor dasatinib only induced p27<sup>kip1</sup> expression to suppress cell proliferation of LR clones in the presence of lapatinib (Figure 6G,H),



**FIGURE 6** Src inhibition overcomes lapatinib resistance by decreasing miR-221 and increasing p27<sup>kip1</sup> expression. A, B, Activation and protein levels of Src kinase family in SKBr3 or BT474 cells and their LR clones were determined in western blot analysis with indicated antibodies. C, D, Inhibitory effect of Src inhibitor dasatinib (72 h, 1  $\mu$ mol/L) on the expression of miR-221 in Sk/LR1 (C) and Sk/LR2 (D) cells as measured by RT-qPCR. Bar graph indicates the fold change of miR-221 level as mean  $\pm$  SD (Student *t* test, \*\**P* < .01, \*\*\**P* < .001). E, F The inhibitory effect of Src inhibitor Dasatinib (72 h, 1  $\mu$ mol/L) on the p27<sup>kip1</sup> 3'UTR activity in Sk/LR1 (E) and Sk/LR2 (F) cells was determined in luciferase reporter assays. Bar graph shows the fold changes of p27<sup>kip1</sup> 3'UTR luciferase activity with normalization to  $\beta$ -galactosidase activity as mean  $\pm$  SD (Student *t* test, \**P* < .05). G, H, Sk/LR1 (G) and BT/LR1 (H) cells were treated with Src inhibitor dasatinib (72 h, 1  $\mu$ mol/L) and were then used to determine cell proliferation in IncuCyte analysis and p27<sup>kip1</sup> protein level in western blot analysis. Line graph presents the fold change of cell proliferation as mean  $\pm$  SD (Student *t* test, \**P* < .05, \*\**P* < .01, and \*\*\**P* < .001). I, BT/LR1 tumor xenografts excised from female BALB/c (nu/nu) nude mice after 25-d treatments with lapatinib (20 mg/kg) and/or dasatinib (10 mg/kg) as indicated (left) and the tumor growth rate in 4 treatment groups (right). Data are representative of *n* = 4 with normalization to tumor volume at day 0 of each group as the means  $\pm$  SD (Student *t* test at end point, ns, not significant, \**P* < .05; \*\**P* < .01)

**FIGURE 7** miR-221 confers lapatinib resistance by negatively regulating p27<sup>kip1</sup> expression in breast cancer cells. Inhibition of HER2/Akt axis by lapatinib elevated FOXO3a to mediate the expression of p27<sup>kip1</sup> for cell cycle arrest in parental HER2-positive breast cancer cells (left). However, activation of Src kinase in response to long-term treatment with lapatinib led to p65/NF- $\kappa$ B-dependent upregulation of miR-221, which represses p27<sup>kip1</sup> expression by targeting its 3'UTR and thereby accelerates cell proliferation



while it had no significant effect on parental cells (Figure S5E-G). These data indicated the dependent role of Src in LR cells to upregulate miR-221 expression.

To examine the effect of dasatinib on overcoming lapatinib resistance *in vivo*, we established and randomly assigned BT/LR1 tumor-xenograft nude mice to 4 treatment groups, including control (oil corn), single treatment with lapatinib (20 mg/kg) or dasatinib (10 mg/kg), and the combination treatment. After 25 d of treatments and observation, the combination treatment with lapatinib and dasatinib showed a synergistically inhibitory effect on tumor size and growth (Figure 6I). Additionally, no significant difference in the body weight of mice was found, suggesting no adverse toxicity was caused by the treatments (Figure S5H). Taken together, these data supported the idea that the combination treatment of lapatinib and dasatinib can overcome lapatinib resistance.

In summary, our study revealed that miR-221 upregulation suppressed p27<sup>kip1</sup> expression by targeting its 3'UTR region and thereby attenuated the inducing effect of lapatinib on cell cycle arrest and apoptosis, leading to lapatinib resistance in breast cancer. The induction of miR-221 in LR clones was associated with Src/p65/NF- $\kappa$ B activation, therefore targeting Src activity by dasatinib inhibitor would be a promising strategy to overcome lapatinib resistance (Figure 7).

## 4 | DISCUSSION

Lapatinib, an EGFR/HER2 dual tyrosine kinase inhibitor, is approved for HER2-positive advanced/metastatic breast cancer patients for blocking its well known tyrosine kinase activity and subsequent PI3K/Akt and MAPK pathways, leading to cell growth inhibition and cell apoptosis induction.<sup>43</sup> Unfortunately, many patients develop

acquired resistance to lapatinib. Several underlying molecular mechanisms have been proposed, including: (a) activation of compensatory signaling pathways such as RTK, RTK-activating ligand, and intracellular kinase activity,<sup>44,45</sup> (b) L755S and T798I mutations in HER2 tyrosine kinase domain,<sup>46,47</sup> and (c) activation of the NF- $\kappa$ B signaling pathway due to gene amplification of IKK $\beta$ -binding protein NIBP.<sup>48,49</sup> These mechanisms further activate the Akt signaling pathway against cell apoptosis. However, in our study, we demonstrated an Akt-independent pathway involving miR-221 upregulation and p27<sup>kip1</sup> downregulation in conferring lapatinib resistance.

Upregulation of miR-221, an onco-miRNA, has been described in several cancer types and has been considered to cause tumor progression by targeting the expression of cell cycle regulators such as p27<sup>kip1</sup> and p57.<sup>50-52</sup> In hepatocellular carcinoma, c-Met increased miR-221 expression to regulate c-Jun expression.<sup>53</sup> However, in comparison with parental cells, the increases in c-Met expression and its phosphorylation were not commonly observed in LR clones (data not shown), suggesting that c-Met is not the central regulator of miR-221 upregulation in response to lapatinib resistance. Although ERK1/2 activation has been reported to regulate miR-221 expression positively,<sup>54</sup> both Akt and ERK, downstream signaling pathways of HER2, were inhibited in the LR clones used in this study. Nevertheless, consistent with the previous findings that miR-221 is upregulated by p65 in prostate cancer and glioblastoma cells,<sup>38</sup> Src-activated NF- $\kappa$ B also mediated miR-221 upregulation in LR clones. Interestingly, miR-16 and miR-630 have also been reported to play a factor in determining the sensitivity of HER2-positive breast cancer cells to lapatinib.<sup>55,56</sup> MicroRNA-16 is also involved in NF- $\kappa$ B pathway regulation.<sup>57</sup> The interplay among miR-221, miR-16, and miR-221 in regulating the therapeutic efficacy of lapatinib would be worthy of further study.

p27<sup>kip1</sup> is an inhibitor of cell cycle progression from G1 to S phase by inhibiting cyclin E-CDK2.<sup>58</sup> The level of p27<sup>kip1</sup> expression is a prognostic factor of patients with breast cancer.<sup>59</sup> Expression of p27<sup>kip1</sup> can be downregulated at post-transcriptional levels through ubiquitination and proteasomal degradation during G1/S phase transition.<sup>60</sup> Overexpression of Src family and HER family activated p27<sup>kip1</sup> proteolysis in various human cancer types.<sup>61</sup> Src phosphorylates p27<sup>kip1</sup> at Tyr88 and Tyr74 to decrease p27<sup>kip1</sup> stability and reduce p27<sup>kip1</sup>-cyclin E-CDK2 complex formation during G1 progression.<sup>62</sup> Moreover, phosphorylation of p27<sup>kip1</sup> at Tyr88 by Src further induces the phosphorylation of p27<sup>kip1</sup> at Thr187 by the cyclin E-CDK2 complex, leading to SCF<sup>SKP1</sup> E3 ubiquitin-dependent ubiquitination and degradation of p27<sup>kip1</sup>.<sup>63</sup> Although our findings indicated the involvement of miR-221, but not the ubiquitination-proteasomal degradation pathway, in the downregulation of p27<sup>kip1</sup> in the LR cells, Src kinase seems to be the common upstream regulator of these mechanisms and is a potential therapeutic target to restore p27<sup>kip1</sup> expression and sensitivity to lapatinib.

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#### DISCLOSURE

The authors have no conflict of interest.

#### ORCID

Wei-Chien Huang  <https://orcid.org/0000-0001-6467-8716>

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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