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KRAS gene silencing inhibits the activation of PI3K-Akt-mTOR signaling pathway to regulate breast cancer cell epithelial-mesenchymal transition, proliferation and apoptosis

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Abstract. – OBJECTIVE: Our study was performed to investigate the effect of KRAS gene silencing on epithelial-mesenchymal transition (EMT), proliferation, and apoptosis of breast cancer cells by mediating PI3K-Akt-mTOR signaling pathway.

MATERIALS AND METHODS: The positive rate of KRAS protein expression was detected in tissues collected from breast cancer patients, associated with the analysis of the relationship between KRAS protein expression and clinicopathological features of patients. The expression of KRAS in breast cancer cell lines was tested to screen the suitable cell line. After cell transfection and grouping, qRT-PCR and Western blot were then used to detect the mRNA and protein expression in each group. MTT assay and flow cytometry detected cell proliferation, cell cycle, and apoptosis, respectively.

RESULTS: The expression of KRAS in cancer tissue was much higher than that in paracancerous normal tissue, and its high expression was correlated statistically with lymph node metastasis, distant metastasis, and tumor infiltration level of patients (all $p<0.05$). KRAS was highly expressed in T47D and thus selected as suitable cell lines for subsequent analysis (all $p<0.05$). Compared with Blank group and NC group, there were significantly reduced mRNA and protein expression of KRAS, PI3K, Akt, mTOR, N-cadherin and Vimentin, increased PTEN and E-cadherin, decreased cell proliferation activity, and increased apoptosis in si-KRAS and Wortmannin groups (all $p<0.05$); while opposite trends were found in HA-KRAS group and Recilisib group (all $p<0.05$). However, there was no significant difference when compared si-KRAS+Recilisib with that in Blank and NC groups (all $p>0.05$).

CONCLUSIONS: Silencing of KRAS gene expression may inhibit the activation of PI3K-Akt-mTOR signaling pathway, and thus inhibit EMT, proliferation and apoptosis of breast cancer cells. By contrast, activation of the studied signaling pathway can reverse the positive effect of KRAS gene silencing.

Key Words:
KRAS, PI3K-Akt-mTOR signaling pathway, Epithelial-mesenchymal transition, Breast cancer, Cell biological characteristics.

Introduction

Breast cancer is one of the most common malignant tumors in women, which is a local manifestation of systemic disease[1]. The age of onset is 40-60 years old commonly and it shows a trend of higher prevalence in young populations recently[2,3]. Surgery is the main therapeutic approach for breast cancer, which is frequently applied with chemotherapy, radiotherapy, endocrine therapy, and immunotherapy[4,5]. It deserves attention that the prognosis of patients with breast cancer is not so well, and there is a need to find out potential effective ways for its treatment. In the study of molecular biology, it has been found that the levels of Akt and mTOR gradually increased from normal mammary epithelial tissue to atypical hyperplasia, malignant transformation and tumor invasion, suggesting that phosphatidylinositol 3-kinase(PI3K)-Akt (also termed protein kinase B, PKB)-mammalian target of rapamycin (mTOR) signaling pathway is highly activated and may be essential in the progression of breast cancer[6,7]. It is one of the signaling pathways closely related to cell apoptosis and cell proliferation[8]. This pathway is usually highly activated in a variety of common malignant tumors such as breast cancer, which is critical in the regulation of cell cycle, apoptosis, angiogenesis etc.[9,10]. Therefore, inhibiting the function of this signaling pathway can help to eliminate cell proliferation signal, block cell cycle, and inhibit tumor growth, which provide us some implications to explore the

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pathogenesis, as well as diagnosis and treatment of breast cancer.

The ability of tumor cells to recur or transfer to other sites depends on tumor microenvironment[11]. Tumor microenvironment is such an internal environment in which tumor cells produce various cells, such as fibroblasts, immune, and inflammatory cells around them, as well as intercellular substance, micro-vessels and infiltrating cytokines in the nearby area[12-14]. Tumor cells can break away from cancer tissue, penetrate basement membrane, enter blood vessel or lymphatic system, which displays the increased cell motility[15,16]. As early as the 1980s, when some scholars studied chicken embryos, they found that epithelial cells can downregulate the characteristics of epithelium and transform them into stroma, which was described as the phenomenon of epithelial mesenchymal transition (EMT)[17,18]. EMT is defined as the process in which cells gradually lose markers of epithelial cells and acquire markers of interstitial cells[19]. In the course of EMT, epithelial cells gradually lose the intercellular connection, recombine the cytoskeleton and rearrange gene expression, resulting in a series of changes in signal transduction pathway and cell morphology[20]. After EMT, the expression of cell adhesion related genes such as E-cadherin may be downregulated, while N-cadherin and Vimentin expressions are upregulated[21,22]. It in turn may increase the mobility and invasiveness of cancer cells, which is conducive to cell deformation, and further smooth passage through the basement membrane and into the blood vessels, lymphatics, and body cavity[23]. EMT usually occurs during tumor progression or normal tissue repair[24]. The core of the interaction between tumor cells and microenvironment is EMT and epithelial transformation[25]. Therefore, it is hypothesized in the current study that EMT plays an important role in promoting local invasion and distant metastasis of breast cancer cells.

Considering that there are few reports concerning the role and mechanism of KRAS in breast cancer EMT *via* intervening PI3K-Akt-mTOR signaling pathway, the present study was thus carried out to investigate the effect of KRAS gene silencing on EMT, proliferation and apoptosis of breast cancer cells by mediating the proposed signaling pathway

Materials and Methods

Objects of Study and Sampling

From January 2014 to January 2017, cancer tissue and paracancerous normal tissue specimens from 84 cases of breast cancer were collected from our hospital, of which all eligible patients were confirmed by pathological examination. Diagnosis of patients with breast cancer was conducted based on the American Joint Committee on Cancer/International Union for Cancer Control Breast Cancer Staging System[26]. Of the enrolled patients, the age ranged from 29 to 71 years old, with a mean age of (50.44±7.25) years old. All patients were confirmed to have no other system tumors, no chemotherapy, radiotherapy, biotherapy, and other anti-tumor treatment before operation. Besides, the clinical data of all selected patients were complete. This study was carried out in accordance with the principle of voluntariness and in agreement with the Declaration of Helsinki, besides, the experimental protocol has been approved by the Local Ethics Committee of our hospital.

Immunohistochemistry

Specimens from each group were used for immunohistochemical analysis. Tissues were fixed by formaldehyde, embedded in paraffin, cut into continuous sections of 4 μm in thickness, followed by conventional dewaxing for 10 min with xylene I and II respectively, gradient ethanol dehydration, treatment with 3% H_2O_2 inactivating endogenous enzyme, and phosphate-buffered saline (PBS) rinsing three times (5 min each). After that, the normal goat serum was added for incubation at 37°C for 15 min. An amount of 20-30 μL PBS diluted KRAS (the primary antibody, 1:50, Abcam, Cambridge, MA, USA) was added and incubated overnight at 4°C. Following rinsing in 0.1 M PBS three times, the tissue slices were added with goat anti-rabbit IgG secondary antibody at 37°C for 20 min, followed by rinsing in PBS three times. Afterwards, streptomyces ovalbumin working solution labeled with horseradish peroxidase was added at 37°C for 20 min, followed by another rinsing in 0.1 M PBS three times. Then, 3,3'-diaminobenzidine tetrahydrochloride (DAB; Fuzhou Maixin Biotech. Co., Ltd., Fuzhou, Fujian, China) was used as chromogenic agent for signal recognition of the antigen for 5-10 min, followed by re-staining with hematoxylin for 5 min; re-bluing by 1% ammonia; dehydration with gradient ethanol at certain concentrations, xylene-transparent treatment and neutral resin sealing. For observation under the microscope, five high power visual fields were randomly selected. In tissue sections, the positive cells showed that the cytoplasm or membrane was stained from light yel-

low to brown, with positive staining determined, according to previous study[27]. The results of immunohistochemistry were scored blindly by two persons independently.

Cell Culture and Screening

Human breast cancer cells MCF-7, T47D, MDA-MB-231, and MDA-MB-453 were provided by Shanghai Institutes for Biological Science (Chinese Academy of Sciences, Shanghai, China). Each cell line was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Grand Island, NY, USA). The medium was supplemented with 10% fetal bovine serum (FBS) for subculture. Cells were routinely cultured, sub-cultured once every 2-3 d, and cultured to the 3rd-5th generations. The cells at logarithmic growth phase were taken for experiment. In order to determine the expression of KRAS in different breast cancer cell lines, qRT-PCR was used to detect the expression of KRAS in different breast cancer cell lines.

Cell Transfection and Grouping

There is difference in the effectiveness of the overexpression vector and small interfering RNA (si-RNA) of target gene designed according to different target gene and different site. In this research, the silencing expression (si-KRAS) and overexpression (HA-KRAS) sequences of KRAS were synthesized by Sangon Biotechnology Co., Ltd. (Xiangmin Road, Songjiang District, Shanghai, China) The recombinant plasmid was constructed by conventional method, and was transfected into human breast cancer cells by packaging retrovirus. After 48 h of transfection, the supernatant was collected for virus titer identification and stored in refrigerator at -80°C. The expression of si-KRAS and HA-KRAS was detected at protein level by Western blot to test the transfection efficiency.

Cells growing to logarithmic growth phase were selected for further inoculation in the 6-well plate (1×10^5 cells per well) with fresh and complete culture medium until the degree of cell confluence was close to 70%-80% for transfection. In the next step, transfection was carried out according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The screened cells were divided into Blank group (human breast cancer cells without transfection), NC group (transfection of blank vector plasmid), HA-KRAS group (transfection of KRAS overexpression vector plasmid), si-KRAS group (transfection of KRAS silencing vector plasmid), Wortmannin group (transfection of pathway antagonist), Recilisib group (transfection of pathway agonist), si-KRAS+Recilisib group (transfection of pathway antagonist and KRAS silencing vector plasmid), respectively.

qRT-PCR

After collection and treatment, the total RNA in cells was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) method. Following the reverse transcription of RNA to cDNA, the cDNA obtained from the above step was diluted with 65 μl DEPC solution to mix well. Real Time-PCR reaction system was prepared, and PCR amplification conditions were described as follows: pre-degeneration at 95°C for 1 min, degeneration at 95°Cfor 30 s, annealing at 58°C for 5 s, in a total of 30 cycles, followed by final extension at 72°C for 5 s. The sequence for PCR was synthesized by BGI Biotechnology Co., Ltd. (Wuhan, Hubei, China) (Table I). With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference, each gene of each sample was set with 3 replicates. The CT value (inflection point of amplification dynamic curve) was taken for calculation by using $2^{-\Delta\Delta Ct}$ method, with three repeats of the experiment. The detection procedure in tissues was similar as that in cells.

Western Blot

Extraction of total protein was achieved by radioimmunoprecipitation assay buffer (RIPA) lysate containing PMSF, followed by homogenized breaking in ice bath (intermittent breaking, 6 s×6 times) and centrifugation, and the supernatant was the extraction of total protein. After 5 min of boiling water bath, the samples were stored at -80°C for further use. An equal amount of total cell protein was taken for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then, transferred to polyvinylidene difluoride (PVDF) membrane, with the membrane washed once by Tris-Buffered Saline and Tween-20 (TBST). After sealing with 5% skimmed milk powder, shaking in the table concentrator for 2 h, and TBST washing three times, the pre-diluted primary antibody was added, including rabbit polyclonal antibody KRAS (1:200, ab180772), rabbit monoclonal antibody PI3K (1:1000, ab32089), rabbit polyclonal antibody Akt (1:500, ab38449), rabbit polyclonal antibody mTOR (1:2000, ab2732), rabbit polyclonal antibody PTEN (1:2000, ab137337), rabbit monoclonal antibody E-cadherin (1:10000, ab40772), rabbit polyclonal antibody N-cadherin

Table I. Primer sequences of KRAS, PI3K, Akt, mTOR, PTEN, E-cadherin, N-cadherin, Vimentin, and GAPDH for qRT-PCR.

Genes	Primer sequences
KRAS	F: 5'-GGCCTGCTGAAAATGACTGAATAT-3' R: 5'-CCTCTATTGTTGGATCATATTCGT-3'
PI3K	F: 5'-CGTTTCTGCTTTGGGACAAC-3' R: 5'-CCTGATGATGGTCGTGGAG-3'
Akt	F: 5'-TGAGAGAAGCCACGCTGTC-3' R: 5'-CGGAGAACAAACTGGATGAA-3'
mTOR	F: 5'-ATTTAAAGCACCAGCCATCG-3' F: 5'-TCTTTAGGAAGGCACGCAGT-3'
PTEN	F: 5'-AGACCATAACCCACCACAGC-3' R: 5'-ACACCAGTTCGTCCCTTTCC-3'
E-cadherin	F: 5'-GAGTG CCAACTGGACCATTCAGTA-3' R: 5'-AGTCACCCACCTCTAAGGCCATC-3'
N-cadherin	F: 5'-CCGGAGAACAGTCTCCAACTC-3' R: 5'-CCCACAAACAGCACCAGTC-3'
Vimentin	F: 5'-ACGCCATCAACACCGAGTT-3' R: 5'-CTTTGTCGTTGGTTAGCTGGT-3'
GAPDH	F: 5'-ACCAGCCTCTGGCTTCTACA-3' R: 5'-CCCTAGCTGTGTGCCTTCTC-3'

(1:1000, ab76057), rabbit monoclonal antibody Vimentin (1:2000, ab92547) and rabbit polyclonal antibody GAPDH (1:2500, ab9458), followed by overnight incubation at 4°C. All the primary antibodies were provided by Abcam (Cambridge, MA, USA). After recovery to 37°C by table concentrator and 3 times TBST washing, the pre-diluted HRP labeled second antibody was added for 2 h of incubation at 37°C. After three times TBST washing, the solution A and solution B in the enhanced chemiluminescence (ECL) fluorescence detection kit (Amersham, UK) of the same amount were taken for mixing in the dark, and dropped onto the membrane for photography by Gel Imaging System (E-Gel® Imager, Beijing Liuyi Biotechnology Co., Ltd., Beijing, China) following chemiluminescence. The gray value was measured by Quantity One v4.6.2 software (Bio-Rad, Hercules, CA, USA), and the gray value of target gene/gray value of GAPDH was taken as the relative expression of the target protein. The detection procedure in tissues was similar as that in the cells.

MTT Assay

After transfection, the cells in the logarithmic growth phase were diluted to prepare single cell suspension. With the adjustment of cell concentration to 2.0×10^7/L, the cells were inoculated to the 96-well plate with three replicates per group to adjust cell density to about 5×10^3 cells per well. Then, the cells were cultured in a CO_2 incubator at 37°C for 24 h, 48 h, 72 h, and 96 h, respectively, followed by the addition of 10 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) per well in the dark for 4 h. With careful absorption of the culture medium in the 96-well plate, 100 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added, and the absorbance (OD) value was measured at wavelength of 570 nm by Microplate Reader (BioTek, Winooski, VT, USA) for reflecting cell viability to evaluate cell proliferation.

Annexin V-FITC/PI Double Parameter Flow Cytometry

After transfection, the cells were digested by trypsin without ethylenediamine tetraacetic acid (EDTA) and collected in flow cytometry tube (dead cells and apoptotic cells in supernatant and solution were both included in the statistics). After centrifugation and discarding of the supernatant, the cells were washed with pre-cooled PBS repeatedly three times, and the supernatant was discarded after centrifugation. According to Annexin V-FITC/PI double staining cell apoptosis detection kit (Dojindo Molecular Technologies, Inc., Beijing, China), the apoptosis was tested following the addition of 150 µl binding buffer and 5 µl Annexin-V-fluorescein isothiocyanate (FITC). No Annexin V was added in the control as the correction factor. Apoptosis was detected by flow cytometry within 1 h as much as possible. The results of flow cytometry was displayed in four grid diagram[28]. The experiment was repeated three times.

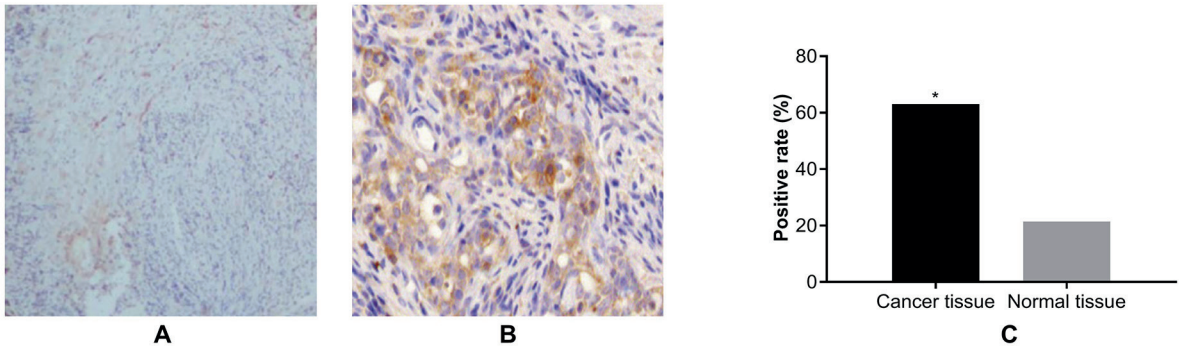

Figure 1. The difference of KRAS expression between human breast cancer and normal tissue. **A,** Immunohistochemical detection of KRAS protein in cancer tissues (× 200). **B,** Immunohistochemical detection of KRAS protein in normal tissues (× 200). **C,** The difference of KRAS protein expression between cancer and normal tissues; *Compared with the normal tissues, $p < 0.05$.

Statistical Analysis

Data were processed by SPSS 22.0 (IBM Corp., Armonk, NY, USA) statistical software. The measurement data following normal distribution were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used for variance analysis and significance test among multiple groups, and the Bonferroni post-hoc test was used to assess pairwise comparisons. The counting data was expressed by percentage and analyzed by chi-square test. A p-value of <0.05 meant that the difference was statistically significant.

Results

Positive Rate of KRAS Expression and Relationship with Clinicopathological Features in Breast Cancer Patients

The expression of KRAS protein was located in the cytoplasm, and the positive cells were yellow to brownish yellow granules, which were diffusely distributed in the cytoplasm, some of which were distributed in mass and in cluster (Figure 1). The results of immunohistochemistry showed a positive expression of KRAS in 53 cancer tissues, and the positive rate was 63.10%; while the positive expression of KRAS was found in 18 normal tissues, with the positive rate of 21.43%. The positive rate of KRAS in cancer tissues was significantly higher than that in normal tissues ($p < 0.05$).

According to the results of the relationship between KRAS positive expression and clinicopathological features in patients with breast cancer, it was revealed that KRAS protein expression was related to lymph node metastasis, distant metastasis, and tumor infiltration level in breast cancer patients (all $p<0.05$). However, the expression of KRAS protein showed no correlation with patients' age, ER status, PR status, Ki-67 proliferation index, local recurrence, and diameter of primary tumor (all $p>0.05$) (Table II).

Screening of Suitable Breast Cancer Cell Line by Detecting the Expression Level of KRAS Gene

According to qRT-PCR results, KRAS mRNA expression was the highest in T47D cell line ($p < 0.05$). There was no evident difference in mRNA expression of the remaining cell lines (all $p>0.05$). The results of Western blotting further confirmed the protein expression of KRAS in these breast cancer cells, which was basically consistent with the mRNA expression trend of KRAS mRNA. Considering the highest expression of KRAS in T47D (Figure 2), it was selected for subsequent transfection.

KRAS Gene Silencing Inhibited the Activation of PI3K-Akt-mTOR Signaling Pathway in Breast Cancer Cells

In T47D cell line, the mRNA and protein expression of KRAS, PI3K, Akt, mTOR, and PTEN in cells of each group were detected by using qRT-PCR and Western blotting (Figure 3). According to the results, compared with Blank group, the mRNA and protein expression of KRAS, PI3K, Akt, and mTOR were significantly decreased in the si-KRAS group and Wortmannin group, and there was an increase in the expression of PTEN

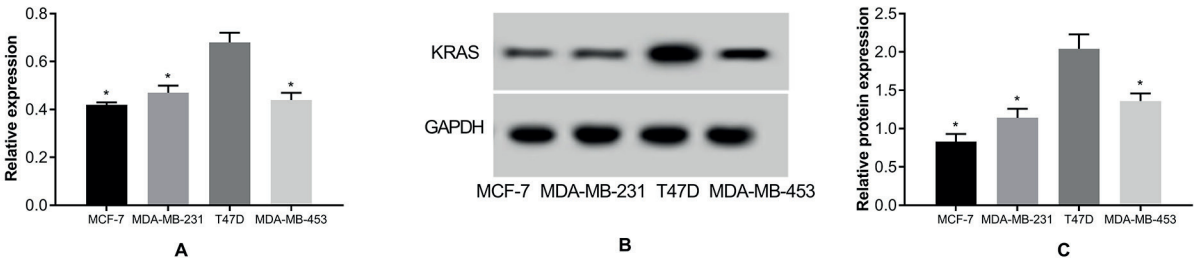

Figure 2. Expression of KRAS in human breast cancer cell lines. **A,** Relative expression of KRAS mRNA in cancer cell lines by qRT-PCR. **B,** Protein expression of KRAS in cancer cell lines by Western blotting. **C,** Statistical analysis of protein expression of KRAS in cancer cell lines by Western blotting. *Compared with T47D cells, $p<0.05$.

(all $p<0.05$). While there were opposite trends in the detection of the above indexes in HA-KRAS group and Recilisib group (all $p<0.05$). Furthermore, KRAS expression was decreased in si-KRAS+Recilisib group ($p<0.05$), but no evident statistical difference was found in the remaining indexes (all $p>0.05$). Nevertheless, no evident difference was measured between Blank group and NC group, or between si-KRAS group and Wortmannin group (all $p>0.05$). The above results suggested that KRAS gene silencing could inhibit the activation of the studied pathway.

Table II. The relationship between KRAS protein and clinicopathological characteristics of patients with breast cancer.

Factors		Cases	KRAS		χ^2	*p*-value
			Positive	Negative		
Age (years)					0.145	0.704
	<45	51	33	18		
	≥45	33	20	13		
ER status					1.027	0.311
	-	44	30	14		
	+	40	23	17		
PR status				2.529	0.112	
	-	50	35	15		
	+	34	18	16		
Lymph node metastasis				8.928	0.012	
	N0	34	15	19		
	N1-N4	23	18	5		
	≥5	27	20	7		
Ki-67 proliferation index (%)				0.588	0.443	
	≤50	61	40	21		
	>50	23	13	10		
Local recurrence				0.327	0.567	
	Without	60	39	21		
	With	24	14	10		
	Distant metastasis				7.489	0.006
	Without	46	23	23		
	With	38	30	8		
	Diameter of primary tumor				0.753	0.686
	<2 cm	20	11	9		
	2-5 cm	43	28	15		
	≥5 cm	21	14	7		
	Tissue infiltration level				8.868	0.031
	I	27	11	16		
	II	42	31	11		
	III	14	10	4		
	IV	1	1	0		

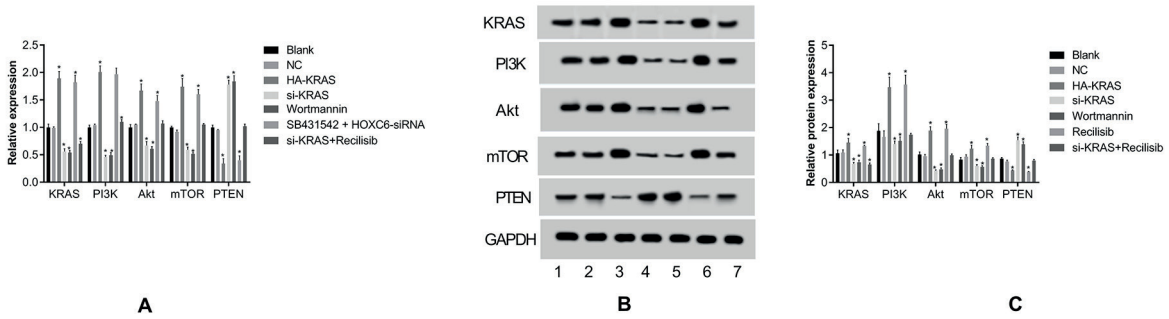

Figure 3. Detection of mRNA and protein expression of KRAS and indexes related to PI3K-Akt-mTOR signaling pathway in T47D cell line after transfection. **A,** Relative expression in T47D cell line by qRT-PCR. **B,** Protein expression in T47D cell line by Western blotting. **C,** Statistical analysis of protein expression in T47D cell lines by Western blotting; *compared with Blank group, $p<0.05$. (1, Blank; 2, NC; 3, HA-KRAS; 4, si-KRAS; 5, Wortmannin; 6, Recilisib; 7, si-KRAS+Recilisib).

KRAS Gene Silencing Inhibited the EMT of Breast Cancer Cells

Compared with Blank group, mRNA and protein expression of N-cadherin and Vimentin were evidently increased in HA-KRAS group and Recilisib group, and the those of E-cadherin were markedly decreased (all $p<0.05$). Besides, N-cadherin and Vimentin were significantly lowly expressed in si-KRAS group and Wortmannin group, and E-cadherin was highly expressed (all $p<0.05$). No statistical difference was found in the si-KRAS+Recilisib group when compared with Blank group and NC group (all $p>0.05$). Meanwhile, there was no statistical difference between Blank group and NC group, or between si-KRAS group and Wortmannin group (all $p>0.05$) (Figure 4).

Inhibition of Proliferation of Breast Cancer Cells by KRAS Gene Silencing

MTT assay was used to detect cell viability of T47D cells in each group after transfection. The results showed that the cell viability of each group showed an upward trend in each time of the test. Compared with the Blank group, there was significant increase in cell viability of HA-KRAS group and Recilisib group (all $p < 0.05$), while it was significantly inhibited in the si-KRAS group and Wortmannin group (all $p < 0.05$), with no difference when compared with NC group ($p > 0.05$). Besides, there was no statistical difference in si-KRAS+Recilisib group when compared with Blank group and NC group (all $p > 0.05$). No statistical difference was found between si-KRAS group and Wortmannin group ($p > 0.05$, Figure 5). The results show that the proliferation of breast cancer T47D cells can be inhibited by silencing KRAS gene through the activation of PI3K-Akt-mTOR signaling pathway.

Promotion of Apoptosis of Breast Cancer Cells by KRAS Gene Silencing

Flow cytometry was used to detect cell apoptosis. The results (Figure 6) showed that compared with the Blank group, there was significant de-

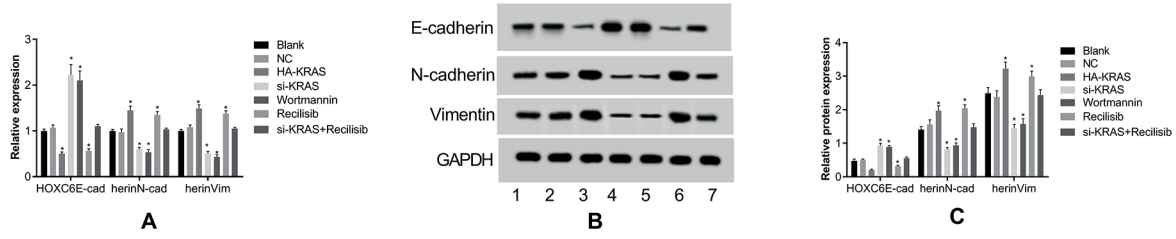

Figure 4. Detection of EMT related indexes E-cadherin, N-cadherin and Vimentin in T47D cell line after transfection. **A,** Relative expression of E-cadherin, N-cadherin and Vimentin in T47D cell line by qRT-PCR. **B,** Protein expression of E-cadherin, N-cadherin and Vimentin in T47D cell line by Western blotting. **C,** Statistical analysis of protein expression of E-cadherin, N-cadherin and Vimentin in T47D cell lines by Western blotting; *compared with Blank group, $p<0.05$. (1, Blank; 2, NC; 3, HA-KRAS; 4, si-KRAS; 5, Wortmannin; 6, Recilisib; 7, si-KRAS+Recilisib).

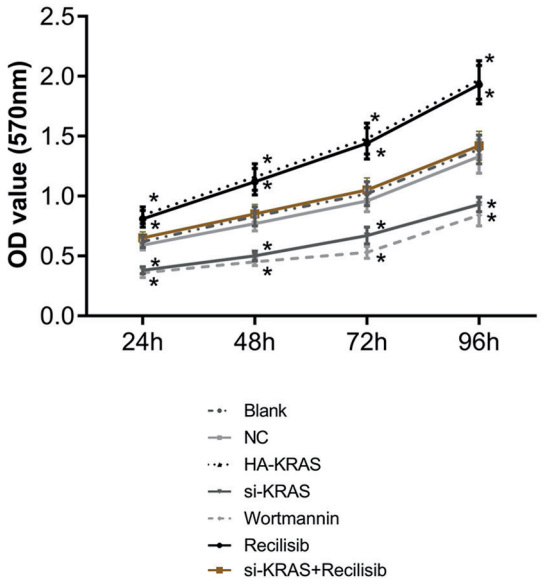

Figure 5. Cell proliferation in T47D cell lines after transfection to reflect the effect of KRAS on the proliferation of breast cancer cell line T47D. Note: OD: optical density. *Compared with Blank group, $p<0.05$.

crease in the apoptosis rate of HA-KRAS group and Recilisib group (all $p < 0.05$), while it was significantly increased in the si-KRAS group and Wortmannin group (all $p < 0.05$), with no difference when compared with NC group ($p > 0.05$). Besides, there was no statistical difference in the si-KRAS+Recilisib group when compared with Blank group and NC group ($p > 0.05$). No statistical difference was found between si-KRAS group and Wortmannin group ($p > 0.05$). The above results showed that silencing of KRAS gene and inhibition of PI3K-Akt-mTOR signaling pathway can induce the apoptosis of tumor cells, whereas the activation of PI3K-Akt-mTOR signaling pathway may reverse the positive role of KRAS gene silencing.

Discussion

EMT is a dynamic process manifested by the decrease of intercellular adhesion, the enhancement of cell mobility, and the change of intercellular connections and cell polarity[29]. EMT is an important process in the early stage of tumor invasion and metastasis[30]. E-cadherin is located on the cell surface of epithelial tissue, which mediates cell-cell adhesion and makes cells unite in normal epithelial tissue[31]. Its expression is opposite to that of EMT and tumor invasion[32]. Meanwhile, N-cadherin and Vimentin are related to EMT and the development of aggressive tumors from well differentiated tumors[33]. Significantly, microenvironment stimuli may regulate EMT by regulating certain signaling pathways[34,35].

Our study was carried out with the expectation of exploring the effect of KRAS gene silencing on EMT, proliferation and apoptosis of breast cancer cells by mediating PI3K-Akt-mTOR signaling pathway. Firstly, KRAS expression was detected in cancer tissues associated with the analysis of its relationship with the clinicopathological features of patients. The results revealed that the positive rate of KRAS in cancer tissues was significantly higher than that in paracancerous normal tissue. Besides, positive expression of KRAS was related to lymph node metastasis, distant metastasis, and tumor infiltration level in breast cancer patients, suggesting that abnormal high expression of KRAS may aggravate the development of breast cancer. In this regard, our study focused on exploring the effect of KRAS gene silencing on the progression of breast cancer.

In our next step, with the screening of suitable breast cancer cell line for further *in vivo* experiment, it was found that KRAS mRNA expression was the highest in T47D cell line, which was selected for subsequent transfection. Notably, to explore the relationship of KRAS gene silencing with the activation of PI3K-Akt-mTOR signaling pathway, different cell transfection groups were constructed. In the process, our experiment used Wortmannin and Recilisib for the inhibition and activation of PI3K-Akt-mTOR signaling pathway. To be specific, Wortmannin is a commonly applied inhibitor of PI3K, which is known to mediate DNA double strand break repair[36]. It can penetrate cells and combine with the 110kD catalytic subunit of PI3K to specifically inhibit PI3K and PI3K/Akt signaling pathway[37]. In practical research, it can be used for *in vitro* research, *in vivo* research, and animal experiment to clarify the role of PI3K-Akt-mTOR pathway for mechanism exploration[38]. Meanwhile, Recilisib is a radiation protective agent, which can activate the activity of Akt and PI3K in cells, exhibiting its role in the activation and mediating of PI3K-Akt-mTOR signaling pathway[39], opposite to that of Wortmannin. According to the results, compared with Blank control, there were evident decrease in the mRNA and protein expression of KRAS, PI3K, Akt, and mTOR, while increase in the ex-

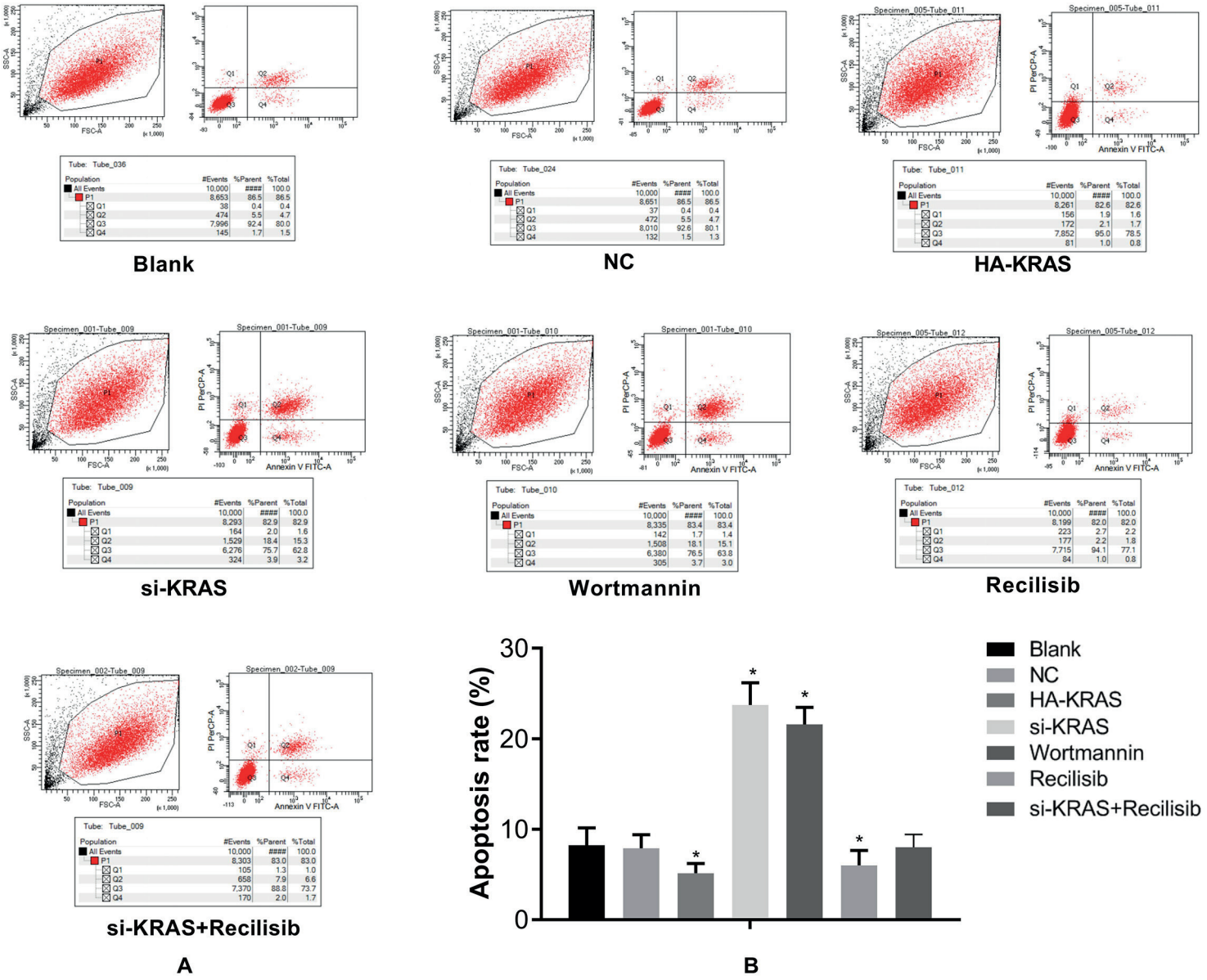

Figure 6. Changes of apoptosis ability in each group after transfection to reflect the effect of KRAS on the apoptosis of breast cancer cell line T47D. **A,** Flow cytometry detection of cell apoptosis in T47D cell line after transfection. **B,** Percentage of apoptosis in T47D cell line after transfection; *Compared with Blank group, $p<0.05$.

pression of PTEN after KRAS gene silencing and inhibited activation of the studied pathway using Wortmannin. While an opposite trend was found with the overexpression of KRAS and activation of the studied pathway using Recilisib. Furthermore, except for a decrease in KRAS expression following treatment with KRAS gene silencing and pathway activation, no significant difference was found in the remaining indexes. The above results suggested that KRAS gene silencing could inhibit the activation of the studied pathway. After the identification of the association of KRAS gene silencing with the role of the studied pathway, subsequent experiment identified the role of KRAS gene silencing in the EMT of breast cancer cells. The expression of N-cadherin and Vimentin were evidently increased, and that of E-cadherin was markedly decreased with the overexpression of KRAS and pathway activation. While these expression trends were reversed after KRAS gene silencing and inhibited pathway activation. These results revealed that the expression changes of molecules related EMT by silencing KRAS gene was speculated to be related to the inhibited activation of the studied pathway. Besides, no significant difference was found under KRAS gene silencing combined with pathway activation. It indicates that the activation of the studied pathway reversed the beneficial role of KRAS gene silencing, which also supports our hypothesis concerning the role of KRAS gene silencing and the auxiliary effect of inhibited activation of the above pathway from another side.

On the basis of the above-mentioned investigation, our study further explored the biological effect of KRAS gene silencing which inhibited the activation of the studied pathway on the proliferation and apoptosis of breast cancer cells. Consequently, it was discovered that there was a decrease in cell proliferation activity and increase in the apoptosis after KRAS gene silencing and inhibited pathway activation; while an opposite finding was found after KRAS overexpression and pathway activation. It supports the positive role of KRAS silencing mediating PI3K-Akt-mTOR in regulating biological properties of breast cancer cells. Besides, an aggravation was found after KRAS gene silencing combined with pathway activation, which also proves the synergistic reversal effect of KRAS gene silencing and inhibited pathway activation. Considering possible mechanism of action, it was speculated that KRAS is an oncogene, and its silenced expression may exert anti-tumor effect possibly, which may be realized by the inhibition of the activation of the studied pathway. Specifically, silenced expression of KRAS may inhibit the over-activation of PI3K, Akt and mTOR, while stimulating the role of tumor suppression gene PTEN, and regulating EMT related genes to suppress the progression of EMT, which may hence reduce tumor cell motility, while increasing cell adhesion, so as to significantly weak the invasion of tumor cells. Our study findings were also consistent with previous research. Dong et al[40] reported the role of PI3K-Akt activation in inducing EMT in endometrial cancer cells and proposed that the method of introducing tumor suppressive or knockdown of oncogenic target could be a promising approach to inhibit the PI3K-Akt pathway in endometrial cancer. In addition, Guo et al[41] also reported potential mechanism of certain oncogene to induce EMT through activation of the studied pathway, which supports the positive role of silenced expression of oncogene in inhibiting EMT through the inhibited activation of the PI3K-Akt-mTOR. They provide our study with molecular biological basis[41].

Conclusions

The present research reports for the first time that the silencing of KRAS gene may inhibit the activation of PI3K-Akt-mTOR signaling pathway, and thus inhibit the EMT, proliferation and apoptosis of breast cancer cells. By contrast, activation of the proposed pathway can reverse the positive effect of KRAS gene silencing. As for the novelty of our study, in addition to *in vivo* clinical experiment and *in vitro* cell experiment to explore the role of gene silencing and overexpression in breast cancer, this investigation also explores the role of PI3K-Akt-mTOR signaling pathway inhibitor and agonist, with the establishment of si-KRAS+Recilisib group to further verify the role of KRAS gene silencing in breast cancer by inhibiting the proposed pathway activation. Collectively, we indicate a promising therapeutic approach of targeting key signaling components or silencing oncogene by intervening signaling pathways to suppress EMT and cell biological properties in breast cancer.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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