Research Article
AK4 Promotes the Progression of HER2-Positive Breast Cancer by Facilitating Cell Proliferation and Invasion

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Breast cancer (BC) is a type of malignant tumor originating from the epithelial tissue of the mammary gland, and about 20% of breast cancers are human epidermal growth factor receptor 2 positive (HER2+), which is a subtype with more aggression. Recently, HER2-positive breast cancer is often accompanied by poor prognosis of patients, and targeted therapy showed a promising prospect. To combat this disease, novel therapeutic targets are still needed. Adenylate kinase 4 (AK4) is a member of the adenylate kinase family and is expressed in the mitochondrial matrix. AK4 is involved in multiple cellular functions such as energy metabolism homeostasis. Interestingly, AK4 was observed highly expressed in several tumor tissues, and the involvement of AK4 in cancer development was generally revealed. However, the possible role of AK4 on the growth and development of breast cancer is still unclear. Here, we investigated the possible functions of AK4 on the progression of HER2-positive breast cancer. We found the high expression of AK4 in HER2-positive breast cancer tissues from patients who received surgical treatment. Additionally, AK4 expression levels were obviously correlated with clinical-pathological features, including pTNM stage (P = 0.017) and lymph node metastasis (P = 0.046). We mechanically confirmed that AK4 depletion showed the obvious impairment of cell proliferation and invasion in MCF7 and MDA-MB-231 cells. AK4 also facilitates tumor growth and metastasis of HER2-positive breast cancer in vivo. In conclusion, we identified and mechanically confirmed that AK4 is a novel therapeutic target of HER2-positive breast cancer.

1. Introduction

Breast cancer (BC) is a type of malignant tumor that occurs in the epithelial tissue of the mammary gland, and approximately 99% of breast cancers occur in women [1, 2]. Breast cancers are usually classified into estrogen receptor-positive (ER+), human epidermal growth factor receptor 2-positive (HER2+), or triple-negative breast cancer (TNBC) [3]. About 20% of breast cancers are an overexpression of HER2, a gene correlated with more aggression compared with other subtypes [4, 5]. Despite advances in the treatment of patients with this disease, it is often accompanied by poor prognosis of patients [6]. Traditional treatment of HER2-positive breast cancer relies on aromatase inhibitors and antiestrogens [7, 8]. Recently, targeted therapy showed a promising prospect on the treatment of this disease [9, 10]. Several targeted drugs
have achieved good clinical results [11]. However, to get a better clinical outcome, novel therapeutic targets are urgently needed.

Adenylate kinases are a class of nucleoside monophosphate kinases involved in various cellular functions, such as the regulation of energy metabolism homeostasis [12, 13]. Adenylate kinase 4 (AK4) is a member of the adenylate kinase family and is expressed in the mitochondrial matrix [14]. Previous studies indicated that AK4 was enzymatically inactive in vitro, but could still retain the capacity of nucleotide binding, so that it could interact with the ADP/ATP translocator and promote cell proliferation and survival [15, 16].

Interestingly, the involvement of the adenylate kinase family in the progression of multiple cancers was observed, and previous studies also reported that adenylate kinases were obviously highly expressed during tumor metastasis progression [17]. In fact, AK4 was markedly overexpressed in highly invasive tumors, such as lung cancer and esophageal cancer [17, 18]. AK4 is involved in the regulation of hypoxia tolerance and mitochondrial activity, further affecting cancer development [17]. Additionally, AK4 promotes the metastasis and recurrence of lung cancer through an Activating Transcription Factor 3- (ATF3-) dependent manner [19]. AK4 could mediate adenosine 5'-monophosphate-(AMP-) activated protein kinase (AMPK) signaling and further promote the survival of glioma patients, suggesting possible effects on the progression of glioma [20]. Despite the extensive involvement of AK4 in tumorigenesis, the possible role of AK4 on the growth and development of breast cancer is still unclear.

Herein, we evaluated the potential effects of AK4 on the progression of HER2-positive breast cancer, and this study is aimed at exploring the function of AK4 in HER2-positive breast cancer.

2. Materials and Methods

We followed the methods of Li et al. in 2019 [21]: the IHC assay, cell culture and transfection, quantitative PCR assay, immunoblot assay, colony formation assay, wound closure assay, transwell assay, and in vivo study.

2.1. Antibodies, Primers, and shRNA Plasmids. Rabbit anti-AK4 was used at 1:200 dilution for IHC assays and at 1:1000 dilution for immunoblot assays (ab131327, Abcam, Cambridge, UK); rabbit anti-β-actin was used at 1:1000 dilution (ab8227, Abcam, Cambridge, UK).

The quantitative PCR primer sequences of AK4 are as follows: forward, 5'-ATGGACCGTCGCTGCTGAAAGT-3' and reverse, 5'-CTCGAAACTTCTCTTGCTGGCTC-3'. The quantitative PCR primer sequences of β-actin are as follows: 5'-CAGCTCACCATGGATGATGATATC-3' and reverse, 5'-AAGCCGGGCTTTGCACAT-3'.

Ready-to-package AAV AK4 shRNA plasmids were bought from Addgene pLC. The shRNA sequence targeting AK4 are as follows: sense, 5'-AACCTTTGGTCTCCAGCATCTCTC-3'.

Table 1: Relationships of AK4 and clinicopathological characteristics in 98 patients with HER2 overexpression breast cancer.

| Features                  | All n = 98 | AK4 expression | χ²   | P     |
|---------------------------|------------|----------------|------|-------|
| Age (year)                |            |                |      |       |
| <50                       | 50         | 18             | 32   |       |
| ≥50                       | 48         | 20             | 28   |       |
| Tumor grade               |            |                |      |       |
| Low                       | 54         | 19             | 35   | 0.653 | 0.419 |
| High                      | 44         | 19             | 25   |       |
| Tumor size                |            |                |      |       |
| <2                        | 56         | 24             | 32   | 0.917 | 0.338 |
| ≥2                        | 42         | 14             | 28   |       |
| pTNM stage                |            |                |      |       |
| I-II                      | 62         | 30             | 32   | 5.674 | 0.017*|
| III-IV                    | 36         | 8              | 26   |       |
| Lymph node metastasis     |            |                |      |       |
| Yes                       | 46         | 12             | 34   | 3.967 | 0.046*|
| No                        | 52         | 22             | 26   |       |
| Vascular invasion         |            |                |      |       |
| Yes                       | 42         | 20             | 22   | 2.421 | 0.120 |
| No                        | 56         | 18             | 38   |       |

2.2. Human Tissue Samples and Analysis. The 98 human HER2-positive breast cancer tissues and adjacent non-tumor tissues examined in this study were collected from patients receiving surgical treatment in the Sun Yat-sen Memorial Hospital and Academy of Medical Sciences & Sichuan Provincial People’s Hospital and Chinese Academy of Sciences Sichuan Translational Medicine Research Hospital from 2008 to 2018. Patients were classified according to the TNM staging. All patients had no preoperative radiotherapy and/or chemotherapy and neoadjuvant chemotherapy. All cases were clearly diagnosed by postoperative pathology, and no serious complications such as cardiovascular and cerebrovascular diseases occurred before and after surgery. The clinical characteristics, such as patient age, tumor grade, tumor size, and pTNM stage are recorded and listed in Table 1.

To explore the correlation between AK4 expression levels and the progression of HER2-positive breast cancer, IHC assays were performed. Briefly, sample sections were fixed with 4% polyfluoralkoxy (PFA) for 30 minutes and subsequently blocked with 2% bovine serum albumin (BSA) for 30 minutes. Slides were incubated with AK4 antibodies at room temperature for 2 hours. Subsequently, the sections were incubated with biotinylated secondary antibody for 1.5 hours, and diaminobenzidine was used as a chromogen substrate.

According to the staining results, AK4 was mainly located in the cytoplasm of HER2-positive breast cancer tissues. The score methods are as follows. Briefly, the proportion of positively stained cells was graded as follows: 0=0%
stained cells; 1=1–30% stained cells; 2=31–60% stained cells; and 3=61–100% stained cells. The staining intensity was evaluated on a score of 0 (no), 1 (low), 2 (modest), and 3 (strong). AK4 expression levels were detected based on the staining index: score of staining intensity × score of stained-cell percentage. Staining index < 4 was considered as AK4 low expression, while staining index > 4 or =4 was considered as AK4 high expression.

2.3. Cell Culture and Transfection. The 2 types of human HER2-positive breast cancer cell lines, MCF7 and MDA-MB-231, were purchased from ATCC. MCF7 and MDA-MB-231 cells were incubated in Eagle’s Minimum Essential Medium (EMEM) and Dulbecco’s Modified Essential Medium (DMEM), respectively, supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA Bio-Rad, CA, USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B in a humidified atmosphere in a 5% CO2 incubator.

The AK4 shRNA plasmids were transfected into MCF7 and MDA-MB-231 cells by the use of Lipofectamine 2000 (11668019, Invitrogen, Carlsbad, CA, USA). The stable depletion of AK4 MCF7 cells was screened by its shRNA lentivirus infection and used for the in vivo assays. The control group was the control-shRNA cell line transfected by Lipofectamine 2000.

2.4. Quantitative PCR Assay. TRIzol Reagent (15596026, Invitrogen, Carlsbad, CA, USA) was used to extract total mRNA from human HER2-positive breast cancer cells. Then, the RNA was reverse-transcribed by M-MLV reverse transcriptase (M1701, Promega, Madison, Wisconsin, USA).

Total mRNA was reverse transcribed to produce cDNA by the cDNA synthesis system. Quantitative PCR was performed using the SYBR Ex Taq kit (638319, Takara, Japan), and the expression levels of AK4 were normalized to the expression of β-actin.

2.5. Immunoblot Assays. Tumor cells or tissues of HER2-positive breast cancers were lysed in RIPA Buffer (9800, Cell Signaling Technology, Danvers, MA). Then, the total proteins were analyzed by SDS-PAGE. Subsequently, the polyvinylidene fluoride (PVDF) membranes were blocked with 5% milk buffer and then incubated with the primary antibodies for the detection of AK4 and β-actin at room temperature for 1.5 hours. Then, the PVDF membranes were incubated with horseradish peroxidase- (HRP-) conjugate secondary antibodies for 45 minutes. Blots were detected with an enhanced chemiluminescence (ECL) kit. Image Pro software was used in this assay to evaluate the intensity of each blot.

2.6. Colony Formation Assay. Approximately 500 MCF7 or MDA-MB-231 cells were added into a 6-well culture plate and transfected with control or AK4 shRNA plasmids and maintained in a 37°C, 5% CO2 incubator. After 14 days, cells were fixed with methanol at -20°C for 10 minutes and stained with 0.1% crystal violet at room temperature for 30 minutes and washed with PBS. Then, the number of colonies was manually counted and analyzed.

2.7. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. MCF7 and MDA-MB-231 cells were plated into 96-well plates at a density of approximately 1000 cells each well, transfected with control or AK4 shRNA plasmids, and incubated for 24 hours. Cells were then incubated with MTT for 3 hours and the medium was subsequently removed. Cells were then washed with PBS and 150 μL dimethyl sulfoxide (DMSO) was added into each well to extract the staining cells. The absorbance value was measured with a microplate reader at 570 nm.

2.8. Wound Closure Assay. MCF7 and MDA-MB-231 cells were transfected with control or AK4 shRNA plasmids and grown as confluent monolayers. Then, a wound was mechanically made using a 20 L pipette tip to generate the wound. Cell debris was washed by PBS, and the medium was added to induce wound healing. The wound was photographed at 0 hour and 24 hours, and the extent percentage of wound closure was measured.

2.9. Transwell Assay. MCF7 and MDA-MB-231 cells were transfected with control or AK4 shRNA plasmids for 48 hours and then trypsinized and resuspended in serum-free medium. The upper chambers of Transwell filters with 8.0 μm membrane pores were subsequently coated with 20% matrigel and incubated at 37°C for 30 minutes. A total of 105 cells in 150 μL of medium were then added to the upper chambers of the inserts and were induced to migrate toward the bottom chambers containing medium with 10% FBS. After 24 hours, cells in the top chamber were removed, and cells on the underside were fixed in 4% parafomaldehyde for 25 minutes and stained with 0.1% crystal violet for 30 minutes. Quantification of migrated cells was performed by dissolving crystal violet with 10% acetic acid, and the cell number was calculated.

2.10. Tumor Growth Assay. All animal assay processes were approved by our Institutional Animal Care and Use Committee. Briefly, MCF7 cells were stably transfected with control or AK4 shRNA lentivirus. About 106 control or AK4 knockdown cells were subcutaneously implanted into athymic nude mice. After 2 weeks, tumors were removed and photographed and the volume was measured every 3 days.

2.11. Tumor Metastasis Assay. All animal assay procedures were approved by our Institutional Animal Care and Use Committee. Nude BalB/c mice (6-8 weeks, 18-22 g) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). MCF7 cells were infected with control or AK4 shRNA lentivirus. About 5 × 105 cells were implanted into the tail vein of athymic nude mice. After 8 weeks, tumors were isolated from mice in each group and weighted.

2.12. Statistics. GraphPad 5.0 software was used for statistical analysis in this study. The link between clinical characteristics and protein levels was analyzed by χ2 analysis. Student’s t-test was used for statistical comparisons in vitro and in vivo, and the results were represented as...
mean ± standard deviation. ∗ indicates $P < 0.05$. Also, $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1. AK4 Expression Is Highly Expressed in Human HER2-Positive Breast Cancer Tissues. To assess the possible role of AK4 in the progression of HER2-positive breast cancer, the expression levels of AK4 in HER2-positive breast cancer tissues of patients who underwent surgical resection were detected by IHC assays. According to the staining results, we found that AK4 was mainly expressed in the cytoplasm of tumor cells (Figure 1(a)).

To further assess the effects of AK4 on HER2-positive breast cancer, we compared the expression levels of AK4 in tumor tissues and the adjacent nontumor tissues by IHC assays. As we expected, tumor tissues showed an obviously higher expression level of AK4 compared with adjacent nontumor tissues (Figures 1(a) and 1(b)).

3.2. AK4 Expression Levels Are Related to the Clinical Features of Patients with HER2-Positive Breast Cancer. A total of 98 tumor tissue samples from HER2-positive breast cancer patients who underwent surgical resection were classified into AK4 low and high expression groups based on the staining intensity (Figure 1(a) and Table 1). According to our scoring standards, 38 patients showed low expression of AK4, while 60 showed high AK4 expression (Table 1).

We subsequently evaluated the clinical significance of AK4 in HER2-positive breast cancer patients. Clinical characteristics such as patient age and pTNM stage were recorded and evaluated. No clinical significance was found in features such as patient age and tumor grade between AK4 low and high expression groups (Table 1). Interestingly, data showed that the expression levels of AK4 were obviously correlated with pTNM stage ($P = 0.017$) and lymph node metastasis ($P = 0.046$) in patients with HER2-positive breast cancer (Table 1).

In conclusion, we found that AK4 expression levels were associated with the clinical features of patients with HER2-positive breast cancer.

3.3. Construction of AK4 Stable Knockdown Cell Lines of MCF7 and MDA-MB-231. MCF7 and MDA-MB-231 cells were transfected with shRNA of AK4 expression in cancer cells. Through RT-qPCR and western blotting validation, we generated stable cell lines with both AK4 mRNA and protein level decreased significantly when compared with control cells (Figure 2). Using this model, we could efficiently study the AK4 role in cancer cells.

3.4. AK4 Ablation Impaired Cell Proliferation and Invasion of HER2-Positive Breast Cancer In Vitro. Next, colony formation assays were performed to evaluate cell proliferation of HER2-positive breast cancer. The data of the colony formation assays showed that knockdown of AK4 markedly reduced colony number (Figure 3(a)). Similarly, an obvious decrease absorbance value at 570 nm was detected in both MCF7 and MDA-MB-231 cells through MTT assays (Figure 3(b)).

We then performed wound healing and transwell assays to evaluate the effects of AK4 on the motility of MCF7 and MDA-MB-231 cells. Interestingly, our results revealed that AK4 depletion obviously reduced the extent of wound closure in both MCF7 and MDA-MB-231 cells (Figure 3(c)). Additionally, the depletion of AK4 significantly inhibited the invasion of both MCF7 and MDA-MB-231 cells through membranes, reflected by the obvious decrease in cell number (Figure 3(d)).

Collectively, we demonstrated that AK4 promotes cell proliferation and invasion of HER2-positive breast cancer in vitro.
3.5. AK4 Contributes to Tumor Growth and Metastasis of HER2-Positive Breast Cancer in Mice.

We further investigated the possibility that AK4 contributes to tumor growth and metastasis of HER2-positive breast cancer in mice. To verify our expectation, MCF7 cells were infected with control or AK4 shRNA lentivirus and subcutaneously injected into nude mice. After 2 weeks, the tumor was isolated from mice and photographed, and the volume was measured every week. Representative photographs were shown (Figure 4(a)). The growth curve was analyzed and shown in Figure 4(a). As we expected, the volume of tumors isolated from AK4 depletion groups was remarkably smaller than that in the control (Figure 4(a)).

Furthermore, MCF7 cells were infected with AK4 shRNA lentivirus to stably deplete the expression of AK4. Subsequently, control or AK4 knockdown MCF7 cells were injected into the caudal vein of nude mice. After 8 weeks, we found that the incidence of lung metastasis for MCF7 cells were significantly reduced compared with control (Figure 4(b)).

On this basis, IHC assays were performed and showed the effective silencing of AK4 in tumors isolated from mice in AK4 depletion groups (Figure 4(c)).

In conclusion, animal assays confirmed the involvement of AK4 in the progression of HER2-positive breast cancer in mice.

4. Discussion

Through immunohistochemistry (IHC) assays, we found that AK4 was highly expressed in human HER2-positive breast cancer tissues and investigated the link between the expression levels of AK4 and clinical features of patients who underwent HER2-positive breast cancer. AK4 depletion also blocked the cell proliferation and invasion of HER2-positive breast cancer both in vitro and in vivo. We therefore believe that AK4 could serve as a novel therapeutic target for the treatment of HER2-positive breast cancer.

HER2 is an important prognostic factor for breast cancer [22]. The development and metastasis of HER2-positive (overexpressed or amplified) breast cancer is different from other subtypes of breast cancer and is treated differently [23]. Recently, targeted therapy had good effects on this cancer [24, 25]. Some targeted drugs, such as Herceptin and Lapatinib, specifically target the HER2 gene and have shown efficacy in early and late (metastatic) HER2-positive breast cancer [26, 27]. Although HER2 is the driving gene of breast cancer, several genes are still involved in the progression of HER2-positive breast cancer. As potential therapeutic targets, it is necessary to develop relevant targeted drugs and participate in combined therapy to improve the prognosis of patients with HER2-positive breast cancer [28]. Interestingly, we found that AK4, as a member of the adenylate kinase family, was involved in the progression of HER2-positive breast cancer. AK4 was highly expressed in tumor tissues. Additionally, it was correlated with the clinical features of patients. Therefore, AK4 could serve as a novel therapeutic target for the treatment of HER2-positive breast cancer.

From the analysis of 82 human HER2-positive breast cancer samples and the adjacent tissue samples through IHC assays, we found that AK4 was highly expressed in...
Figure 3: AK4 facilitates cell proliferation and invasion of HER2-positive breast cancer in vitro. (a) Colony formation assays were then performed using MCF7 and MDA-MB-231 cells transfected with control or AK4 shRNA plasmids, and colony number was counted. (b) The results of MTT assays showed the inhibition of cell proliferation caused by AK4 knockdown. (c) AK4 depletion resulted in the lower migration degree in MCF7 and MDA-MB-231 cells. Photographs showing that at the 0 and 24th-hour time point migrated cells were present. (d) Transwell assays using both MCF7 and MDA-MB-231 cells transfected with control or AK4 shRNA plasmids, and the degree of invasion was quantified by the invasion cell number. Results are presented as mean ± SD, *P < 0.05.
tumor tissues. Additionally, AK4 expression was significantly correlated with clinical characteristics including pTNM stage and lymph node metastasis. These clinical analyses confirmed the potential effects of AK4 in the progression of HER2-positive breast cancer. Further investigations proved our hypothesis that AK4 could serve as a trigger of cancer progression through the regulation of cancer cell proliferation and invasion. Similarly, previous studies indicated the contributory role of AK4 in tumorigenesis [18]. AK4 was highly expressed in multiple cancer cells and promoted the proliferation and invasion of cancer cells [17]. These studies, together with our findings, confirmed the important role of AK4 in cancer progression.

In addition, AK4 promotes the development and metastasis of lung cancer through an ATF3-dependent manner [19]. Interestingly, the involvement of ATF3 in the growth of different tumors has been widely reported [29]. ATF3 contributes to cell migration and invasion in breast cancer [30, 31]. In this study, we found that AK4 promoted the proliferation and invasion of HER2-positive breast cancer cells in vitro and promoted tumor growth and metastasis in mice. We thus suspect that in HER2-positive breast cancer, AK4 promotes cell proliferation and invasion through an ATF3-dependent manner.

As was known, AK4 was involved in metabolism regulation, and its expression levels were markedly correlated with several metabolic processes [32]. Cancer metabolism is a critical factor because tumor cells could produce and use energy from multiple sources more efficiently, compared to normal cells [33, 34]. Whether AK4 affects the progression of HER2-positive breast cancer through metabolic regulation remains to be studied further. Additionally, AK4 is located in the mitochondrial matrix but has no adenylate kinase activity [35]. Previous studies also indicated that interaction with the mitochondrial inner membrane protein Adenine Nucleotide Translocase (ANT) is a requirement for several functions of AK4 [15]. We should next explore whether the interaction is needed for the development of HER2-positive breast cancer.

In summary, we found the high expression of AK4 in human HER2-positive breast cancer tissues. We also investigated the link between the expression levels of AK4 and clinical characteristics of patients with HER2-positive breast cancer. Additionally, AK4 facilitated the proliferation of HER2-positive breast cancer cells in vitro and contributed to tumor growth and metastasis in mice. We therefore have a mechanical conclusion of AK4 in the development and metastasis of HER2-positive breast cancer and provide a potential therapeutic target for the treatment of this cancer.

**Data Availability**

The dataset supporting the conclusions of this article is included within the article.

**Ethical Approval**

All applicable international, national, and/or institutional guidelines for the care and use of human tissues and animals were followed.
Conflicts of Interest
The authors declare that they have no conflicts of interest.

Authors' Contributions
Jie Zhang, Yan-Tao Yin, and Zhi-Xi Li conceived the idea and designed the experiments. Jie Zhang, Yan-Tao Yin, Wu Chi-Hua, and Rong-Lin Qiu performed the experiments. Wen-Jun Jiang and Xiao-Geng Deng contributed reagents, materials, and analysis tools. Jie Zhang, Yan-Tao Yin, and Zhi-Xi Li wrote the paper. Jie Zhang and Yan-Tao Yin contributed equally to this study.

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