Cytoplasmic translocation of p16 could be associated with CDK4 to promote progression of breast cancer

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Abstract
Background: p16 protein was up-regulated in breast cancer with translocation from nucleus to cytoplasm. In this study, the mechanism of p16 cytoplasmic translocation and its role in progression of breast cancer were investigated.

Methods: The expression and subcellular redistribution of p16 and CDK4 were detected by immunohistochemistry and immunofluorescence in 93 cases of breast cancer. Regulation of different CDK4 status on p16 and its influence on the progression of breast cancer were analyzed with western blotting, CCK8, flow cytometry and transwell assays.

Results: Both p16 and CDK4 proteins mainly expressed and co-localized in cytoplasm or nucleoplasm either in breast cancer tissues or in cell lines. Further analysis indicated that p16 was mainly in cytoplasm when CDK4 was over-expressed. However, it located in nucleus when CDK4 was mutated at position 24 (R24C) or knocked down. In addition, cytoplasmic p16 could promote proliferation, invasion and metastasis of breast cancer.

Conclusion: The aberrant accumulation of p16 in cytoplasm could be associated with up-regulated CDK4, which could promote progression of breast cancer cells, indicating a poorer prognosis.

Background
Breast cancer is the most commonly diagnosed and main reason of cancer-related death in women worldwide [1]. The St. Gallen International Expert Consensus has proposed to sub-classify breast cancer into five subtypes based on immunohistochemistry: luminal A-like, luminal B-like, Hormone receptor-positive and HER2-positive, Hormone receptor-negative and HER2-positive, and triple-negative breast cancer (TNBC) [2]. Molecular typing plays a quite important role in the treatment and prognosis evaluation of breast cancer patients. As is known, patients with positive hormone receptor can receive endocrine therapy, and those with HER2 over-expression can receive targeted therapy of Herceptin. However, TNBC is a sub-group with much higher histological grade, younger onset age, shorter recurrence time and higher risk of metastasis. Moreover, due to lacking therapeutic targets,TNBC patients mainly receive surgery, chemotherapy and/or radiotherapy with limited efficacy but significant toxicity. Therefore, it is very necessary to study the biological behavior of breast cancer,
especially TNBC, to find other potential novel therapeutic targets.

The p16 tumor suppressor, encoded by CDKN2A at 9p21, negatively regulates cell cycle progression. Cytoplasmic p16 in tumor cells has already been observed and reported. But the specificity and validity have been questioned. Evangelou and Nilsson et al. verified that cytoplasmic p16, observed by immunohistochemistry in both cell lines and tumor tissues, was not an artifact but specific [3, 4]. However, the mechanism remains unclear. Several hypotheses to explain the presence of p16 in cytoplasm have been described. And it seems that its cytoplasmic accumulation is not caused by mutations of p16 gene [5]. Some proteins such as cyclin-dependent kinase 4 (CDK4) or anion exchanger 1 (AE1) are assumed to be responsible for the aberrant accumulation. It was reported that p16 could interact with CDK4 through electrostatic attraction [6]. Based on the above, we hypothesized CDK4 might interact with and sequestrate p16 in cytoplasm with co-accumulation of these proteins in breast cancer [7].

In this study, we preliminary investigated whether aberrant cytoplasmic accumulation of p16 was associated with CDK4 and its role in progression of breast cancer.

Materials And Methods

Ethics statement

This study was approved by the Dalian Medical University Institutional Review Board (IRB) before the start. Samples and data collection were approved for informed consent waiver by the institutional review board of our hospital.

Patients And Tissue Specimens

A total of 93 surgically resected cases of breast cancer (non-specific) were collected, and none of the patients received preoperative chemotherapy. 4% buffered formalin-fixed and paraffin-embedded sections (4 µm thick) were stained with hematoxylin and eosin for histologic evaluation, diagnosis, typing, and grading. The diagnoses were re-evaluated independently by two pathologists according to the WHO classification [8].

Immunohistochemistry (ihc) And Staining Evaluation

The operation of immunostaining and the interpretation of p16 staining have been described in our previous article [9]. The expression of CDK4 in cancer cells was defined by cytoplasmic staining with
or without nuclear staining in more than 10% cells, similar to that of p16. Stained slides were evaluated separately by two independent pathologists blinded to the clinical data.

Cell Culture And Treatment

MCF7 and BT549 cells were cultured in RPMI 1640 (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, USA). Cells were incubated in a humidified atmosphere with 5% CO2 at 37°C.

Plasmid Construction And Transfection

CDK4 gene and its mutant (named CDK4R24C) were cloned into the eukaryotic expression vector pEGFP-C1, and corresponding plasmids were obtained. CDK4 was generated by cloning RT-PCR amplified cDNA and then it was cloned into pEGFP-C1 (named pEGFP-CDK4), whereas CDK4R24C was constructed by site-directed mutagenesis (resulting in the replacement of an arginine residue at position 24 by a cysteine, R24C) of the pEGFP-CDK4 plasmid (named pEGFP-CDK4R24C). Both of the two recombinant plasmids were confirmed by sequencing. Four interfering sequences (shown in Table 1) against CDK4 were designed and cloned into pGPU6. The above plasmids were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

| Table 1 | The interfering sequences against CDK4 |
|---------------------------------|-------------------------------------|
| GPU6-CDK4-574: 5'-GCATGTAGACCAGGCCTAAG-3' |
| GPU6-CDK4-657: 5'-GCCAGTTTCTAAGAGGCCTAG-3' |
| GPU6-CDK4-764: 5'-GACTTTGGCCTGGCCAGAATC-3' |
| GPU6-CDK4-997: 5'-GGATGACTCCCTGCTCGAGATGT-3' |
| NC (Negative control): 5'-GTTCTCCGAACGTGTCACGT-3' |

Immunofluorescent Microscopy

Cells grown on cover slides were fixed in ice-cold acetone for 10 minutes, blocked with serum for 20 minutes, and then incubated with primary antibodies (diluted in blocking buffer) at 4°C overnight. After three PBS washes, the slides were incubated with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC)–conjugated antibodies (Sigma, St Louis, MO, USA) at room temperature for 60 minutes. Nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) for 3 minutes and mounted with anti-fade solution. Results were observed and recorded using a fluorescence microscope (Model CX51; Olympus), and Photoshop version 7.0 (Adobe Systems Inc.) was used for image processing.

Western Blotting


Proteins were extracted either using whole-cell lysates or Nuclear and Cytoplasmic Extraction Reagents. Protein separation and detection were performed using an automated capillary electrophoresis system (Simple Western system and Compass software; Proteinsimple, California, USA). The following antibodies were used: anti-p16 (Abcam, Cambridge, UK), anti-lamin B (Abcam), and anti-β-tubulin (Proteintech, Chicago, IL, USA). Signals were detected by HRP-conjugated secondary and were visualized using proteinsimple software.

**Statistical analysis**
All analysis was performed using SPSS statistics software (Version 17.0, IBM, Chicago, IL, USA).

Relationships between tumor markers and other parameters were tested using χ² test, Fisher exact test, Continuity Correlation or the independent t test when appropriate. A P value of less than 0.05 was considered to be of statistical significance, and the level of significance was set at < 0.05 (*), < 0.01 (**), or < 0.001 (***)

**Results**
**p16 proteins co-localized with CDK4 in breast cancer**
In normal breast tissues adjacent to cancers, p16 expressed only in nuclei of individual cells. But aberrant staining was shown in 66.67% (62/93) of breast cancers, generally presenting in both nuclei and cytoplasm. Even in some cases, staining was mainly in cytoplasm (Fig. 1a). This is consistent with our previous study [9]. Further analysis showed that the positive rates of p16 in nucleoplasm, only in cytoplasm and only in nuclear were 70.97% (44/62), 17.74% (11/62), and 11.29% (7/62), respectively. (Table 2)

CDK4 protein in the 93 cases presented a similar pattern to that of p16 (Fig. 1a). The positive rate of CDK4 in breast cancer was 84.95% (79/93), with the rates in nucleoplasm, only in cytoplasm and only in nuclear being 56.96% (45/79), 26.58% (21/79) and 16.46% (13/79), respectively. (Table 2)

|        | +       | Total | Positive rate |
|--------|---------|-------|---------------|
|        | Nucleus | Cytoplasm | Nucleoplasm |
| P16    | 31      | 7      | 44            | 93 | 66.67% |
| CDK4   | 14      | 13     | 21            | 45 | 84.94% |

Further analysis showed that p16 co-expressed with CDK4 in 60 out of 93 cases (64.52%, 60/93). The
co-positive rates of the two in nucleoplasm, only in cytoplasm and only in nuclear were 61.67\% (37/60), 18.33\% (11/60), 11.67\% (7/60), respectively. Only 5 cases showed inconsistent subcellular localization (8.3\%, 5/60). (Table 3)

Table 3
The relationship of subcellular localization between p16 and CDK4 in breast cancer

|       | CDK4+ |       | Total | P     |
|-------|-------|-------|-------|-------|
|       | Nucleus | Cytoplasm | Nucleoplasm |       |
| P16+  | 12     | 5     | 6     | 31    | 0.0001 |
|       | 0      | 7     | 0     | 7     |       |
|       | 2      | 1     | 4     | 11    |       |
| Total | 14     | 13    | 21    | 45    | 93     |

Then immunofluorescence staining on tissue and cell slides of breast cancer further confirmed that both p16 and CDK4 mainly located in nucleoplasm or only in cytoplasm and they were co-localized (Fig. 1b), indicating that cytoplasmic p16 in breast cancer could be associated with CDK4.

Subcellular Localization Of P16 Associated With Cdk4
To verify the above hypothesis, we cloned CDK4 and CDK4R24C into eukaryotic expression vector pEGFP-C1 and obtained pEGFP-CDK4 and pEGFP-CDK4R24C, which were stably introduced into BT549 cells. Compared with the control, the expression of p16 protein reduced significantly in nucleus but increased in cytoplasm when cells over-expressed CDK4. However it increased markedly in nucleus but reduced in cytoplasm in cells over-expressing CDK4R24C (Fig. 2).

Next, four different shRNAs (Table 1) against CDK4 gene were constructed and transfected into BT549 cells. Real-time PCR and western blotting were used to detect the expression of CDK4. Results showed that pGPU6-CDK4-997 could markedly reduce the expression of CDK4 at both mRNA and protein levels (Fig. 3). A cell line stably expressing pGPU6-CDK4-997 was constructed to detect the expression of p16. And it was found that p16 in CDK4 depleted BT549 cells presented a similar expression to that when CDK4 was mutated position 24 (Fig. 2).

Aberrant expression of p16 promoted proliferation, invasion and migration of breast cancer cells
Our previous study has shown that cytoplasmic p16 was associated with poorer biological behavior and prognosis of TNBC [9]. Therefore, in this study we checked the influence of cytoplasmic p16 on proliferation, invasion and migration of tumor cells. Subcellular localization of p16 was induced by
different CDK4 status. CCK8, flow cytometry, scratch test and transwell assays with or without Matrigel were carried out. In CCK8 test, the proliferating rate in cells over-expressing CDK4 was faster than that of the control, whereas it reduced significantly when CDK4 was mutated or knocked down (Fig. 4a). Meanwhile, G1 phase, a main regulation point of CDK4, in the above groups was detected. Results demonstrated a significantly shortened G1 in cells over-expressing CDK4, but an obviously prolonged one when CDK4 was knocked down. No obvious change of G1 was observed when CDK4 was mutated. (Fig. 4b). Transwell assays were used to determine the influence on migration and invasion and similar results to that in CCK8 were illustrated (Fig. 5).

To sum up, these above demonstrated that cytoplasmic p16 could promote proliferation, invasion and migration of BT549 cells

**Discussion**

As an important tumor suppressor, p16 negatively regulates cell proliferation through inhibiting the activity of cyclin-dependent kinase 4/6 (CDK4/6), which promotes phosphorylation and, therefore, inactivate Rb. The p16-cyclinDs-CDK4/6-Rb-E2Fs pathway is abrogated frequently in multiple types of human tumors, either through inactivation of Rb or p16, or through over-expression of cyclin D1 or CDK4. Loss of p16 activity has been widely found in many human cancers due to gene mutation or promoter methylation [10, 11]. However, its aberrant expression and changes in subcellular localization have also been found in some types of cancers, especially in precancerous lesions of cervix and cervical cancers [9, 12, 13]. Evangelou et al. verified that cytoplasmic p16 in non-small cell lung cancer was specific using Electron Microscope Technology [3]. Nilsson et al. showed that p16 indeed expressed in cytoplasm as well as in nucleus by subcellular fractionation of Rb-functional and Rb-inactivated cell lines [4]. Moreover, they also indicated that p16 might have functions in cytoplasm. Studies have demonstrated that cytoplasmic p16 was correlated with the development and prognosis of some tumors. To be exact, in normal breast tissue and fibroadenoma, p16 was present only in nuclei of individual cells, whereas aberrant staining demonstrated in cancer cells, generally in nucleoplasm or only in cytoplasm [9, 14]. In colorectal cancer, p16 presented a similar expression pattern [15]. At present, little was known about the reason for aberrant accumulation of
cytoplasmic p16 and its significance in the development of breast cancer.

CDK4, locating at 12q13, plays vital roles in proliferation of mammalian cells. In particular, several tumorigenic events ultimately drive proliferation through CDK4 complexes, underscoring CDK4 as an important therapeutic target in cancer treatment [16]. The importance of interaction between CDK4 and p16 has become apparent with identification of a mutation in CDK4 in patients with familial melanoma [17, 18]. This germline mutation results in the replacement of an arginine residue at position 24 by a cysteine (R24C), making CDK4 lose its affinity for p16 without affecting its ability to bind cyclin D and then form a functional kinase. However, this mutation does not affect the interaction of CDK4 with P21CIP or P27KIP inhibitors.

In this study, we first confirmed co-localization of p16 with CDK4 either in breast cancer tissue or cell lines by immunohistochemistry and immunofluorescence. Further, we observed that subcellular localization of p16 changed significantly with different CDK4 status. p16 protein was mainly in cytoplasm when CDK4 was over-expressed, whereas it mainly located in nucleus when CDK4 was mutated at position 24 (R24C) or knocked down. The above preliminarily confirmed that cytoplasmic p16 could be associated with CDK4, which caused retention of p16 in cytoplasm when CDK4 was up-regulated in some cases. However, more detailed mechanisms need to be further studied.

p16 gene mutations have complicated effects on its subcellular localization. Some studies have shown that various mutants of p16 expressed predominantly in cytoplasm [19–21]. On the other hand, at least one study has shown that cytoplasmic p16 observed in tumor-derived cell lines was not linked to its gene mutations [5]. Therefore, in this study we selected BT549 cell line harboring no p16 mutations to eliminate the influence of mutation on its localization. Besides, as a triple-negative breast cancer cell, BT549 can realistically simulate the expression and subcellular localization of p16 in triple-negative breast cancer tissues. In this way, we can better understand the biological behavior of breast cancer, particularly TNBC, and provide some guidance for the treatment and prognosis of breast cancer patients.

p16, as a tumor biomarker, has been widely used in the pathological diagnosis of many tumors. As is known, abnormal expression of p16 is helpful in differential diagnosis of cervical squamous
intraepithelial lesions [12]. Besides, cytoplasmic p16 staining of tumor cells further demonstrated the prognostic value. In primary breast carcinoma, a strong cytoplasmic expression of p16 was associated with a highly malignant phenotype [8], whereas in malignant melanoma, no association between cytoplasmic p16 and patient outcome was indicated [22]. In our study, cytoplasmic p16 was found to promote proliferation, invasion and metastasis of BT549 cells, indicating a poorer prognosis. However, there are some limitations in this study. Given the heterogeneity of breast cancer, the clinical cohort in this study is relatively modest. Only Chinese ethnicity and one center were involved with no randomization or controls. Besides more detailed mechanisms were needed. Therefore, further investigation is necessary to overcome all these limitations.

Conclusions
In this study, aberrant accumulation of p16 in cytoplasm could be associated with up-regulated CDK4, which could promote proliferation, invasion and metastasis of breast cancer cells, indicating a poorer prognosis.

Abbreviations
TNBC: triple-negative breast cancer. CDK4: cyclin-dependent kinase 4. AE1: anion exchanger 1. IHC: immunohistochemistry. FITC: fluorescein isothiocyanate. TRITC: tetramethyl rhodamine isothiocyanate. DAPI: 4, 6-diamidino-2-phenylindole

Declarations

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Authors’ contributions
HW and HG designed the study. SX and RZ performed the cellular and molecular experiments. SX, RZ and LZ collected and analyzed the data. WQ and JH performed the IHC experiments and prepared the figures. QZ and LZ reviewed the histopathology and IHC results. HW, SX and HG drafted the manuscript. HW and LZ provided the
funding. HW and HG provided technical or material support and critical comments on the manuscript. All authors have read and approved the final manuscript.

**Availability of data and materials**

All data are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

This study was approved by the Ethics and Research Committees of Dalian Medical University, China.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests

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Figures

A  p16  CDK4
| Case 1 | Case 2 | MCF7 | BT549 |
|--------|--------|------|-------|
| p16    | CDK4   | DAPI | Merged|

Figure 1
Subcellular staining of p16 correlated with CDK4 in breast cancer tissues and cell lines. a. Representative images of subcellular staining of p16 and CDK4 by immunohistochemistry in breast cancer tissues (X200). b. Immunofluorescence staining verified that p16 and CDK4 were co-localized in both breast cancer tissues (Representative images of 2 cases of breast cancer) and cell lines (MCF7 and BT549) (X1000).
Cytoplasmic p16 in breast cancer could be associated with CDK4. The expressions of p16 in cytoplasm (a) and nucleus (b) measured by western blotting when pEGFP-CDK4, pEGFP-CDK4R24C and pGPU6-CDK4-997 were introduced into BT549 cells, respectively. Tubulin and lamin B were used as internal standards. All experiments were repeated at least three times and the results were displayed as a histogram of means ± SEM. in the right panel. *, p < 0.05, **, p < 0.01 and ***, p < 0.001, respectively.
pGPU6-CDK4-997 could significantly reduce the expression of CDK4 at both mRNA and protein levels.

Four different shRNAs against CDK4 were constructed and transfected into BT549 cells. Then the expression of CDK4 was detected. A, Quantitative real-time PCR analysis of CDK4 in BT549 cells transfected with different shRNAs. GAPDH was used for normalization. The results represented three
independent experiments and were expressed as mean ± SEM in the right panel. *, p < 0.05, **, p < 0.01, respectively. B, Western blotting was used to detect protein level of CDK4. Tubulin was used as internal standard. Multiple western blottings were analyzed by densitometry and the results are displayed as a histogram of means ± SEM. in the right panel. *, p < 0.05, **, p < 0.01, respectively.
CCK8 (A) and flow cytometry (B) were carried out to detect proliferation ability of BT549 cells stabling expressing pEGFP-CDK4, pEGFP-CDK4R24C and pGPU6-CDK4-997. All experiments were repeated at least three times.
Transwell assays with or without Matrigel were carried out to determine invasion (a) and migration (b) of BT549 cells with different CDK4 expressing status. The results represented three independent experiments and were expressed as mean ± SEM in the lower panels. **, p < 0.01.