Effect of p21^{Waf1} and p27^{Kip1} on centrosome replication and proliferation of breast cancer cell

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Abstract

Aberrant centrosome numbers are detected in virtually all cancers increasing the risk for cell division errors and chromosomal instability.

Deregulation of the centrosome duplication cycle is considered as the major contributing factor for abnormal amplification of centrosomes. $p21^{Waf1}$ and $p27^{Kip1}$, general CDK inhibitors by inhibiting cyclin-dependent kinase 2 (CDK2)/cyclin E and cyclin A complexes, controlled the initiation and progress of centrosome duplication . We transfected $p21^{Waf1}$, $p27^{Kip1}$ or $p21^{Waf1}$- $p27^{Kip1}$ genes into MCF-7 cells by lipofection to explore the effect of the genes on centrosome duplication and proliferation of breast cancer cell. The result shows that the cell growth was obviously inhibited after being transfected, resulting in an accumulation of cells in G_1 and the proportion of cells which contained abnormal centrosomes was obviously decreased. Comparing with $p21^{Waf1}$or $p27^{Kip1}$, the effects of $p21^{Waf1}$- $p27^{Kip1}$ genes are more significative. These results suggest that $p21^{Waf1}$ and $p27^{Kip1}$ genes could inhibit the growth of human breast cancer

cells and reverse abnormal duplication of centrosomes. $p21^{Waf1}$and $p27^{Kip1}$ cooperate to regulate centrosome duplication and cell cycle progress, indicating $p21^{Waf1}$- $p27^{Kip1}$ combined gene might be potential therapeutic agents of breast cancer which reveals suppressed $p21^{Waf1}$ and $p27^{Kip1}$ expression.

Key words: Centrosome; Cell Proliferation; $p21^{waf1}$ gene; $p27^{kip1}$ gene; Breast neoplasms

Introduction

The centrosome is the major microtubule organizing center during interphase and mitosis in most animal and human cells [1]. Each centrosome consists of a pair of centrioles and a surrounding protein matrix referred to as pericentriolar materials [2].

During interphase, centrosomes organize the cytoplasmic microtubule network, which is involved in vesicle transport, proper distribution of small organelles, and establishment of cellular shape and polarity. During mitosis, duplicated centrosomes direct formation of bipolar spindles, which is critical for accurate segregation of chromosomes and cytokinesis [3]. The centrosome duplicates once every cell cycle, which starts during the G_1-S transition, coincident with the onset of DNA replication. Abrogation of regulatory mechanisms governing centrosome duplication leads to generation of amplified centrosomes (more than two centrosomes), which in turn leads to mitotic aberration (multipolar spindles) and unequal segregation of chromosomes[3]. Destabilization of chromosomes by centrosome amplification aids acquisition of further malignant phenotypes, hence promoting tumor progression. Recent studies have shown

that centrosome hyperamplification is commonly observed, and is the major contributing factor for chromosome instability in human tumors [4–7]. Moreover, a significant reduction of $p21^{Waf1}$ and $p27^{Kip1}$ expression, which occurs at a high frequency in human cancer [8-12], strongly correlates with the occurrence of centrosome hyperamplification. $p21^{Waf1}$ and $p27^{Kip1}$, general CDK inhibitors, mainly inhibits cyclin/CDK2 complexes, thereby arresting the cell cycle[13,14]. Some reports have shown that the activation of CDK2/cyclinE (and cyclinA) is essential for the initiation of centrosome duplication[15]. It is not difficult to consider the role of $p21^{Waf1}$ and $p27^{Kip1}$ in centrosome homeostasis. In one of the first related studies, cells deficient of p21 and exposed to ionizing radiation were found to accumulate abnormal numbers of centrosomes following abortive mitoses[16]. Similar results were obtained in p27–/–

and p27+/– mouse embryonic fibroblasts and p27-silenced human cells [17]. Thus, both CDK inhibitors regulate centrosome duplicatin and cooperation of p21 and p27 might be important for accurate chromosome duplication. Furthermore, inhibitory effects of CDK inhibitors, $p21^{Waf1}$ and $p27^{Kip1}$, on repeated centrosome reproduction were reported in frog embryos and Xenopus egg extracts[18-20]. But whether $p21^{Waf1}$ and $p27^{Kip1}$ can inhibite the abnormal duplication of centrosomes in breast cancer cells is not well known.

In this study, we transfected $p21^{waf1}$, $p27^{kip1}$ and $p21^{waf1}$- $p27^{kip1}$ genes into MCF-7 cells by lipofection to explore the role of $p21^{Waf1}$ and $p27^{Kip1}$ as a therapeutic target in breast cancer and the mechanism of interation between $p21^{Waf1}$ and $p27^{Kip1}$.

Materials and Methods

Reagents and antibodies.

pIRES-p21^{waf1} pIRES- p27^{kip1} and pIRES-p21^{waf1}- p27^{kip1} were generous gifts from LIU Xiang-ping. A human breast carcinoma cell line MCF-7 was purchased form Chinese Academy of Science. p21^{Waf1} and p27^{Kip1} antibodies were purchased form Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-mouse was obtained from Boster(Wuhan,China). The -tubulin antibody and a FITC conjugated secondary antibody were purchased form BD Pharmingen. All fine chemicals were purchased from Sigma. The ECL detection system was obtained from Amersham Biosciences (Piscataway, NJ). All cell culture products were purchased from

Jinuo(Hangzhou, China). Llipofectamine 2000 was purchased from Invitrogen (Grand Island, NY).

Cell line and transfection.

The human breast carcinoma cell line MCF-7 was grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 in 5% CO_2 incubator. The recombinant plasmids pIRES-p21 ^{Waf1}, pIRES- p27 ^{Kip1} and pIRES-p21 ^{Waf1}- p27 ^{Kip1} were generously provided by Dr. Liu(center laboratory Qingdao, China). MCF-7 cells were transfected with these plasmids using lipofectamine.

Western blot analysis.

Cells were lysed in RIPA buffer [20 mM Tris–HCl, pH8.0, 1 mM EDTA, 1mM

EGTA, 150 mM NaCl, 0.1% (w/v)SDS, 1.0% (v/v) NP-40], which contained Completek-Mini protease inhibitors. The amount of proteins was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA). An aliquot of 50 g proteins from a total lysate was electrophoresed on SDS-PAGE gel and then transferred to Immobilon-P (Millipore, Bedford, MA). After blocking with 5% nonfat dry milk in Tris-buffered saline (TBS), the membrane was incubated with a primary antibody at an appropriate dilution overnight at 4 and then washed three times in TBST. The membrane was then incubated with a secondary antibody at a dilution of 1:5,000 at room temperature for 1h and again washed three times in TBST. The blots were visualized with ECL (Amersham).

Flow cytometry.

The cells were fixed in 70 % ethanol for 1 hour at 4 °C. The fixed cells were incubated with PBS with 300 µg/mL RNase A at room temperature and stained with 50 µg/mL propidium iodide. Flow cytometric analysis was done with FACSC alibur and Cell Quest software (Becton Dickinson, San Jose, CA).

Indirect immunofluorescence.

Cells grown on slides were fixed with 4 % formalin for 20 min at 25 . The cells were permeabilized with 0.2 % Triton X-100 for 30 min, followed by incubation with blocking solution (10 % normal goat serum in PBS) for 1h. Cells were then probed with primary antibody for 1h and antibody-antigen complexes were detected with FITC-

conjugated goat secondary antibody by incubation for 1 h at 25 . The samples were washed three times with PBS after each incubation, and then counterstained with 4', 6-diamidino-2-phenylindole (DAPI).

Statistical analysis.

Differences between two groups were compared using the two-tailed unpaired Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Arrest of cell cycle and restoration of the normal centrosome duplication cycle by high expression of re-introducted $p21^{Waf1}$

Several reports have shown that breast cancer cells lacking $p21^{Waf1}$ exhibit centrosome amplification after DNA damage. And after induced DNA damage, the level of $p21^{Waf1}$ is gradually increases and centrosome amplification is suppressed. We decided to test whether re-introduction of $p21^{Waf1}$ gene into MCF-7 can induce cell cycle arrest or restore the normal centrosome duplication cycle. The MCF-7 cells were transfected with a pIRES-$p21^{Waf1}$, generating a MCF-7- $p21^{Waf1}$ cell line that constitutively expresses P21 protein. As a control, a pIRES vector was transfected into the MCF-7 cells (MCF-7-pIRES .

After transfected 48 hours, a statistical significant increased $p21^{Waf1}$ expression was detected by immunoblot analysis (Fig 1). At the same time, cells were analysed by flow cytometry(Fig 2). MCF-7- $p21^{Waf1}$ cells showed a different cell cycle phase distribution

from the control MCF-7-pIRES. MCF-7- $p21^{Waf1}$ cell increased the number of cells in G_1 phase $P<0.01$ and decreased the number of cells in S phase, indicating that $p21^{Waf1}$ overexpression can inhibit cell cycle progression resulting in an accumulation of cells in G_1.

The centrosome in the MCF-7- $p21^{Waf1}$ cell line was examined using antibody against - tubulin, a major component of centrosomes (Figure 3). As the control, MCF-7-pIRES cells were also immunostained. MCF-7-pIRES cells showed an expected abnormal centrosome profile, 13% of the cells contained abnormally amplified centrosomes (n 3). In contrast, MCF-7- $p21^{Waf1}$ cells showed a partial restoration of centrosome profile; an increase in the number of cells with one or two centrosomes and a decrease in the number of cells with n 3 centrosomes(4%), demonstrating that re-

introduction of physiological high level of $p21^{Waf1}$ re-established the normal centrosome profile.

These observations demonstrate that $p21^{Waf1}$ is directly involved in the controlling the coordinated initiation of centrosome and DNA duplication. $p21^{Waf1}$ overexpression can induce cell cycle attest and restoration of the normal centrosome duplication cycle in breast cancer.

Arrest of cell cycle progression and restoration of the normal centrosome duplication cycle by high expression of re-introduced $p27^{Kip1}$

$p27^{Kip1}$, as the related CDK inhibitor, also exhibited a significant reduction in most human aggressive tumors(16). We transfected pIRES-$p27^{Kip1}$ into the MCF-7 cells to

explore effect of transfected $p27^{Kip1}$ gene on the duplication of centrosomes and the proliferation of human breast carcinoma cells.

After transfected 48 hours, a statistical significant increase $p27^{Kip1}$ expression was detected by immunoblot analysis (Fig 1). At the same time, cells were analysed by flow cytometry(Fig 2) MCF-7- $p27^{Kip1}$ cells showed a different cell cycle phase distribution from the control MCF-7-pIRES. MCF-7- $p27^{Kip1}$ cell increased he number of cells in G_1 phase $P<0.01$ and decreased the number of cells in S phase, indicating that $p27^{Kip1}$ overexpression can inhibit cell cycle progression presumably during the G_1/S transition period, resulting in an accumulation of cells in G_1.

The centrosome in the MCF-7- $p27^{Kip1}$ cell line was examined using antibody against

- tubulin (Fig3). Comparing with the controlled MCF-7-pIRES cells, MCF-7-p27^{Kip1} cells showed a partial restoration of centrosome profile; an increase in the number of cells with one or two centrosomes and a decrease in the number of cells with n 3 centrosomes(6%), suggesting re-introduction of physiological high level of p27^{Kip1} re-established the normal centrosome profile.

The effect of cooperation of p21^{Waf1} and p27^{Kip1} on the duplication of centrosome and the proliferation of human breast cancer cell

The above results demonstrated that both p21^{Waf1} and p27^{Kip1} can inhibit cell cycle transition and restore centrosome duplication. As the related CDK inhibiters, the cooperation of both might be important for the progression of cell cycle and centrosome

duplication. Therefore we test whether $p21^{Waf1}$- $p27^{Kip1}$ combination get more effective inhibit effects.

The recombinant pIRES-$p21^{Waf1}$- $p27^{Kip1}$ was constructed and transfected into the MCF-7 to generate a MCF-7- $p21^{Waf1}$- $p27^{Kip1}$ cell line that constitutively expresses P21 and P27 proteins.

After transfected 48 hours, a similar significant increased P21 and P27 proteins expression were detected as the same as the expression in MCF-7- $p21^{Waf1}$ cells and MCF-7- $p27^{Kip1}$ cells(Fig 1). By flow cytometry, MCF-7- $p21^{Waf1}$- $p27^{Kip1}$ cells showed a different cell cycle phase distribution from the controls, MCF-7- $p21^{Waf1}$ and MCF-7- $p27^{Kip1}$. MCF-7- $p21^{Waf1}$- $p27^{Kip1}$ cells exhibited an increase in the number of cells in G_1 phase $P<0.01$ and a decrease in the number of cells in S phase (Fig 2),

indicating that $p21^{Waf1}$ and $p27^{Kip1}$ combination exhibited more evident inhibit effects on cell cycle progression than $p21^{Waf1}$ or $p27^{Kip1}$ single gene.

As anticipation**,** in contrast to the controls, MCF-7- $p21^{Waf1}$- $p27^{Kip1}$ cells showed a statistical increase in the number of cells with one or two centrosomes and a decrease in the number of cells with n 3 centrosomes(2%) (Fig 3), further demonstrating that $p21^{Waf1}$-$p27^{Kip1}$ combination is a more effective inhibitor than $p21^{Waf1}$ or $p27^{Kip1}$ single gene.

Several studies have suggested that both $p21^{Waf1}$ and $p27^{Kip1}$, general inhibitors of cyclin-dependent kinases in G_1 and S phase, can interact with many different cyclin-CDK complexes. This interaction is mediated by a homologous domain. Our results indicated the association between $p21^{Waf1}$ and $p27^{Kip1}$ and suggested that the association

of $p21^{Waf1}$ and $p27^{Kip1}$ plays a crucial role to assure the accurate cell cycle transition and centrosome duplication. But the specific mechanism is still poorly understood.

Discussion

Defect in the fidelity of chromosome segregation is a common characteristic of cancer cells and are likely to be important in the progression of a cancerous phenotype. Studies of cultured cells and tumor tissues have shown that tumorgenesis associated with suppressed $p21^{Waf1}$ and $p27^{Kip1}$ is primarily attributed to the deregulated centrosome duplication cycle and the consequential centrosome hyperamplification.

CDKs, a family of serine/threonine kinases, control the onset of the major cell cycle events such as DNA synthesis and mitosis, and also the accurate duplication of

centrosome. The activity of CDKs is in part regulated by association of different cyclins, which are temporally expressed at specific cell cycle stages. CDK inhibitors $p21^{Waf1}$ and $p27^{Kip1}$ interact with these complexes and thereby inhibit CDKs activity [19]. Labaer[21] and Nomura[22] found the differences between $p21^{Waf1}$ and $p27^{Kip1}$, when they study the function of CDK inhibitors. $p21^{Waf1}$ inhibits the assembly of cyclinD/CDK4 complex which is the mainly active kinase complexes in mid-earlier period of G_1, and is considered as the major inhibitor in earlier G_1. $p27^{Kip1}$ firstly binds to cyclinD/CDK4 in earlier G_1. After cyclinE/CDK4 complex formation, $p27^{Kip1}$ is visible to bind to cyclinE/CDK4 complex in later G_1 and inhibits the cell cycle transition. The accurate regulation of $p21^{Waf1}$ and $p27^{Kip1}$ is the major contributing factor for cell normal growth and development.

$p21^{Waf1}$, a general inhibitor of cyclin-dependent kinases in G_1 and S phase, can bind to many different cyclin-CDK complexes, inhibit activity of nuclear cyclin-CDK complexes. Recent reports suggested that $p21^{Waf1}$ controlled the initiation and progress of centrosome duplication by regulating cyclin/CDK2 activity[15]. One of the first related investigation was that cells lacking $p21^{Waf1}$ were prone to centrosome amplification following a prolonged S phase arrest [23]. Additional support for a role of $p21^{Waf1}$ in centrosome duplication came from studies that The human papillomavirus type 16 E7 (HPV-16 E7) oncoprotein, which inactivates $p21^{Waf1}$ [24,25] was found to stimulate centrosome overduplication[26]. In keeping with these findings, depletion of $p21^{Waf1}$ in human hematopoietic cells was found to cause abnormal centrosome numbers together with a deformed nuclear architecture and polyploidy[27]. In our studies, we

found the abnormal duplication of centrosome in MCF-7 breast cancer, following a suppressed expression of $p21^{Waf1}$. This is consistent with previous research rusult that centrosome aberrations in primary invasive breast cancer are associated with nodal status and hormone receptor expression[28]. Furthermore, after transfected exogenous $p21^{Waf1}$, MCF-7 breast cancer cells with $p21^{Waf1}$ overexpression, exhibited the arrest of cell cycle progression and restoration of the normal centrosome duplication cycle. These results are consistent with our prior studies and supported by a report that $p21^{Waf1}$ overexpression can inhibit centrosome overduplication of cells treated with hydroxyurea for a long time[15,29].

$p27^{Kip1}$, similar with $p21^{Waf1}$, was initially identified as a negative regulator of G_1 progression in growth-arrested cells [30-32] and required to ensure the coordinated

progression of centrosome duplication and cell cycle progress. Some reports have suggested that abnormal mitotic cells with amplified centrosomes were frequently observed in p27-silenced cells [17]. Furthermore, silencing of Skp2 by siRNA leads to P27 accumulation, reduction of CDK2 activity and suppression of centrosome amplification in lung cancer cells [33]. We observed that MCF-7 breast cancer cells transfected with $p27^{Kip1}$ presented cell cycle inhibition and abnormal centrosome amplification suppression, suggesting that $p27^{Kip1}$ could suppress cell cycle transition and restore centrosome amplification in breast cancer cells.

We show here that both $p21^{Waf1}$ and $p27^{Kip1}$ contribute to suppress CDK2 activity, arresting cell cycle progress and centrosome amplification. Either defection of $p21^{Waf1}$ or $p27^{Kip1}$ can induce cell cycle disorder and centrosome amplification. Thus, there

remained a possibility that $p21^{Waf1}$ and $p27^{Kip1}$ cooperate to regulate cell cycle progress and centrosome duplication depending on the differences between $p21^{Waf1}$ and $p27^{Kip1}$ in molecular structure, control methods, pathway activity and mode of action. Our study found that $p21^{Waf1}$- $p27^{Kip1}$ combination had more significant inhibition of cell cycle progress and centrosome amplification than $p21^{Waf1}$ or $p27^{Kip1}$ single gene. These results lend support to the hypothesis that the cooperation of $p21^{Waf1}$ and $p27^{Kip1}$ play a synergia role in regulating the cell cycle progress and centrosome duplication.

The tumorigenesis and progression of breast cancer refer to a variety of oncogenes, tumor suppressor genes forming a network-control. Therefore study of a single gene is difficult to see the whole picture, and that of multiple genes and their linkages is closer to the body the actual situation.Our findings suggest that $p21^{Waf1}$ and

$p27^{Kip1}$ overexpression can inhibit cell proliferation through G_1 cell cycle arrest and restore centrosome duplication through centrioles separation supression in breast cancer. Possibly because of the differences in molecular structure, control methods, pathway activity and mode of action, $p21^{Waf1}$ and $p27^{Kip1}$ cooperate to inhibit cell proliferation and centrosome duplication. Then $p21^{Waf1}$- $p27^{Kip1}$ combination might have potential clinical significance as therapeutic agents of breast cancer with suppressed $p21^{Waf1}$ and $p27^{Kip1}$ expression. Multi-gene therapy may provide a new idea for breast cancer gene therapy, but the mechanism of the synergy between $p21^{Waf1}$ and $p27^{Kip1}$ remains to be further explored.

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Conflict of interest statement

None declared.

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