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

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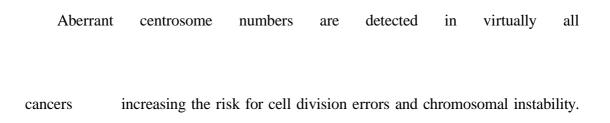
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Abstract

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 <u>significative</u>. 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.

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$^{\text{Waf1}}$

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$^{\text{Waf1}}$ re-established the normal centrosome

profile.

These observations demonstrate that p21$^{\text{Waf1}}$ is directly involved in the controlling the

coordinated initiation of centrosome and DNA duplication. p21$^{\text{Waf1}}$ 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$^{\text{Kip1}}$

p27$^{\text{Kip1}}$, as the related CDK inhibitor, also exhibited a significant reduction in most

human aggressive tumors(16). We transfected pIRES-p27$^{\text{Kip1}}$ 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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