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CYR61 controls p53 and NF-κB expression through PI3K/Akt/mTOR pathways in carboplatin-induced ovarian cancer cells

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ABSTRACT

CYR61 over-expression promotes cell proliferation by inhibiting carboplatin-induced apoptosis, decreasing Bax expression, and increasing Bcl-xL, Mcl-1, and Bcl-2. At the same time, down-regulating p53 expression, while up-regulated NF-κB expression. Additionally, p21 and p53 promoter activities were reduced, while NF-κB and Bcl-2 activities increased. In parallel, CYR61-expressing cells, during carboplatin-induced apoptosis, resulted in an increase of Akt phosphorylation, while rapamycin-treated cells were not affected. Carboplatin effectively inhibited the activation of mTOR signaling cascade, which includes mTOR, 4E-BP1, p70S6K, HIF-1α, and VEGF. These results provide evidence that CYR61 promotes cell proliferation and inhibits apoptosis.

1. Introduction

Cysteine-rich 61 (CYR61), one of the tissue growth factors in the CCN family (CYR61/CTGF/NOV), is highly expressed in various cancer tissues and cell lines, including breast cancer, endometrial cancer, MCF-7 cells, SKOV-3 cells, gastric cancer cells, benign prostatic hyperplasia, gliomas, and melanomas [1–7]. In contrast, resting cells were down-regulated, but various agents including growth factor are involved in regulating the resting cells [8]. The various agents are also responsible for regulating multiple cellular functions, such as cell proliferation, inflammation, cell adhesion, migration, embryogenesis, and wound healing [6,9–12]. Interestingly, the over-expression of CYR61 inhibits apoptosis, which is mediated by paclitaxel and cisplatin, and may also contribute to cancer cell viability [3,13,14]. In addition, CYR61 protein promotes angiogenesis and migration, as well as adhesive signaling by interacting with αvβ3 and α6β1 [15,16].

Carboplatin (*cis*-diammine-1,1-cyclobutanedicarboxylate-platinum II) is widely used clinically on various human carcinomas, including ovarian, lung, head, and neck carcinomas [17–19]. Carboplatin, an analog to cisplatin, has fewer adverse toxic effects, unlike cisplatin, which generally involves serious side effects, such as nephrotoxicity, neurotoxicity, myelosuppression, nausea, and vomiting [20,21]. As such, carboplatin has been suggested as a means to suppress cell growth in cancer cells through the death receptor [22,23].

Apoptosis plays an important role by which cells undergo death to control cell proliferation. Generally, regulation

Abbreviations: CYR61, cysteine-rich 61; Carboplatin, *cis*-diammine-1,1-cyclobutanedicarboxylate-platinum II; DAPI, 4,6′-diamidino-2-phenylindole; mTOR, the mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-^{2}H-tetrazolium bromide; FITC, fluorescein isothiocyanate; PI, propidium iodide.

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in this form of cell death comprises the participation of p53 and Bcl-2 family genes. Bcl-2 family proteins are known as important regulators of apoptosis [24,25], and involve anti- or pro-apoptotic members, such as Bcl-2, Bcl-xL, Mcl-1, and Bax [26,27]. The activation of Bcl-2 protein can be regulated by post-translational modifications, such as phosphorylation to Akt, mTOR, and p70S6K [28,29]. The Akt family is a critical mediator of cell growth. Akt promotes cell survival via various molecular mechanisms that include phosphorylation and the inactivation of pro-apoptotic proteins, such as Bad, glycogen synthase kinase-3 (GSK-3), Forkhead, and caspase 9 [30–32]. Interestingly, this down-stream effector for PI3K/mTOR is constitutively activated in many types of human tumors, including ovarian cancer [33–35].

In this study, we showed that the over-expression of CYR61 clearly prevented carboplatin-induced apoptosis in ovarian cancer cells, and also determined the underlying molecular mechanisms. Furthermore, we showed that the NF-κB and PI3K/Akt pathways are affected by CYR61 and the pattern of expression of Bcl-2 family proteins are regulated by CYR61 expression under siRNA (CYR61) or by carboplatin-induced apoptotic conditions in OVCAR-3 cells. With these observations, we elucidated that the possible intracellular mechanisms of CYR61 have a protective role on carboplatin-induced apoptosis.

2. Materials and methods

2.1. Cell line, culture, chemicals, and antibodies

Human ovarian cancer cell lines (OVCAR-3) were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and grown in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 units/ml) in a 37 °C atmosphere containing 5% CO_2.

Rapamycin was purchased from Cell Signaling Technology (Beverly, MA). Other anti-cancer drugs and chemicals were purchased from Sigma (St. Louis, MO). Carboplatin was dissolved in distilled water. The following antibodies were used in this study: anti-CYR61, anti-Bax, anti-Bcl-xL, anti-Mcl-1, anti-Bcl-1, anti-p53, anti-NF-κB, anti-CDK4, anti-cyclin D1, anti-Akt, anti-phospho-specific Akt, anti-PI3K, anti-phospho-specific PI3K, anti-mTOR, anti-phospho-specific mTOR, anti-p70S6K, anti-phospho-p70S6K, and anti-VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-caspase-3 (BDPhamingen, San Diego, CA); anti-p27, anti-p21, and anti-p16 (Oncogene, San Diego, CA); anti-4E-BP1 and anti-phospho-4E-BP1 (Cell Signaling Technology); and anti-β-actin (Sigma).

2.2. Analysis of cell proliferation

Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-^{2}H-tetrazolium bromide (MTT) assay. OVCAR-3 ovarian cancer cells were briefly plated in DMEM containing 10% FBS. The cells were seeded at a density of 4.5×10^3 cells/well in 96-well plates. After 24 h, fresh cell culture medium containing 10% FBS and 20 μl of MTT solution (5 mg/ml; Sigma) was added to each well. Each well was then incubated for an additional 4 h at 37 °C. The amounts of MTT-formazan generated were measured as the absorbance with the use of a microplate reader at 540 nm. Each sample was assayed in triplicate and the experiments were repeated three times.

2.3. Apoptosis and cell distribution analysis

OVCAR-3 cells were distributed onto 6-well plates and treated with different concentrations of carboplatin (0, 5, 10, 15, 20, 25, and 50 μM) for 24 h, or transfected with the expression vector containing human *Cyr61* cDNA. To evaluate apoptotic cells, the nuclei were fixed in methanol, stained with 2 μg/ml of 4,6′-diamidino-2-phenylindole (DAPI; Boehringer Mannheim, Mannhein, Germany) at 37 °C for 15 min, rinsed twice with PBS, and monitored under a fluorescence microscope. Three independent experiments were conducted in triplicate. The DNA content was subsequently analyzed by flow cytometry. OVCAR-3 cells were seeded at a density of 3.6×10^5 cells in 60-mm plates. After treatment/transfection with carboplatin, CYR61, or siCYR61, the cells were harvested, rinsed with ice-cold PBS, and fixed with ice-cold 70% ethanol. The cells were centrifuged at 1,000*g* for 5 min, and re-suspended in PBS containing 5 mM of EDTA and RNase A (1 mg/ml). After incubation at 37 °C for 1 h, the cells were treated for 15 min with fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (PI), according to the supplier's directions (Boehringer Mannheim), then the data was analyzed with a flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ).

2.4. Construction of small interfering RNA (siRNA)

The siRNA oligonucleotide sequence targeting *Cyr61* (5′-AACATCAGTGCACATGTATTG-3′) corresponded to nucleotides 561–581 in the human sequence. The siRNA was synthesized using a siRNA construction kit (Ambion, Austin, TX, USA), and was then transfected by oligofectamine (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's directions. After transfection of over-expressing *Cyr61* cDNA and siRNA of *Cyr61*, the mRNA and levels of protein expression of human CYR61 were compared to mock transfectants (empty vector only without insert) by RT-PCR and immunoblot, as described below.

2.5. Measurement of caspase-3 activity

For detection of caspase-3 activity, cells (2.2×10^6) were grown in the absence or presence of carboplatin for 24 h at 37 °C. Caspase-3 activity was quantified by use of an actyl-DEVD-7-amino-4-trifluoromethyl coumarin as the substrate, according to the supplier's instructions (BDPharmingen). The cells were briefly incubated for 24 h with VP-16 (100 μg/ml), lysed in lysis buffer, and centrifuged at 12,000*g* at 4 °C for 25 min. Enzyme activity was measured in the supernatant fraction according to the proteolytic cleavage of the colorimetric substrate using a Spectramax 340 microplate reader (Molecular Devices, Sunnyvale, CA) in the fluorescence mode, with excitation