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Original research

P53 suppresses cell proliferation, metastasis, and angiogenesis of osteosarcoma through inhibition of the PI3K/AKT/mTOR pathway

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HIGHLIGHTS

- P53 potently inhibited cell proliferation in osteosarcoma cell line (MG63) and in human normal osteoblasts (hFOB1.19) *in vitro.*
- An inhibitory effect of P53 on metastasis was observed in osteosarcoma cell line MG63.
- P53 suppresses cell proliferation and angiogenesis of osteosarcoma through inhibition of the PI3K/AKT/mTOR pathway.

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ABSTRACT

Objective: To investigate the role of P53 in the pathogenesis of osteosarcoma and the possible mechanism involved in it.
Methods: The anti-proliferative effect of P53 was assessed using the cell counting Kit-8 assay. The migration and invasion potential were analyzed using wound-healing and transwell assays, respectively. The Matrigel capillary tube formation assay was performed to mimic *in-vivo* angiogenesis. Immunoblotting and immunofluorescence were used to observe protein levels and distribution of actin fibers. Finally, S2448p-mammalian target of rapamycin (mTOR) expression was detected on osteosarcoma tissues using immunohistochemistry.
Results: Firstly, P53 potently inhibited cell proliferation in osteosarcoma cell line (MG63) and in human normal osteoblasts (hFOB1.19) *in vitro* at the IC50 ranged from 50 to 500 nmol/l. Then, an inhibitory effect of P53 on metastasis was observed in osteosarcoma cell line MG63, along with the cytoskeletal rearrangements and suppression of the phosphorylation of PI3K downstream factors including AKT and mTOR.
Conclusion: These results show that P53 suppresses cell proliferation and angiogenesis of osteosarcoma through inhibition of the PI3K/AKT/mTOR pathway, which might be an effective novel therapeutic candidate against osteosarcoma in the future.
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1. Introduction

Osteosarcoma is the most common bone malignancy affecting children, adolescents, and young adults in the world [1], and for which no appreciable improvement in outcome has been attained in the past 20 years, indicating that we have reached the limits of classical non-targeted therapies. Tumor cell metastasis is thought to be controlled by molecular processes that are different from those which control tumor initiation and growth [2]. Support for this hypothesis comes from the observation of human cancer lesions as well as several mouse models in which tissue-specific oncogene expression led to tumor initiation, yet tumor progression was not observed [3–5]. The metastatic process is complex because it involves several distinct steps such as tumor cell dispersal from the epithelium, invasiveness, intravasation into lymph or blood vessels, dissemination, and extravasation into a remote organ and colonization of this organ [6].

The mammalian target of rapamycin (mTOR) is a serine/threonine downstream mediator in the phosphatidylinositol 3-kinase signaling pathway, which is a central controller of eukaryotic cell growth and plays a critical role in regulating important cellular functions, including proliferation, growth, survival,

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mobility, and angiogenesis [3,4]. Aberrant activation of mTOR signaling has been reported in many cancers including human osteosarcoma, which could be attributed to several mechanisms including mutational activation of PIK3CA and loss of the phosphatase–tensin homolog deleted from chromosome 10 (PTEN) [5,6]. It exists in two distinct complexes –mTORC1 and mTORC2. mTORC1 controls cell autonomous growth in response to nutrient availability and growth factors, whereas mTORC2 is considered to mediate cell proliferation and cell survival [7,8]. When active, mTORC1 promotes cell growth by directly phosphorylating the translational regulators ribosomal protein S6 kinase (P70S6K1) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). Phosphorylation of 4E-BP1 inhibits its binding to eukaryotic initiation factor 4E (eIF4E), which enables the cap-dependent translation of multiple mRNAs such as Bcl-2 and vascular endothelial growth factor, thereby increasing cell proliferation, survival, and angiogenesis [9,10]. This provides a strong rationale for targeting mTORC1 in cancer and this led to clinical trials of several rapamycin analogues [11]. However, the first-generation mTOR inhibitor everolimus as a single agent was thwarted in advanced osteosarcoma patients possibly in part by strong mTORC1-dependent negative feedback loops [7,8].

Although mTORC1 is in the vanguard of mTOR research, mTORC2 is emerging as a pivotal player in many cancers.

It was discovered that mTORC2 directly phosphorylates PI3K, which is required for maximal activation of the anti-apoptosis kinase, leading to enhanced cell survival, proliferation, and migration [12]. This led to efforts to develop selective ATP-competitive small-molecule mTOR inhibitors with the expectation of completely blocking both mTORC1 downstream effectors and mTORC2 substrates, which may outperform rapamycin analogues as anticancer drugs [7,13]. For these reasons, we thereby investigate the role of P53 in the pathogenesis of osteosarcoma and the possible mechanism involved in regulating mTOR.

2. Materials and methods

2.1. Cell culture and transfection

Human osteosarcoma cell line MG63 and normal human osteoblasts cell line hFOB1.19 were purchased from the American Type Culture Collection and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) with 100 ug/ml penicillin/streptomycin at 37 °C with 5% CO_2. Culture medium was Dulbecco modified Eagle medium and Ham F12 medium (DF12) containing 40% MCDB-201 medium complete with trace elements (MCDB) (Sigma), 2% fetal calf serum (FCS; Gibco Life Technologies, Paisley, United Kingdom), 1 × insulin transferrin selenium (Gibco Life Technologies), 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 20 ng/mL interleukin-6 (Sigma), 10 ng/mL epidermal growth factor (Sigma), 10 ng/mL platelet-derived growth factor BB (Sigma), 50 ng/mL fetal liver tyrosine kinase 3 (Flt-3) ligand (Sigma), 30 ng/mL bone morphogenetic protein-4 (Sigma), 100 U/mL penicillin and 100 ug/mL streptomycin (Gibco Life Technologies) at 37 °C and a 5% CO_2 humidified atmosphere. Culture media were changed every 4–6 days. Transfection was performed with Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. Total RNA and protein were prepared 48 h after transfection and were used for qRT-PCR or Western blot analysis.

2.2. IHC

Staining of chronic meyloid leukemia specimen sections for phosphorylated mTOR was performed with an antibody specific for mTOR when it was phosphorylated on S2448 (clone 49F9 used at a 1:50 dilution; Cell Signaling Technologies). Paraffin-embedded sections of 5 μm thickness were deparaffinized with xylene and rehydrated through a graded alcohol series. Endogenous peroxidase activity was then blocked by incubation in 3% hydrogen peroxide–methanol for 10 min. Antigen retrieval was achieved by microwaving (0.1 mol/l citrate buffer, pH 6.0). EnVision (Dako, Carpinteria, California, USA) was used as the secondary antibody. Antibody binding was visualized by a standard streptavidin immunoperoxidase reaction, followed by chromogen detection with diaminobenzidine for 30 s and hematoxylin counterstaining. Immunoreactivity in the membrane was evaluated. Each histological section was examined at × 40 magnification to identify areas of maximum tumor positivity. At × 200 or × 400 magnification, cells were analyzed from five areas of maximum tumor positivity in each case and the average percentage of positive cells was recorded. The specificity of phospho-mTOR staining was validated in serial negative control sections.

2.3. Immunofluorescence assay

To determine the effect of P53 on cell morphology and actin stress fibers, MG63 and hFOB1.19 cells (8×10^4 cells per well) were plated in six-well plates and grown for 24 h so that they attached to the surface of the plates completely. P53 was added to cells grown at 37 °C for 24 h. After the exposure period, media were removed and cells were washed with PBS. Cells were then fixed with 3.7% paraformaldehyde in PBS for 15 min and incubated with 0.1% Triton X-100/in PBS for 30 min. Cells were incubated with 1% BSA in PBS for 30 min (blocking) and then with 70 nmol/l rhodamine phalloidin for 20 min to stain the actin filaments. Nuclei were stained with 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) in PBS for 1 min and the culture plates were examined and photographed by immunofluorescence microscopy.

2.4. Western blot

Cells were harvested, washed twice in PBS, and lysed in lysis buffer (protease inhibitors were added immediately before use) for 30 min on ice. Lysate was centrifuged at 10,000 rmp and the supernatants were collected and stored at −70 in aliquots. All procedures were carried out on ice. Protein concentration was determined using BCA assay kit (Tianlai Biotech).

2.5. Cell viability assay

Cells were seeded into 96-well plates (2×10^3 cells/well) directly or 24 h after transfection and allowed to attach overnight. Freshly prepared cisplatin was then added with different final concentrations. Forty-eight hours later, cell viability was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay as described previously [13].

2.6. Wound-healing assay

A wound-healing assay was used to assess capacity for tumor cell motility. Briefly, cells (1×10^6/well) were seeded in six-well plates, cultured overnight. On reaching confluency, the cell layer was scratched with a sterile plastic tip and then washed with culture medium twice and cultured again for up to 48 or 72 h with serum-reduced medium containing 1% FBS. Photo images of the plates were taken under a microscope. The gap closure was measured at 48 or 72 h and the data were summarized based on sextuple assays for each experiment.

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