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SCIENCE DIRECT
Colloids and Surfaces B: Biointerfaces 46 (2005) 188–196
COLLOIDS AND SURFACES B
www.elsevier.com/locate/colsurfb

The co-transfection of $p16^{INK4a}$ and $p14^{ARF}$ genes into human lung cancer cell line A549 and the effects on cell growth and chemosensitivity

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Received 11 September 2005; accepted 10 October 2005

Abstract

Two functionally and structurally different proteins, $p16^{INK4a}$ and $p14^{ARF}$, encoded by the gene INK4a/ARF located at 9p21 are cyclin-dependent kinase (cdk) inhibitors and important cell cycle regulators. More and more evidences have been accumulated to show that the exogenous $p16^{INK4a}$ or $p14^{ARF}$ can inhibit the cell growth and/or induce the apoptosis. But it is still unclear if they can play positive role when combine with the conventional chemotherapy in cancer treatment. Here we show that cationic liposome-mediated gene transfection of INK4a/ARF into lung cancer cell line A549, in which the INK4a/ARF locus was lost, suppressed the growth and induced apoptosis. When treated with five different chemotherapy drugs with different mechanism after the transfection, A549 got an increased chemosensitivity for adriamycin and cisplatin and an unchanged result for topotecan, taxol or vinorelbine. The results indicated that cell cycle redistribution and increased apoptosis index after transfection might be the main explanation for the enhanced chemosensitivity. The combination of gene therapy with conventional chemotherapy is not always better than single chemotherapy. This trial will be of benefit to the treatment of lung cancer when combine the conventional chemotherapy and gene therapy in the future.

Keywords: INK4a/ARF; $p16^{INK4a}$; $p14^{ARF}$; Gene therapy; Chemosensitivity; Lung cancer; A549

1. Introduction

The recent completion of the human genome project and advances in functional genomics have launched the war against cancer by seeking novel therapeutic interventions based on the most fundamental aspect of cancer pathogenesis: gene. INK4a/ARF, one gene located at chromosome 9p21, is a major target in the tumorigenesis of various human malignant diseases. This locus harbors two tumor suppressor genes, INK4a and ARF, which encode two functionally and structurally distinct proteins, $p16^{INK4a}$ and $p14^{ARF}$ respectively [1–4]. Both genes include three exons and share the same exons 2 and 3. The first exon is different, 1α for $p16^{INK4a}$ and 1β (located about 15 kb upstream of 1α) for $p14^{ARF}$. Proteins $p16^{INK4a}$ and $p14^{ARF}$ consist of 156 amino acid (aa) and 138 aa respectively and the primary aa sequences of the two proteins are completely unrelated due to the different reading frame. Both $p16^{INK4a}$ and $p14^{ARF}$ are cell cycle-regulatory proteins and simultaneously play active roles through two most important cell cycle regulation pathways so far identified, p16-CDK4/6-cyclinD-Rb-E2F and $p14^{ARF}$-MDM2-p53 respectively (Fig. 1) [5–9].

$p16^{INK4a}$ is a member of the INK4 family, which also includes the p15INK4B, p18INK4C, p19INK4D and p21WAF1 [10]. Since it was firstly reported in 1994, $p16^{INK4a}$ has been considered as one of the most inactivated genes in a wide variety of malignant human tumors by several molecular mechanisms including homozygous deletions, point mutations, and hypermethylations in CpG islands [11–15]. The trials both in vivo and in vitro showed that p16 function through inhibiting the cyclin-dependent kinases CDK4 and CDK6, which promote the cell cycle progression through G_1 phase by phosphorylation of the retinoblastoma gene product (Rb). When $p16^{INK4a}$ overexpresses, the cell cycle will be arrested at the checkpoint G_1 to S phase [16,17]. In addition, the adenovirus/plasmid-mediated

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doi:10.1016/j.colsurfb.2005.10.006

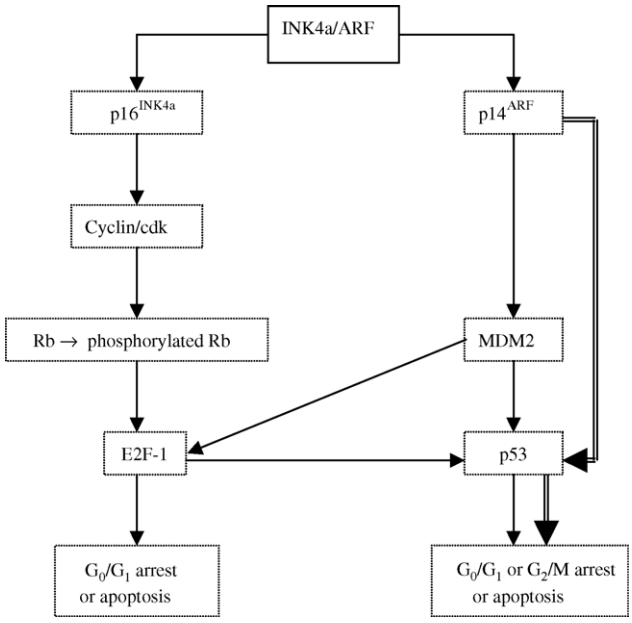

Fig. 1. The INK4a/ARF network.

overexpression of $p16^{INK4a}$ in mammalian cells showed that this protein also plays a very important role in the regulation of apoptosis [18,19]. So, it was proposed that $p16^{INK4a}$ will be of benefit to the treatment of malignant diseases in the future.

Despite the completely different structure with $p16^{INK4a}$, $p14^{ARF}$ can also arrest the cell cycle at both G_0/G_1 and G2/M checkpoints in a p53-dependent manner. The mechanism of this inhibitory function is that $p14^{ARF}$ binds with MDM2 protein and accelerates its degradation. Thus the tumor suppressor p53 can stabilize and accumulate to inhibit the cell proliferation or induce apoptosis [20,21]. At the same time, more and more evidences are counting to show that ARF was also a tumor suppressive gene. The exogeneous overexpression of $p14^{ARF}$ could inhibit the growth of some human cancer cell lines including lung cancer, colon cancer, liver cancer and so on. In addition, $p14^{ARF}$ could also induce apoptosis in some experiments [22–24].

Recently, the further research demonstrate that MDM2 can stimulate E2F transactivation activity and inhibits Rb growth suppression function by p21-independent manner and E2F-1 can mediate apoptosis by inducing the phosphorylation of p53 [25–27]. So, the pRb and p53 pathways make up of a cell growth regulation network and the protein $p16^{INK4a}$ and $p14^{ARF}$ are the upstream elements. The absent of protein $p16^{INK4a}$ and/or $p14^{ARF}$ will lead to the dysfunction of the cell cycle control.

Here we co-transfected wild-type (wt) $p16^{INK4a}$ and $p14^{ARF}$ into the lung adenocarcinoma cell line A549 in which the INK4a/ARF gene site is lost but the cyclin D, cdk4/6, Rb, E2F, MDM2 and p53 genes are normally expressed. As a control, the parental cell and negative control cell with blank control plasmid pcDNA3-LacZ were used. By comparing the cell survival curves, clonal survival rates, apoptosis index, cell cycle redistribution and the effects on the chemosensitivity of five chemotherapy drugs with different mechanisms, we concluded that combination of some gene therapy with conventional chemotherapy is not always better than single chemotherapy. The possible reason was discussed.

2. Materials and methods

2.1. Cell line and cell culture

The established human lung adenocarcinoma cell line A549 bought from Shanghai Institutes for Biological Sciences, CAS (SIBS) was used in our experiment. A549 has a deletion in the INK4a/ARF locus but retains intact wild-type p53, RB, CDK4/6, cyclin D, E2F and MDM2. The cells were cultured in RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and sodium bicarbonate at 37 °C in an incubator with 95% air and 5% CO_2.

2.2. Cytotoxic drugs

Five cytotoxic drugs with different mechanisms were used, adriamycin (ADM, HISUN Pharmaceutical Co. Ltd., China), cisplatin (CDDP, F.H. Faulding & Co. Ltd., Australia), vinorelbine (NVB, Pierre Fabre, France), topotecan (TPT, Runkang Pharmaceutical Co. Ltd., China) and paclitaxel (Anzatax, F.H. Faulding & Co. Ltd., Australia). Every drug was initially dissolved in 0.9% sodium chloride and was diluted with fresh medium immediately before each experiment. The concentration of each drug varied from 0.001 to 10 times of the peak plasma concentration (PPC).

2.3. Plasmids and transfection

The eukaryotic expression plasmids pcDNA3-$p16^{INK4a}$ containing the wild-type INK4a gene was a gift from Dr. Frank Furnari (Ludwig Institute For Cancer Research, USA), pcDNA3.1-$p14^{ARF}$ containing the wild-type $p14^{ARF}$ was from Dr. Sylvie Gazzeri (Groupe de Recherche sur le Cancer du Poumon, FRANCE) and the blank control plasmid pcDNA3-LacZ was from Dr. Rui Hong-bing.(Hematology Department of Fujian Medical University, China).

Transfection was performed according to the liposomal formulation of DOTAP transfection reagent (Roche, USA) in six-well plates. The day before transfection, A549 cells were subcultured and replated at a plating density yielding approximately 50% confluence at the time of the transfection. DOTAP and the plasmids pcDNA3-$p16^{INK4a}$ and pcDNA3.1-$p14^{ARF}$ were mixed 20 min before transfection and then culture medium was replaced with fresh medium containing the DOTAP/plasmids mixture. Cells were incubated for 10 h before the medium containing DOTAP/plasmid was replaced with normal medium and the cells were cultured for another 48 h. Afterwards, normal culture with additional 400 mg/ml G418 (Roche) was used for 14 days to select the transfected cells. The cells containing both plasmids pcDNA3-$p16^{INK4a}$ and pcDNA3.1-$p14^{ARF}$ by RT-PCR and ICC were used for the following experiments and named as A549-$p16^{INK4a}$-$p14^{ARF}$. The control cells transfected with pcDNA3-LacZ was named A549-vector.