TRANSFER OF p14ARF GENE IN DRUG-RESISTANT HUMAN BREAST CANCER MCF-7/Adr CELLS INHIBITS PROLIFERATION AND REDUCES DOXORUBICIN RESISTANCE

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ABSTRACT

Objective: To elucidate the effect of p14ARF gene on multidrug-resistant tumor cells. Methods: We transferred a p14ARF cDNA into p53-mutated MCF-7/Adr human breast cancer cells. Results: In this report we demonstrated for the first time that p14ARF expression was able to greatly inhibit the MCF-7/Adr cell proliferation. Furthermore, p14ARF expression resulted in decreases in MDR1 mRNA and P-glycoprotein production, which linked with the reducing resistance of MCF-7/Adr cells to doxorubicin. Conclusion: These results imply that drug resistance might be effectively reversed with the wild-type p14ARF expression in human breast cancer cells.

Key words: p14ARF, MDR-1, P-glycoprotein, Doxorubicin, Breast cancer, Tumor suppression

The INK4a/ARF locus on human chromosome 9p21 encodes two different transcripts derived from alternative splicing of upstream exons (E1 α and E1 β) to a common splice acceptor site in exon2.[1-5] The α transcript encodes the p16INK4a tumor suppressor,[6,7] while the β one yields a completely distinct polypeptide that incorporates exon2 coding sequences in an alternative reading frame encoding an unrelated product ARF (human p14ARF, murine p19ARF).[1-5] The p16INK4a protein, recognized as a major tumor suppressor implicated in a wide variety of tumor types, can block cyclin D-dependent kinase (cdk) activities by preventing their association with the D-type cyclins, and cause cells to arrest in G1.[7-10] ARF, functioning also as a growth suppressor,[8,9,11,12] is ubiquitously expressed at low levels detectable only by RT-PCR in most tissues with the exception of the pancreas,[13] and is elevated in cells lacking functional p53.[14] ARF does not bind to cdks or inhibit the activities of cyclin-cdk complexes, however, overexpression of ARF results in cell cycle arrest in both G1 and G2.[1,5,8,9,14] Cell cycle arrest mediated by ARF is abolished if cells lack functional p53, indicating that ARF may act on the upstream of p53.[14-17]

Recent work has shown that the role of ARF in the p53 pathway is to bind to MDM2.[18-25] Some studies confirmed that ARF acted by binding directly to MDM2, resulting in the stabilization of both p53 and MDM2,[18-23] and the other studies also reported that ARF inhibited the ubiquitin ligase activity of MDM2 for p53, suggesting that ARF promotes the stabilization of p53 by inactivating MDM2.[25,26]

However, no study of the effect of p14ARF gene transfer in multidrug resistant tumor cells has been reported so far. In order to investigate the effect of p14ARF gene on multidrug-resistant tumor cells, we transferred a p14ARF cDNA in p53-mutated MCF-7/Adr human breast cancer cells. In this report we demonstrated for the first time that p14ARF expression was able to greatly inhibit the MCF-7/Adr cell proliferation. Furthermore, p14ARF expression resulted in decreases in MDR1 mRNA and p-glycoprotein production, and also led to reduction of resistance in MCF-7/Adr cells to doxorubicin. These results suggest that p14ARF, second to p53, is a potential candidate in gene therapy for clinical multidrug-resistant tumors.

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MATERIALS AND METHODS

Cell lines and Culture Conditions

L02 human liver cells, MCF-7 human breast cancer

cells which express wild-type p53, and its multidrug-resistant derivative MCF-7/Adr which express mutated p53, were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 ng/ml penicillin and 100 ng/ml strptomycin (Gibco-BRL, Grand Island, NY) at 37℃ in 5% CO_2. MCF-7/Adr cells were maintained continuously in the presence of 8μg/ml doxorubicin (Sigma, St. Louis, MO), except that the drug was removed from the medium 7 days prior to use in an experiment.

Construction of expression vectors

Total RNA was isolated from L02 human liver cells using Trizol reagent (Gibco/BRL) as described by the manufacturer. The RNA was reverse transcribed in the presence of random hexamers (Phermacia) using MoMLV reverse transcriptase (Gibco/BRL) in a volume of 40μl as described by the manufacturer. The primers for PCR amplification of p14ARF were: 5'— GGC GGA TCC ATG GTG CGC AGG TTC T—3'(25bp) and 5'— CCG AAT TCT CAG CCA GGT CCA CG—3'(23bp). Cycle parameters consisted of denaturation at 94℃ for 30 sec, primer annealing at 55℃ for 1 min and extension at 72℃ for 2 min. Thirty-five amplification cycles were performed and a final extension at 72℃ for 10 min. The PCR product was digested with BamHI and EcoRI and subcloned into the corresponding sites in the Bluescript KS(+),called pKSARF. After DNA sequencing the p14ARF cDNA were excised from the pKSARF using BamHI and EcoRI, and subcloned into the same sites in the pcDNA3 vector, called pcDNA3ARF.

Transfection and Colony-forming Assay

For transient transfection assays, MCF-7 and MCF-7/Adr cells were plated at a density of 5×10^5 cells per 10 cm dish, and transfected overnight with 10 μg pcDNA3ARF or the control vector pcDNA3 using Lipofectamine™ (Gibco-BRL). Colony formation assays were performed by passaging cells 48h after transfection at a low density into 6cm dishes containing media with 500 μg/ml G418 (Gibco-BRL), and cell colonies were scored by methanol fixation and crystal violet staining 3 weeks later. Stable transfectants were selected from some single colonies with G418. Immunocytochemistry assay using anti-p14ARF polyclonal antibody (kindly provided by Dr. Peters) was performed to identify p14ARF stable transfectants.

Doxorubicin Resistivity Assays

Doxorubicin(DOX) resistivity was measured by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay, for which 200 μl of cell suspension was seeded into 96-well plates at a final concentration of 5×10^3 cells/well, and incubated at 37℃ in a humidified incubator under 5% CO_2 for 16–24 hours, then dilutions of the DOX in PBS was done by using a series of concentrations and adding them into the wells. Then incubation was continued for an additional 72 hours. 20 μl MTT (10 μg/μl) was added into each well for 4 hours further incubated at 37℃. The dye was solubilized with DMSO and cell viability was determined by measure of absorption (570 nm) using an Elx800 (Bio-Tek instruments INC.).

MDR-1 Expression and Western Blot Assay

RT-PCR assay was performed as the above described with 1μg total RNA using gene specific primers. The primers for amplification of MDR1 and β–actin were: MDR-1, 5'-CTG AAG AGG TCT TGG CAG CA-3' and 5'-CAT AGA GCC TCT GCA TCA GC-3'; β–actin, 5'-TAC ATG GCT GGG GTG TTG AA-3', and 5'-AAG AGA GGC ATC CTC ACC CT-3'. β–actin mRNA was amplified as a control separately to avoid any competitive PCR, PCR products were 573 bp and 121 bp for MDR1 and β–actin, respectively, PCR products were separated on a 2% agarose gel. The relative expression of marker mRNA was quantitated by densitometry.

Western blotting was done according to the described previously [14, 26]. Briefly, after separation on a 4–15% polyacrylamid gel containing SDS, the protein was transferred electrophoretically onto a membrane (Immobilon-P, Millipore, Eschbom, Germany) using a mini transblot cell (Bio-Rad Laboratories, Hercules, CA) in towbin-buffer (25 mM Tris-HCl, pH 8.3, 193 mM glycine, 0.1% SDS, 20% methanol) at 150 mA for 1.5 hours. The membrane was blocked with 1% bovine serum albumine and subsequently incubated with the mouse anti-Pgp, P170 (Zymed, San Francisco, CA) at room temperature for 2 hours. After washing with 0.1% Tween 20-containing TBS (100 mM Tris-HCl, pH 8, 1.5 M NaCl) the membrane was incubated with a horseradish peroxidase-linked secondary antibody for 1 hour at room temperature and detected by the ECL Western blotting detection kit (Amersham).

Accumulation of Rhodamine 123

Rhodamine 123, a fluorescent dye, can easily be expelled to outside of the cell by the product of *mdr* 1 gene — Pgp glycoprotein, and the intracellular accumulation of this compound indicated the functional activity of the efflux pump.[27] Cells were centrifuged and resuspended in serum-free DMEM at approximately 1×10^6 cells per ml, Immediately before measurement of fluorescence intensity the cells were pipetted through a

1ml syringe to avoid their aggregation, and rhodamine 123 (Sigma) was added to a final concentration of 2.5 μg/ml and the mixture was incubated at 37% for 30 min. After the cells were washed twice with serum-free DMEM, incubated at 37℃ for 10 min, and washed with culture medium, the fluorescence intensity was measured finally at 488 nm by flow cytometry (FACS440, Becton, Dickinson).

RESULTS

Effect of p14ARF on MCF-7 and MCF-7/Adr Cells Growth

In order to investigate the effect of p14ARF expression on the multidrug-resistant human breast cancer MCF-7/Adr cells ,we cloned the human CDKN2A β transcript (p14ARF) by RT-PCR using RNA obtained from L02 human liver cells, and the result of the cDNA sequencing indicated the same as the previously reported. Then the expression plasmid pcDNA3ARF was generated by inserting the above p14ARF cDNA into the pcDNA3 (See Materials and Methods).

To test whether p14ARF expression in MCF-7/Adr cells suppress their growth, we performed a colony-forming assay by transfecting pcDNA3ARF or empty vector pcDNA3 containing neomycin-resistance gene into MCF-7 and MCF-7/Adr cells. After 3 weeks of G418 selection, the number of G418-resistant colonies was scored. A dramatic reduction in the number of G418-resistant colonies was seen in cells (MCF-7 and MCF-7/Adr) transfected with pcDNA3ARF as compared with that with the empty vector pcDNA3 (Figure 1). The colony-forming efficiency of the pcDNA3ARF-transfected MCF-7 cells to pcDNA3- transfected cells was 26±2.5%. A similar degree of inhibition was found in MCF-7/Adr cells (17±1.5%). These data suggest that p14ARF expression might be associated with antiproliferation and /or proapoptotic activity in breast cancer cells.

To further characterize the biological effects of p14ARF expression on the MCF-7/Adr cells, we attempted to isolate several lines of stably p14ARF-expressing clones. Using immunocytochemistry assay with anti-p14ARF polyclonal antibody, we were able to identify one stably p14ARF-expressing clone (of 20) from each cell line, i.e., MCF-7-ARF and MCF-7/Adr-ARF (Figure 2). The low frequency of p14ARF-expressing clones obtained from the G418-resistant colony supports the idea that p14ARF expression may cause an antiproliferation and/or proapoptotic effect on these cells. Similarly, the p14ARF-expressing stable cells also showed a slower growth rate than the control cells (Figure 3).

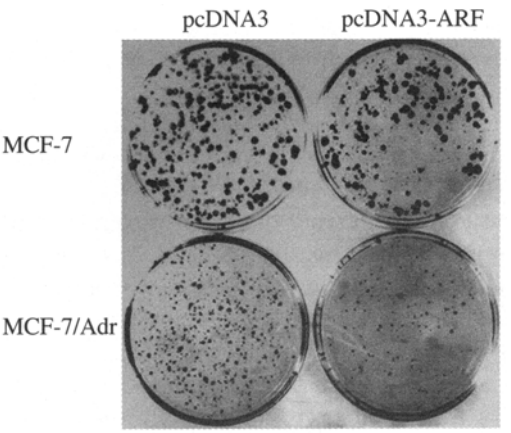

Fig. 1. Expression of p14ARF inhibits the proliferation of MCF-7 and MCF-7/Adr breast cancer cells, G418-resistant cell colonies were scored 3 weeks later by methanol fixation and cystal violet staining. A representative assay is shown from multiple experiments performed in triplicate.

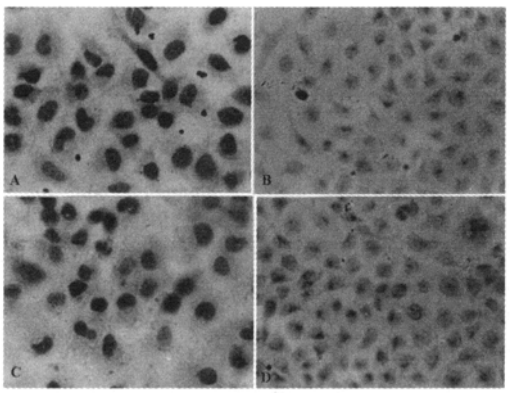

Fig. 2. Immunocytochemistry of MCF-7 transfected cells by (A) pcDNA3 control plasmid and MCF-7/Adr trasfected cells by (C) pcDNA3 ARF plasmid or (D) pcDNA3 control plasmid.

Effect of p14ARF Expression on Doxorubicin-Resistant MCF-7/Adr Cells

In an attempt to examine the effect of p14ARF expression on doxorubicin-resistant MCF-7/Adr cells, we compared the cell viability among these 4 cell lines: MCF-7, MCF-7/Adr cells, MCF-7/Adr-pcDNA3 and MCF-7/Adr-ARF by the MTT assay when exposed to a series of concentrations of doxorubicin, and the results are presented in Figure 4. The concentrations of doxorubicin that inhibited cell survival by 50% (IC_{50}) were 0.0037, 3.7, 0.95 and 0.075μM, respectively (the above IC_{50}'s were calculated from cell survival plots of Figure 4). Our data demonstrated, for the first time, that expression of p14ARF in MCF-7/Adr cells is associated

with the reducing doxorubicin resistance.

The previous studies[28, 29] have shown that the MCF-7/Adr cells overexpress MDR1 which contributes to the drug-resistant phenotype and retain less Rhodamine 123 than the parental MCF-7 cells. Thus, we detected the levels of MDR1 expression and P-glycoprotein production. The results of RT-PCR revealed that MDR1 mRNA was significantly decreased in MCF-7/Adr-ARF cells compared to that of MCF-7/Adr-pcDNA3 cells (Figure 5a), which was also associated with a decrease in the production of P-glycoprotein as indicated by Western blot analysis (Figure 5b), and with an increase in Rhodamine 123 accumulation by FCAS assay (Figure 6). These data suggest that p14ARF expression in MCF-7/Adr cells could down-regulate the MDR1 expression. Those, in turn, enhance its sensitivity to doxorubicin.

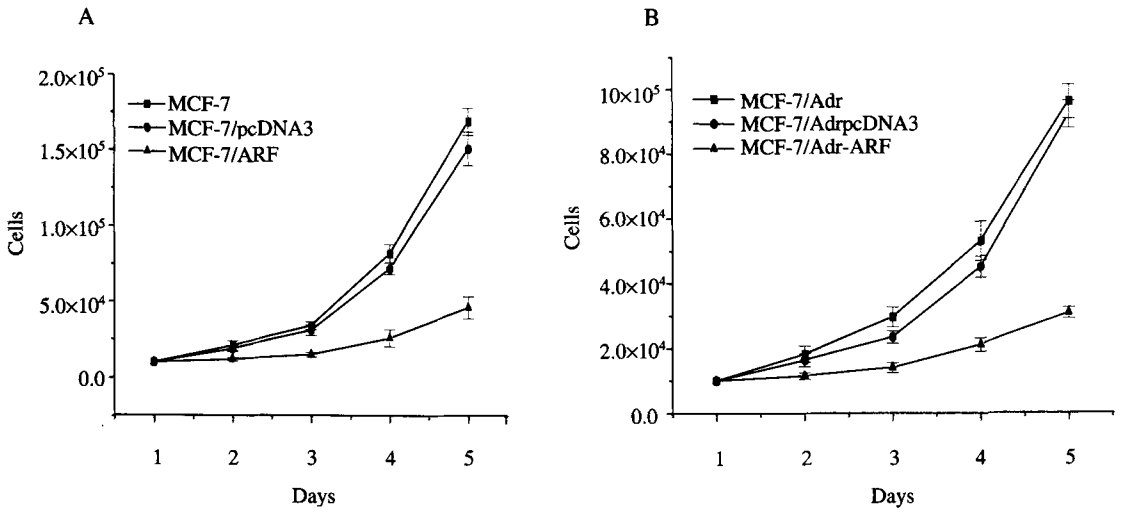

Fig. 3. Effect of p14ARF on breast cancer cells growth ($\bar{x}\pm s$). Cells (1×10^4) were plated in triplicate on 12-well plates, and cell number was counted daily.

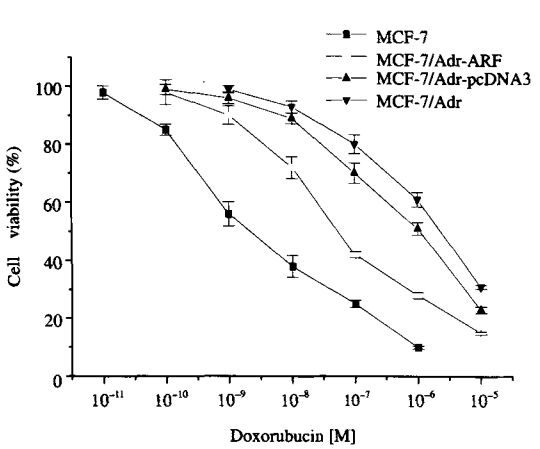

Fig. 4. Cytotoxicity of doxorubicin to MCF-7, MCF-7/Adr-ARF, MCF-7/Adr-pcDNA3, and MCF-7/Adr cells

DISCUSSION

Most of the current information about ARF relates to the mouse homologue p19ARF, and much less is known about the human p14ARF. Stott et al. (1998)[14] reported the mouse and human ARF protein show only 50% identity, but transfection experiments have indicated that p14ARF can also induce cell cycle arrest in G1 and G2/M phases, the same as that of p19ARF.[1] The data we present here about transfection of p14ARF in the breast cancer cells are consistent with such a role for ARF, that is, restrain the breast cancer cells growth. However, an interesting question is raised: the growth suppressor mediated by p14ARF in breast cancer cells seems not correlative with the status of p53, at least in MCF-7 and MCF-7/Adr cells, since the broadly used line MCF-7 cells retain wild-type p53, whereas its multidrug-resistant subline MCF-7/Adr cells express mutated p53.[30] Our work provide additional insights into p14ARF role in tumor suppression.

The predominant view of ARF action centers around its ability to bind directly to MDM2, resulting in the stabilization of both p53 and MDM2,[18-25] and it is clear that ARF plays no part in the p53 mediated response to DNA damage. Some recent works demonstrated that ARF was dispensable for p53 activation in response to ionizing, UV radiations or actinomycin D treatment.[14-16, 31] However, after we attempted to detect the sensitivity

of stably p14ARF-expressing clones to doxorubicin, a surprising phenomenon was found that p14ARF expression can reduce the MCF-7/Adr cells resistance to doxorubicin, as further manifested by increase of the amount of rhodamine 123 accumulated in the stably p14ARF-expressing cells. Moreover, an obviously decrease in the level of MDR1 mRNA in the MCF-7/Adr-ARF cells was observed compared to that of the control cells, MCF-7/Adr-pcDNA3, and was consistent with a decrease in the production of P-glycoprotein as revealed by Western blot analysis. The above observation presents a possible notion that there is an association between p14ARF expression and the down-regulation of the MDR1 expression in the MCF-7/Adr cells. The origin of this effect remains unclear and is the subject of our ongoing research.

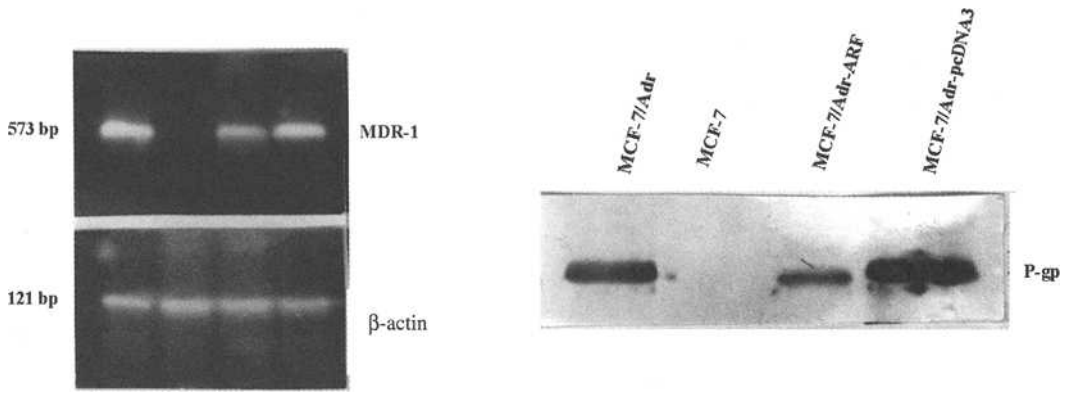

Fig. 5. Expression of MDR1 mRNA and protein is down-regulated in the MCF-7/Adr-ARF cells stably transfected the p14ARF. (a), RT-PCR analysis of MDR1 mRNA expression and (b), Western blot analysis of MDR1 expression in MCF-7/Adr, MCF-7, MCF-7/Adr-ARF and MCF-7/Adr-pcDNA3 cells. RT-PCR assay was performed with 1 μg total RNA using gene specific primers, and Western blot analysis was carried out as described in "materials and methods".

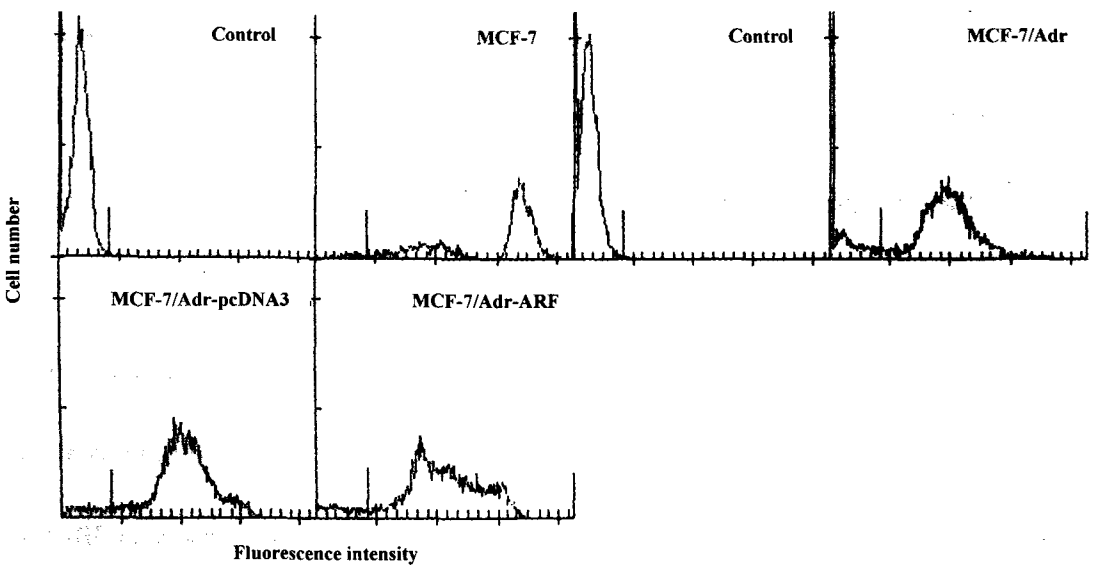

Fig. 6. Intracellular accumulation of rhodamine 123 in (1) MCF-7 cells control, (2) MCF-7 cells, (3) MCF-7/Adr cells control, (4) MCF-7/Adr cells, (5) MCF-7/Adr-pcDNA3 cells and (6) MCF-7/Adr-ARF cells. The respective control cells was not incubated with rhodamine 123.

Drug resistance, whether primary or acquired, is a major obstacle to advances in cancer chemotherapy, and so far, among the tumor suppressor genes, the wild-type p53 was mostly reported to play a role in enhancing the sensitivity of the multidrug cancer cells to drugs,[32-34] while our findings suggest that drug resistance may be effectively reversed with the wild-type p14ARF expression in human breast cancer cells.

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