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Polyethylenimine (PEI)-Mediated E1A Increases the Sensitivity of Hepatocellular Carcinoma Cells to Chemotherapy

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to assess the ability of polyethylenimine (PEI) as an E1A plasmid vector to transfect hepatocellular carcinoma SMMC-7721 cells and to analyze the sensitization effect of E1A on various anti-tumor drugs.

Material/Methods: PEI-mediated recombinant plasmid psv-E1A with high expression of the E1A gene was introduced into hepatocellular carcinoma SMMC-7721 cells, and the effective transfection of E1A gene was determined by RT-PCR and Western blot analysis. The CCK8 method was used to detect the proliferation inhibition of docetaxel, epirubicin, gemcitabine, and 5-fluorouracil on SMMC-7721 cells before and after the transfection of the E1A gene.

Results: RT-PCR and Western blot analysis showed that PEI could transfect plasmid psv-E1A with stable expression. After the transfection of E1A gene, the sensitivity of SMMC-7721 cells to docetaxel, epirubicin, gemcitabine, and 5-fluorouracil was increased ($P<0.05$), and the sensitivity to docetaxel was significantly improved ($P<0.01$).

Conclusions: PEI can transfect plasmid psv-E1A. The E1A gene can increase the sensitivity of hepatocellular carcinoma cells to chemotherapeutic drugs. The mechanism may be related to the increased ability of the E1A gene to inhibit proliferation of hepatocellular carcinoma cells and altering the cell cycle of hepatocellular carcinoma cells.

MeSH Keywords: Adenovirus E1A Proteins • Carcinoma, Hepatocellular • Cell Cycle • Cell Proliferation • Chemotherapy • Polyethyleneimine • Transfection

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Background

Globally, liver cancer is one of the most frequently diagnosed cancers, responsible for approximately 748 300 new cases and 695 900 cancer deaths in 2008, half of which were reported to occur in China [1]. Hepatocellular carcinoma (HCC), which is the most common form of human liver cancer and the fifth most common cancer worldwide, has a high propensity to recurrence and metastasis [2]. If HCCs are detected at an early stage, surgical resection or liver transplantation are potential curative options [3]. However, 70% of patients are inoperable at diagnosis because the tumor is too advance or due to the coexistence of liver cirrhosis [4]. Due to multidrug resistance (MDR) and heterogeneity, HCCs are highly resistant to conventional antineoplastic drugs. Systemic therapies, including sorafenib, are associated with only a minimal or at best a modest benefit in overall survival [5]. The 5-year relative survival rate of HCC is only about 7% worldwide [6]. Therefore, to improve the outcome for patients with intermediate and advanced HCC, novel therapeutic strategies such as gene therapy are urgently needed. Gene therapy has shown promise in the treatment of various malignancies. With the development of vector systems, tumor-specific oncolytic adenoviruses that replicate selectively in tumor cells have become an appealing treatment strategy [7].

Adenovirus type 5 early region 1A (E1A) was the first gene transcribed after infection, and its primary function was to induce the cells to enter S phase, deregulate cellular gene expression to favor viral replication, and activate the expression of viral transcription units [8]. The E1A gene from adenovirus has become a potent tool in cancer research. In this regard, during the 1990s, E1A was used as a model to study cooperation with well-established oncogenes such as *v-H-Ras* [9]. The E1A gene was initially recognized as an oncogene that can promote oncogenic transformation in rodent cells by other oncogenes, such as the adenovirus E1B gene [10]. However, despite intensive research, E1A has not been associated with human neoplasms. Instead, E1A has been shown to have several anti-oncogenic properties, including suppression of cancer genesis, malignant transformation, tumor angiogenesis and metastasis, and induction of apoptosis in various cancer cell lines and animal models [11,12]. Moreover, it was shown that the expression of E1A sensitizes cells to several apoptosis-inducing stimuli, such as chemotherapeutic agents, ionizing radiation, serum starvation, and tumor necrosis factor-α (TNF-α) [13]. The anti-tumor effect of variety classes of drugs can be significantly enhanced by E1A gene therapy, including 5-fluorouracil (5-FU), gemcitabine (GEM), docetaxel, doxorubicin, mitoxantrone, cisplatin, and TNF-related apoptosis-inducing ligand (TRAIL) in hepatocellular, pancreatic, ovarian, colon, prostatic, and breast cancer cells [14–19]. Gene therapy with E1A may enhance the susceptibility of tumor cells to chemotherapy-induced cell death. One of the molecular mechanisms by which E1A induces chemosensitization is downregulation of erb-b2 receptor tyrosine kinase 2/proto-oncogene neu overexpression [20]. It has been reported that the adenovirus E1A gene induces sensitivity to DNA-damaging agents, including cisplatin (CDDP), DOX, and γ irradiation on squamous cell carcinoma cells. Regulation of certain critical tumor suppressors was proposed as being involved in E1A-induced chemo sensitization, including p53 and $p19^{aRF}$ [20]. Proteins encoded by the E1A gene interact with a wide variety of different cellular transcription factors and regulatory proteins, including RB, CBP/p300, p400, P/CAF, YY1, CtBP, and CDK8 [21,22]. Multiple molecular mechanisms may be involved in the chemosensitization induced by E1A. Early studies reported that E1A sensitized ovarian cancer cell lines to cytotoxic agents by suppressing Her-2/neu overexpression [23]. Recently, E1A was shown to overcome the resistance of HCCs to doxorubicin, 5-FU, and TRAIL by downregulation of Mcl-1 [24]. Stabilization of FOXO3a proteins by E1A is considered to be important in the chemosensitization to paclitaxel [25], and repression of Akt activation through upregulation of PP2A may be another pathway of E1A-mediated sensitization to paclitaxel-induced apoptosis [26]. E1A was also shown to stabilize c-Myc protein, the key regulator of cellular proliferation, by binding to p400, and this stabilization is critical for the ability of E1A to sensitize tumor cells to chemotherapy [27]. Moreover, it was demonstrated that E1A induced apoptosis in HCC cells by a p53-dependent pathway, in which E1A upregulated pro-apoptotic factors Bax, caspase-3, and Fas, and down-regulated anti-apoptosis factors survivin and Bcl-2 [28].

As stated above, E1A is a good candidate for cancer gene therapy to enhance the chemotherapeutic effect of HCCs, but a critical challenge is how to efficiently and safely deliver the E1A gene into the target cancer cells. The present study assessed the capability of polyethyleneimine (PEI) as the carrier of E1A plasmid to transfect the HCC cell line SMMC-7721. We also assessed the sensitization effect of E1A to multiple anti-cancer drugs.

Material and Methods

Cell culture and reagents

The human hepatoma cell line SMMC-7721 was obtained from the Liver Cancer Institute of Zhongshan Hospital (Shanghai, China). Cells were cultured in RPMI 1640 (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco), and placed in a 37°C humidified 5% CO_2 incubator.

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Preparation of plasmid DNA

E1A cDNA fragment covering entire coding region (380bp) was excised with *Xho* and *Hind* III from PsuCMV-E1A plasmid obtained from Dr. CQ Su (Orient Hospital, Shanghai, China) and inserted into mammalian expression vector plasmid pcDNA3.1 with an ampicillin-selectable gene. The plasmid pcDNA3.1 without the E1A gene was used as an empty plasmid.

Preparation of PEI-pcDNA3.1 formulations

PEI (25 kDa, branched form) was obtained from Shanghai Institute of Applied Physics (Chinese Academy of Sciences, Shanghai, China). The desired amount of pcDNA3.1 in PBS was commixed with PEI by slowly adding the pcDNA3.1 to the PEI while vigorously stirring the solution. The solution was then incubated at room temperature for 30 min before use. The resulting charge ratio was expressed as a molar ratio of nitrogen atoms of PEI to the phosphate groups of pcDNA3.1 (N: P). PEI- pcDNA3.1 was used at a 10: 1 N: P ratio and a 5: 2 PEI: DNA weight ratio.

Preparation of PEI-pcDNA3.1-E1A complexes

We mixed 8 µl of 10 µg/µl pcDNA3.1-E1A in water with an equal volume of PEI, varying in their concentration, and incubated for 30 min at room temperature. The mixing ratio of PEI and pcDNA3.1-E1A was expressed at a 10: 1 N: P ratio, a 5: 2 PEI: DNA weight ratio.

In vitro transfection

For serum-free transfections, cells were plated at 5×10^5 cells/well in 6-well plates the day before transfection. Cells were approximately 60–70% confluent at the time of transfection. The PEI-DNA formulations were brought up in RPMI1640 supplemented with 10% FBS, as above, to a final DNA concentration of 1 µg/ml. Cells were overlaid with 1 ml of the transfection solution per well. After 6 h, the transfection solution was removed and the cells were rinsed 2 times with PBS and overlaid with RPMI1640 supplemented with 10% FBS.

Reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated from the cultured cells using TRIzol (Invitrogen, USA) following the manufacturer's protocol. Single-stranded cDNA was prepared with the Superscript First-Strand System (Invitrogen) as described previously [29]. Specific oligonucleotide primers were the forward primer 5'-cggaggtgttattaccgaag-3', and backward primer 5'-tcgtcactgggtggaaagcc-3'. The PCR reaction consisted of 94°C for 4 min, 35 cycles at 94°C for 15 s, 60°C for 60 s, and 72°C for 1 min, followed by an extension of 5 min at 72°C. The PCR products were separated by electrophoresis in 1% agarose gels. The length of the expected product was 870 bp.

Western blot analysis

Cells were washed 3 times with PBS and then lysed in lysis buffer. Protein content was determined against a standardized control by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, USA). A total of 50 µg of protein were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Nonspecific binding on the membrane filter paper was minimized with blocking buffer consisting of 5% nonfat dry milk and 0.1% (v/v) Tween 20 in PBS. The treated filter paper was then incubated, first with the primary antibody and then with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse antibody; 1: 5000 dilution; Jackson ImmunoResearch Laboratories, USA). The specific antibodies used were mouse anti-E1A antibody (1: 500 dilution; PharMingen, USA) and rabbit anti-actin antibody (1: 5000 dilution; Sigma, USA).

Cell cycle analysis

Cells were harvested at 24 h after transfection with PEI-pcDNA3.1-E1A; fixed in 75% chilled ethanol, and kept at 4°C for at least 24 h. To measure DNA content, cells were washed twice with PBS and stained with 25 µg/mL PI (Sigma). Cell cycle distribution was determined by flow cytometry analysis. Ten thousand events were acquired for each sample and analyzed.

Cell viability assay

Cells in the experimental group were treated with PEI-pcDNA3.1-E1A. Cells treated with PBS or PEI-empty plasmid were used as blank or negative control. Cells were then plated in a 24-well plate at a density of 5×10^4 cells per well, and cultured at 37°C and humidified 5% CO_2. When cells were approximately 70–80% confluent, they were treated with drugs at the following dose range: docetaxel (0, 20, 40, 80, 160 mg/L), epirubicin (0, 20, 40, 80, 160 mg/L), gemcitabine (0, 100, 200, 400, 800 mg/L), 5-fluorouracil (0, 200, 400, 800, 1600 mg/L). After 24 h of incubation, cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Statistical analysis

All data were calculated with SPSS 16.0 and expressed as the mean ±SD. The statistical significance of differences in cell cycle phases and cell viability between SMMC-7721, SMMC-7721-vector and SMMC-7721-E1A cells was calculated with the *t* test. A value of $P<0.05$ was considered statistically significant.

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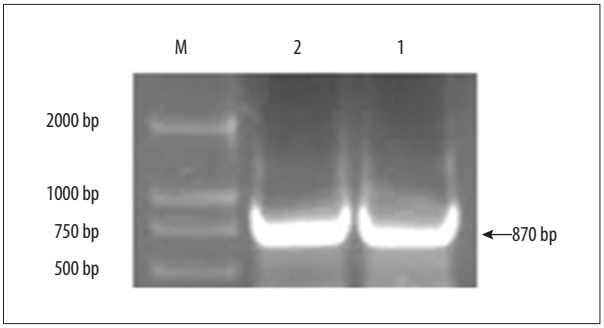

Figure 1. RT-PCR analysis for E1A gene (870 bp) expression in E1A-transfected cells. M – mark; 1, 2 – E1A transfected cells.

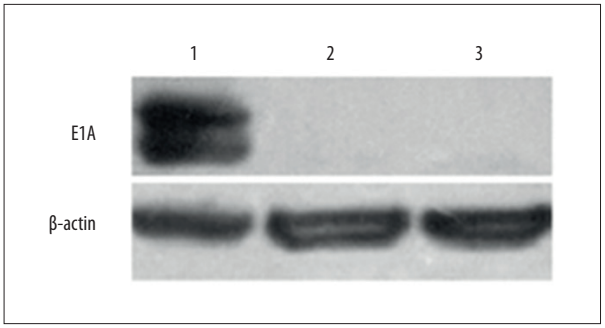

Figure 2. Western Blot analysis of E1A protein expression in SMMC-7721 cells. β-actin expression is shown as a loading control. 1 – PEI-pcDNA3.1-E1A; 2 – PEI-empty plasmid; 3 – PBS.

Results

Transfection of SMMC-7721 cells by PEI-pcDNA3.1-E1A

The expression of E1A was determined by RT-PCR and Western blot analysis. RT-PCR showed that 870 bp E1A gene expression was detected in E1A-transfected cells (Figure 1). As shown in Figure 2, E1A protein was successfully expressed in PEI-pcDNA3.1-E1A transfected cells, while PEI-empty plasmid cultured cells or blank control cells did not show any band corresponding to E1A protein. These results showed that PEI-pcDNA3.1-E1A was effective in E1A gene transfection.

For the study of cell cycle redistribution, SMMC-7721-E1A cells were analyzed for DNA content by flow cytometry. After transfection of PEI-pcDNA3.1-E1A, there was a significant increase in the proportion of cells in G2/M phase, a modest decrease in G0/G1 phase, and a significant increase in S phase, in comparison with the 2 control groups ($P<0.05$) (Figure 3). E1A redistributed the cell cycle of SMMC-7721 cells, which can alter the sensitivity of SMMC-7721 cells to some chemotherapy drugs.

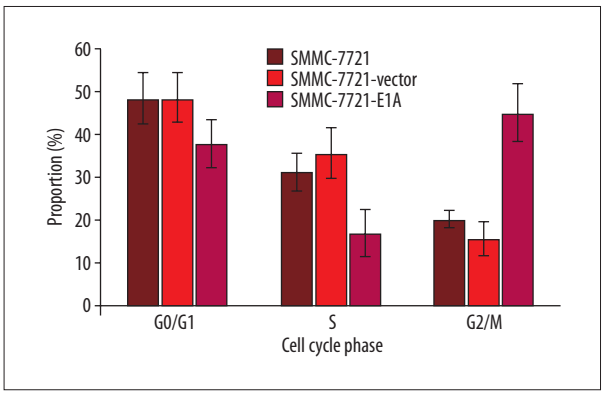

Figure 3. The cell cycle distribution of SMMC-7721, SMMC-7721-vector, and SMMC-7721-E1A cells was measured by flow cytometry.

E1A sensitizes HCC cells to several chemotherapeutic agents

To explore the enhanced toxicity of PEI-pcDNA3.1-E1A plasmid with chemotherapy, SMMC-7721 cells were treated with PEI-E1A-positive or -negative plasmid before treating with different concentrations of docetaxel, epirubicin, gemcitabine, and 5-fluorouracil. The cell viability was measured with MTT assay. As shown in Figure 4A, at a dose of 160 mg/L, E1A resulted in an average 96% decrease in cell viability in comparison with negative and blank controls, in which docetaxel achieved only 49% and 47% decrease, respectively. Transfection with PEI-pcDNA3.1-E1A induced a 13-fold greater cellular sensitivity to docetaxel ($P<0.05$), whereas transfection with PEI-empty plasmid did not alter cell sensitivity. As shown in Figure 4B, 4C, and 4D, cytotoxicity of epirubicin, gemcitabine, and 5-fluorouracil were also enhanced by about 3-, 2-, and 1.4-fold ($P<0.05$), respectively. These results suggest a role of E1A as a chemosensitizer for docetaxel, epirubicin, gemcitabine, and 5-fluorouracil in HCC cells.

Discussion

Due to the overexpression of multidrug-resistance genes and the heterogeneity of HCCs, systemic chemotherapeutic agents, administered alone or in combination, have only minimal survival benefit [5]. As demonstrated previously, E1A gene therapy is effective in overcoming the strong chemoresistance of HCCs. Adenovirus (Ad) is one of the agents extensively investigated and is easily genetically manipulated to produce replication-restricted types for human tumors [30].

The adenoviral vector is an ideal gene transfer system due to its capacity to produce high titers, as well as being genomically stable and having a low rate of integrating its own DNA into the host's genome [31]. Adenovirus is the most studied gene carrier of E1A. The preclinical data of adenovirus-expressing E1A are promising. Phase I and phase II clinical trials in patients

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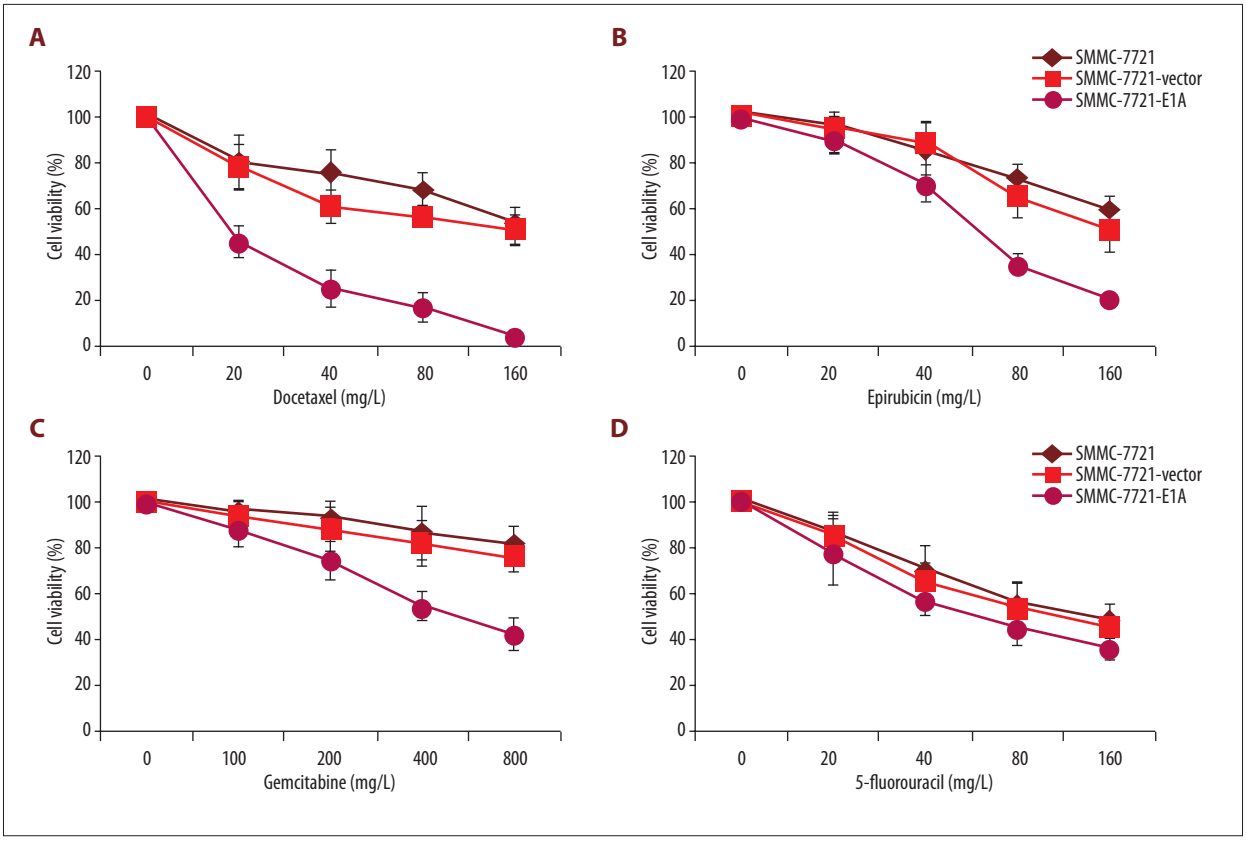

Figure 4. HCC cells treated with PEI-pcDNA3.1-E1A showed enhanced sensitivity to docetaxel, epirubicin, gemcitabine, and 5-fluorouracil. PBS was used as a blank control and PEI-empty plasmid was used as a negative control. Cell viability in response to the indicated concentrations of agents after 24 h culture was analyzed by MTT assay. (**A–D**) All combinations of E1A and agents had significantly greater cytotoxicity than in the control groups. The experiment was done 3 times, with similar results.

with primary liver carcinoma or gastrointestinal tumors metastatic to the liver have also been reported [32,33]. The mechanisms proposed to explain E1A-associated transformation include cell cycle alteration, escape from oncogenic-induced senescence, and blockage of tumor-suppressor genes. Several possibilities have been proposed to explain the anti-tumor behavior of E1A; for example, the effect of E1A on certain oncogenic proteins such as Her2/neu or EGFR have been considered as major mechanisms [9].

However, Ad vectors have strong immunogenicity and robustly generate immune responses, which is the major disadvantage for its use in gene therapy [34]. The adenovirus-related and dose-limiting toxicity in animal models was typically hepatotoxicity [35]. Early deaths due to liver toxicity were shown in mice [36]. Severe systemic inflammatory response has also been reported in early clinical trials [37]. The tragic death of a patient under an Ad gene therapy trial, which was caused by a severe immune response, has put on hold many clinical trials of Ad [38]. Furthermore, Kupffer cells in the liver express high levels of Ad receptors. When delivered in a low dose by intravenous injection, up to 98% of Ad was rapidly accumulated in the liver and captured by Kupffer cells, which led to little hepatocyte transduction [39,40]. A higher dose could improve the transduction efficiency, but toxic adverse effects were simultaneously increased [41]. Finally, when Ad gene therapy is applied for HCCs, liver cirrhosis must be considered, as its sensitive to treatment may make the liver toxicity much more serious.

For the above reasons, the question is raised of whether it is safe to use Ad vectors for treatment of HCCs. Nonviral and noninfectious gene delivery systems may be more appropriate alternatives for HCCs gene therapy. Nonviral vectors with cationic liposomes and polymers have been reported. Cationic vectors are known to exhibit high gene expression through specific mechanisms such as binding to the surface of cells, being taken up *via* the endocytotic pathway, and the plasmid DNA (pDNA) release from endosomes. However, cationic vectors were reported to cause undesirable gene expression and blood aggregation by their cationic charges [42].

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PEI is considered the criterion standard for polymer-based gene carriers because of the high *in vitro* and *in vivo* transfection efficiency [43]. PEI has many advantages, such as good water solubility, good biologic performance, low cost, available in large scale, and easy to develop functionalization [44,45]. In this report, we showed that PEI-pcDNA3.1-E1A can stably and efficiently transfect the E1A gene to SMMC-7721 cells. The expression of E1A altered the cell cycle distribution and sensitized SMMC-7721 cells to docetaxel, epirubicin, gemcitabine, and 5-fluorouracil.

Docetaxel is a semi-synthetic analogue of paclitaxel; both act by stabilizing microtubules, leading to mitotic aberrations and cell death. Docetaxel has a greater affinity for tubulin and is more potent than paclitaxel. An initial report suggested that one of the mechanisms by which E1A enhances the chemosensitivity to paclitaxel was downregulation of Her-2/neu overexpression [46]. Paclitaxel-mediated apoptosis in cancer cells was associated with increased expression of Bim by activated FOXO3a activity [47]. A recent report showed that the upregulation of PP2A/C activity by E1A triggered a cascading intracellular signaling pathway that stabilized FOXO3a, which is important for E1A-mediated sensitization to paclitaxel [25]. In recent research, Ad-E1A was reported to induce a significant accumulation of cells in G2/M [48]. In the present study, we also showed that E1A altered cell cycle progression and induced SMMC-7721 cells to accumulate at G2/M phase. There may be another mechanism by which E1A enhances the cytotoxic effect of docetaxel, in that docetaxel is a cell cycle-specific agent, targets centromere formation, and causes cell damage in the G2 and M phases. Considering that SMMC-7721 is not a type of cell that overexpresses Her-2/neu, E1A-mediated stabilization of FOXO3a and cell cycle arrest may together contribute to the large (13-fold) increase in docetaxel-induced cytotoxicity.

Epirubicin is the 4'-epi-isomer of doxorubicin; they both interfere with the synthesis and function of DNA and RNA by intercalating DNA strands and inhibiting the enzyme topoisomerase IIα. They have similar activity, but epirubicin has less cardiac toxicity than doxorubicin. Doxorubicin is wildly used as a single agent for HCC therapy, but it produces only a low response rate (10–15%), and without any survival benefits [49]. In the present study, we showed that E1A enhanced the cytotoxic effect of epirubicin by 3-fold. As epirubicin is cell-cycle nonspecific, findings by other researchers may explain this enhancement. E2F-1 is a transcription factor that regulates topoisomerase IIα. E1A gene expression can up-regulate the activity of topoisomerase IIα through the E2F-1 pathway [50]. It was demonstrated that in doxorubicin-resistant tumor cells, the expression of topoisomerase IIα was decreased [51]. The elevated levels of topoisomerase IIα proteins enhanced the therapeutic effect of doxorubicin [50].

There was a significant decrease in the number of cells in S phase, a relatively small decrease of cells in G1 phase, and a corresponding increase in the proportion of G2/M phase cells after PEI-pcDNA3.1-E1A transfection. Gemcitabine and 5-fluorouracil are both S phase-specific agents; nevertheless, expression of E1A increased the anti-tumor efficacy of gemcitabine and 5-fluorouracil by 2-fold and 1.4-fold, respectively. One explanation for the increased activity is that E1A can reverse the epithelial-to-mesenchymal transition of tumors and induce the formation of gap junctions, which in turn increases the efficacy of cell-to-cell movement of small-molecule drugs, such as gemcitabine and 5-fluorouracil [52]. In addition, the inactivation of NF-κB is one of the mechanisms by which E1A promotes apoptosis in cancer cells [53]. The expression of poly (ADP-ribose) polymerase (PARP) is also inhibited by E1A, whose major function is DNA damage repair [46]. In gemcitabine-resistant HCC cells, NF-κB and PARP are both induced, and this resistance can be reversed by E1A through suppression of NF-κB and PARP, resulting in sensitization to gemcitabine [54].

Conclusions

PEI-pcDNA3.1-E1A can efficiently transfer E1A into the HCC cell line SMMC-7721. E1A gene therapy enhanced the cytotoxicity efficacy of several chemotherapy drugs, including docetaxel, epirubicin, gemcitabine, and 5-fluorouracil. The increased proliferation inhibition and cell cycle of G2/M redistribution induced by E1A may partly explain the remarkably enhanced effect of docetaxel, but other mechanisms might be involved in all 4 agents, as discussed above. PEI has been widely used in biomedical applications and successfully delivered genes into a variety of cell types *in vitro* and *in vivo*. There is concern about its toxicity due to lack of biodegradability and targeting [55]. In future investigation, we will modify PEI with hydrophilic polymers such as PEO and PEG and with target ligands. The gene transfer efficiency and toxic of PEI will be evaluated in animal models.

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