Efficacy of c-erbB-2 antisense oligonucleotide transfection on uterine endometrial cancer HEC-1A cell lines

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Summary

Objective: Antigene therapy targeting only one oncogene has made much progress although it still has some limitations. To explore the potential for antigene therapy in uterine endometrial cancer, we examined the *in vitro* inhibitory effects of liposmal antisense phosphorothioate oligonucleotides targeting c-erbB-2 in the human uterine endometrial cancer HEC-1A cell line.

Methods: 1) To detect c-erbB-2 protein expression on HEC-1A cell membranes by immunohisto- chemistry. 2) To assay cellular growth inhibition by MTT after transfecting 0.1-0.6 μM ASODN. 3) To observe cellular and ultra-structural changes under transmission electron microscope and to assay the cellular apoptotic rate by flow cytometry and c-erbB-2 mRNA, and protein expression by RT-PCR and Western blot after transfecting 0.3 μM ASODN.

Results: 1) c-erbB-2 protein expression was positive on HEC-1A cell membranes. 2) With the increase of the transfecting ASODN concentration from 0.1-0.6 μM, HEC-1A cellular growth inhibition was also enhanced. The results of MTT showed that when the transfecting concentration of ASODN was 0.3 μM, the HEC-1A cellular growth inhibition rate was 50% while when the transfecting concentration of ASODN was 0.6 μM, the HEC-1A cell growth inhibition rate was 75%. 3) When the concentration of transfecting ASODNs was 0.3 μM, there were obvious vacuolar degenerations in the plasma of HEC-1A cells, disappearance of organelle and nuclear structure and obvious shrinkage of nuclei under transmission electron microscope. The cellular apoptotic rate was 62.80%, while c-erbB-2 mRNA and protein expression were 47.18% and 33.60%, respectively, compared with those of the normal control cells.

Conclusion: Transfecting c-erbB-2 ASODNs can obviously suppress the mRNA and protein expression in HEC-1A cells, cause cellular apoptosis and inhibit cell growth. It may be a more useful gene therapy for endometrial cancer.

Key words: Antisense; Oligodeoxynucleotides; Transfection; Uterine endometrial neoplasms.

Introduction

Endometrial cancer (EC) is the third most common gynecological malignancy in China after cervical and ovarian cancer. The molecular pathogenesis of EC remains poorly understood. However, as in other malignancies, such as colorectal cancer, the transition from normal endometrium to carcinoma is thought to involve a stepwise accumulation of alterations in genes favoring cell proliferation, inhibition of apoptosis and angiogenesis [1].

C-erbB-2(HER-2/neu) is a main oncogene of uterine endometrial cancer, and has been confirmed to have a close relation with EC and play an important role in the biological behavior. Its over-expression is an important factor of bad prognosis [2].

Human epidermal growth factor receptors (HER/erbB) constitute a family of four cell surface receptors involved in transmission of signals controlling normal cell growth and differentiation. A range of growth factors serve as ligands, but none is specific for the HER2 receptor. HER receptors exist as both monomers and dimers, either homo- or heterodimers. Ligand binding to HER1, HER3 or HER4 induces rapid receptor dimerization, with a marked preference for HER2 as a dimer partner. Moreover, HER2-containing heterodimers generate intracellular signals that are significantly stronger than signals emanating from other HER combinations. In normal cells, few HER2 molecules exist at the cell surface, so few heterodimers are formed and growth signals are relatively weak and controllable. When HER2 is overexpressed, multiple HER2 heterodimers are formed and cell signaling is stronger, resulting in enhanced responsiveness to growth factors and malignant growth [3].

Activated c-erbB oncogenes potentiate tumor cell adhesion to endothelial cells and up-regulate VEGF, potentially facilitating angiogenesis and vascular invasion. In addition, cells over-expressing these oncogenes frequently show aberrant cell-cell and cell-matrix interactions, mediated by changes in integrin and cadherin function. Both EGFR and c-erbB-2 signaling can significantly up-regulate specific matrix metalloproteinases, key enzymes involved in angiogenesis and invasion. C-erbB receptors linked to the actin cytoskeleton and highly expressed on invadopodia are thought to assist cell migration. Taken together, these observations suggest that such receptors can act as "master switches" in metastasis, whose activation coordinately controls events normally utilized in development, now subverted by the metastatic cell. As such, they represent ideal targets for therapeutic intervention [4].

Preliminary findings suggest that serum HER-2/neu (c-erbB-2) levels may be used as a tumor marker in a subset of patients with tumors that overexpress the HER-2/neu

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receptor. Receptor-targeted therapeutics currently being studied include the use of receptor antibodies, liposomally delivered antisense DNA, antigen-activated cytotoxic lymphocytes, and adenovirus-mediated E1A delivery to overexpressing tumor cells [2].

Thus we designed this study with the objective of transfecting c-erbB-2 antisense oligonucleotides (ASODN) to endometrial cancer HEC-1A cell lines and assaying the suppression of c-erbB-2 mRNA and protein expression to observe HEC-1A cell ultra-structural changes under transmission electron microscope (TEM), and to detect cellular apoptosis and growth inhibition after transfecting c-erbB-2 ASODN to evaluate the effects of transfecting c-erbB-2 ASODN on uterine endometrial cancer.

Materials and Methods

Cell line and cell culture

Uterine endometrial cancer HEC-1A cells (obtained from the American Type Culture Collection) were cultured in DMEM medium (Gibco) supplemented with 10% heat- inactivated fetal bovine serum (FBS, Gibco), and 50 μg/ml each of penicillin and streptomycin, and 10% CO_2 at a temperature of 37°C. The cultured cells were used for the following studies.

Immunohistochemistry

After HEC-1A cells were cultured on slides for 48 hours and the confluence was approximately 75%, c-erbB-2 protein expression on cell membranes was detected by S-P immunohistochemistry.

C-erbB-2 ASODNs transfection

1) Seeding HEC-1A cells in 96-well plates: HEC-1A cells in log phase growth were digested by 0.25% pancreatin and seeded on 5 x 10^3 cells/well of a 96-well plate with 100 μl DMEM culture medium (each well for 48 hours). The confluence was nearly 90% which was suitable for transfection. 2) Preparation of ASODN/lipofectamine2000 transfection reagent and control reagents: phosphorothioate ASODN(5'-TCC ATG GTG CTC ACT-3') complementary to the initiation codon region of c-erbB-2 mRNA, and phosphorothioate scrambled c-erbB-2 oligonucleotides (SODN,5'-CTA GCC ATG CTT GTC-3') [5] were synthesized by Shanghai Sangon Company (China). ASODN/lipofectamine 2000 complexes, SODN/lipofectamine 2000 complexes and other control reagents were prepared as recommended by the manufacturer using lipofectamine 2000 reagent (Invitrogen). Briefly 40 μl lipofectamine 2000 was diluted into 1 ml of serum- and antibiotic-free medium, depleted medium (DM), and allowed to stand at room temperature for five minutes. The diluted lipofectamine2000 was gently mixed with 1 ml of a 3.5 μM ASODN solution (diluted with DM), incubated for an additional 20 minutes to yield 2 ml 1.75 μM ASODN/lipofectamine 2000 complexes. With the same method, 2 ml 1.75 μM SODN/lipofectamine2000 complexes were also made; 3.5 μM ASODNs was gently mixed with 2 ml DM to yield 2 ml 1.75 μM ASODN control solution; 40 μl lipofectamine2000 was gently mixed with 1 ml DM, incubated at room temperature for five minutes and then diluted with another 1 ml DM to yield 2 ml lipofectamine2000 control solution. The above three ODN solutions were diluted to 0.1-0.6 μM, respectively, with DM, and lipofectamine2000 control solution was also diluted to the proportional concentration. 3) HEC-1A cell transfection: The cells, seeded and grown as above in 96 well plates, were washed twice with 50 μl DM and then overlayed with 50 μl of the above 0.1 μM ~0.6 μM transfecting solutions and control solutions, respectively. The normal control well contained 50 μl DM. After 24-hour incubation, 100 μl DMEM medium supplemented with antibiotics and 10% FBS was added to each well and incubation was continued for an additional 48 hours, and then cellular viability was determined by MTT (Sigma Co.) assay, which was performed as recommended by the manufacturer. The absorbance was measured at 570 nm in a spectrophotometer (Sunrise, Tecan Co., Austria). The percentage of viable cells was calculated as the relative ratio of their absorbance to the normal control cells. All determinations were performed in quadruplicate and each experiment was repeated at least three times.

Observation of cell ultrastructural changes under TEM

Cells in log-phase growth were digested by 0.25% pancreatin to yield single-cell suspension with DMEM medium and seeded in five bottles (A, B, C, D, E; 75 cm^2). Each bottle contained 1 x 10^7 cells and 20 ml DMEM medium. They were incubated for 48 hours, then medium was removed and they were gently washed twice with 5 ml DM. DM was added to bottle A (10 ml) as a normal control. Bottles B, C and D were respectively filled with 10 ml DM diluted with 0.3 μM ASODN/lipofectamine2000 complex, ASODN control solution and SODN/lipofectamine2000 complex. Lipofectamine2000 of proportional concentration (10 ml) was added to bottle E. After incubating for 24 hours, 10 ml of DMEM media containing antibiotics and 10% FBS were added to each bottle and incubation continued for another 48 hours. The treated cells were gently digested by 0.25% pancreatin and transferred into 10 ml test tubes for centrifugation at 1000 rpm for five minutes. The precipitates were transferred into five EP tubes, gently washed with PBS and centrifuged twice. After removing PBS, the pellets were overlayed with 1.5 ml 5% glutaraldehyde, fixed 24 hours for TEM (JEM1200 EX, Japan Electron Co.) examination.

Apoptosis flow cytometric assay

Cultured cells were seeded in five wells of a 6-well plate, marked A1, B1, C1, D1 and E1. Each well contained 1 x 10^6 cells. A1, B1, C1, D1, E1 respectively, served as normal control, ASODN transfection, ASODN control, SODN transfection, and lipofectamine 2000 control. The transfecting concentration was 0.3 μM and transfecting experiments were performed as recommended by the manufacturer of lipofectamine 2000. After transfection, the cells were harvested in five test tubes of 5 ml, centrifuged at 1000 rpm for 5 min and washed with 3 ml PBS twice. The cellular precipitates were fixed with 4 ml of 75% ice ethanol at -20°C for four hours, centrifuged at 1000 rpm for 5 min and then the supernatant was removed. Cellular precipitates were soaked with 3 ml PBS for 5 min and the cells pellets were stained with 50 μg/ml propidium iodide (PI, Sigma Co.) at 4°C for 30 min in the dark. The samples were analyzed by a FACScan flow cytometer at an excitation wavelength of 488 nm (Becton Dickinson Co. USA).

RT-PCR assay

Cell culture, transfection and harvest were the same as the flow cytometry steps. Each sample contained 1 x10^6 cells. After treated as above, total cellular RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions and quantitated by absorbance at 260 nm. For RT-PCR,

the RNA.PCR kit (AMV, TaKaRa Bio., Japan) was used. In brief, RNA 1 μl, 2 × buffer, 25 mM $MgSO_4$ 4 μl, 10 mM dNTPs 1 μl, 22 u/μl reverse transcriptase 1 μl, 5 μM OligodT 1 μl, 40 u/μl RNAse inhibitor 0.5 μl, reaction temperature was set at 65°C 1 for min, 30°C for 5 min, 65°C for 30 min, 98°C for 5 min, 5°C for 5 min to produce cDNA template. The total reaction volume of PCR was 25 μl. Primer sequences for PCR amplification were designed with Primer Premier 5.0 Software (Genebank x 03363), and synthesized by Shanghai Sangon Bioengineering Co. (Shanghai, China). The β-actin control primers were purchased from Sigma Co.. The sense and antisense primers were as follows: c-erbB-2 (450 bp) 5'-AGG AGT GCG TGG AGG AAT-3', and 5'-CTG CCG TCG CTT GAT GAG-3'; β-actin (690 bp) 5'-CAC CCT GTG CTG CTC ACC GAG GCC-3', and 5'-CCA CAC AGA TGA CTT GCG CTC AGG-3'. PCR reaction contained: 10 × buffer, 2.5 μM dNTPs 2 μl, 5 u/μl Taq polymerase 0.2 μl, sense and antisense c-erbB-2 primers 20 pM, respectively, sense and antisense β-actin primers 20 pM, respectively, cDNA 3 μl. Reaction temperature was set at 94°C for 3 min followed by 94°C for 45 sec, 56.6°C for 1 min, 72°C for 1 min, for 30 cycles with a final extension stage at 72°C for 7 min. PCR products (5 μl) were added to 6 × loading buffer, mixed and run on 1.5% agarose gel (0.5 mg/l EB). After electrophoresis at 100 volts for 40 min, the signed intensity of the bands was quantified by a Kodak 1D photograph system. The expression level of c-erbB-2 mRNA was described as the ratio of c-erbB-2/β-actin.

Western blot analysis

The cell culture, transfection, harvest and washing methods were the same as for the TEM steps. Each sample contained 1 × 10^7 cells. The cells were centrifuged at 1000 rpm for 5 min. The precipitates were washed twice in ice-cold PBS and centrifuged, then solubilized in 200 μl lysis buffer (20 mM/l Tris-Hcl, pH 7.5, 0.1 mM/l Na3Vo4, 25 mM/l NaF, 2 mM/l EDTA, 2 mM/l EGTA, 0.5% Triton x-100, 1 mM/l PMSF, 1 mM/LDTT, 1 μg/ml leupeptin/aprotinin), ultrasonication in ice-cold water five times each for 20 sec with 20-second intervals. They were incubated overnight at 4°C, and centrifuged at 12000 rpm 4°C for one hour. The nuclear precipitates were isolated by centrifugation. The supernatant contained the cell protein extract and the protein concentrations were assayed by phenol reagent. The protein samples were adjusted to the same concentration, added to 2 × buffer and boiled for 5 min. Equal amounts of protein (50 μg) were loaded onto 8% SDS-polyacrylamide gels to achieve electrophoresis (150 V, 2 hours). Proteins were separated and electrophoretically transferred to nitrocellulose membranes (50V, 100mA, 2 hours). Membranes were soaked by TBS for 10 min, blocked by 5% skimmed milk powder TBS for one hour, and washed by TTBS for 5 min twice. Then blots were probed with human monoclonal antibody against c-erbB-2 (1:1000, Santa Cruz Biotechnology Inc., USA), and with anti-β-actin antibody (1:1000, Sigma Chemical Co.) to confirm equal amounts of loaded samples, overnight at 4°C. Blots were then incubated with secondary antibody conjugated to horseradish peroxidase (mouse anti-goat, 1:2000, Beijing Zhongshan Biotechnology Co., China) for two hours and then washed with TTBS for 5 min twice and with TBS for 5 min twice. Signals were detected using enhanced chemiluminescence (ECL, Beijing Zhongshan Biotechnology Co., China) according to the manufacturer's instructions. Enhanced chemiluminescence films were digitized using a scanner, and images were processed using Photoshop 5.0.

Results

Immunohistochemistry

As shown in Figure 1, c-erbB-2 protein was positively expressed in HEC-1A cell membranes.

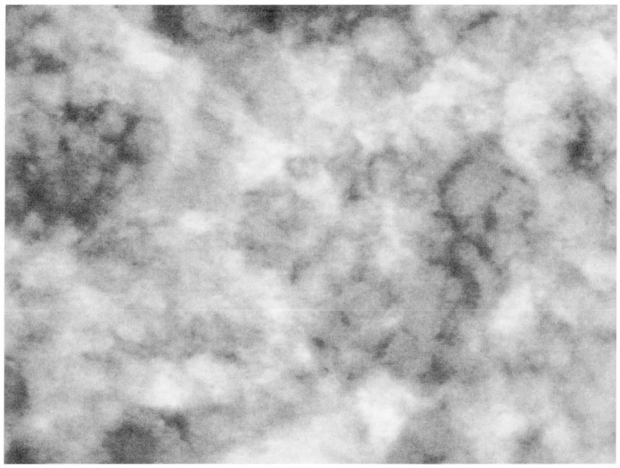

Figure 1. — Immunohistochemistry – Positive staining of c-erbB-2 protein in HEC-1A cell membranes (SP x 400).

TEM observations

After transfecting 0.3 μM ASODN, obvious vacuolar degeneration appeared in HEC-1A cell plasma (Figure 2). The nuclear structure and organelles disappeared, and the obvious shrinkage of the nucleus could be observed under TEM. There was only slight vacuolar degeneration in the cells which were treated only by 0.3 μM ASODN, organelles were present and the nuclear structure was clear. The ultrastructure of the cells which were treated by lipofectamine2000 and by transfecting 0.3 μM SODN had no obvious changes compared to the normal control cells.

MTT assay

As shown in Figure 3, with the increase of the transfecting ASODN concentration, the inhibition of HEC-1A cell growth was also increased. When the transfecting concentration of ASODN was 0.3 μM, the HEC-1A cell growth inhibition rate was 50%; at the concentration of 0.6 μM, the cell growth inhibition rate was 75%. The growth inhibition rate of the cells which were treated only by 0.6 μM ASODN was only 30%, while in the SODN transfection and lipofectamine2000 treatment groups with the increase of the concentrations, cell growth inhibition was not obvious.

Flow cytometric assay

As shown in Figure 4, the apoptotic rate of normal control cells was 1.78%, and the apoptotic rates of the cells which were treated only by 0.3 μM ASODN, lipofectamine2000 and by transfecting 0.3 μM SODN were 24.64%, 12.67% and 16.13%, respectively. The apoptotic rate of the transfecting 0.3 μM ASODN cells was 62.80% which was significantly higher than those of other groups.

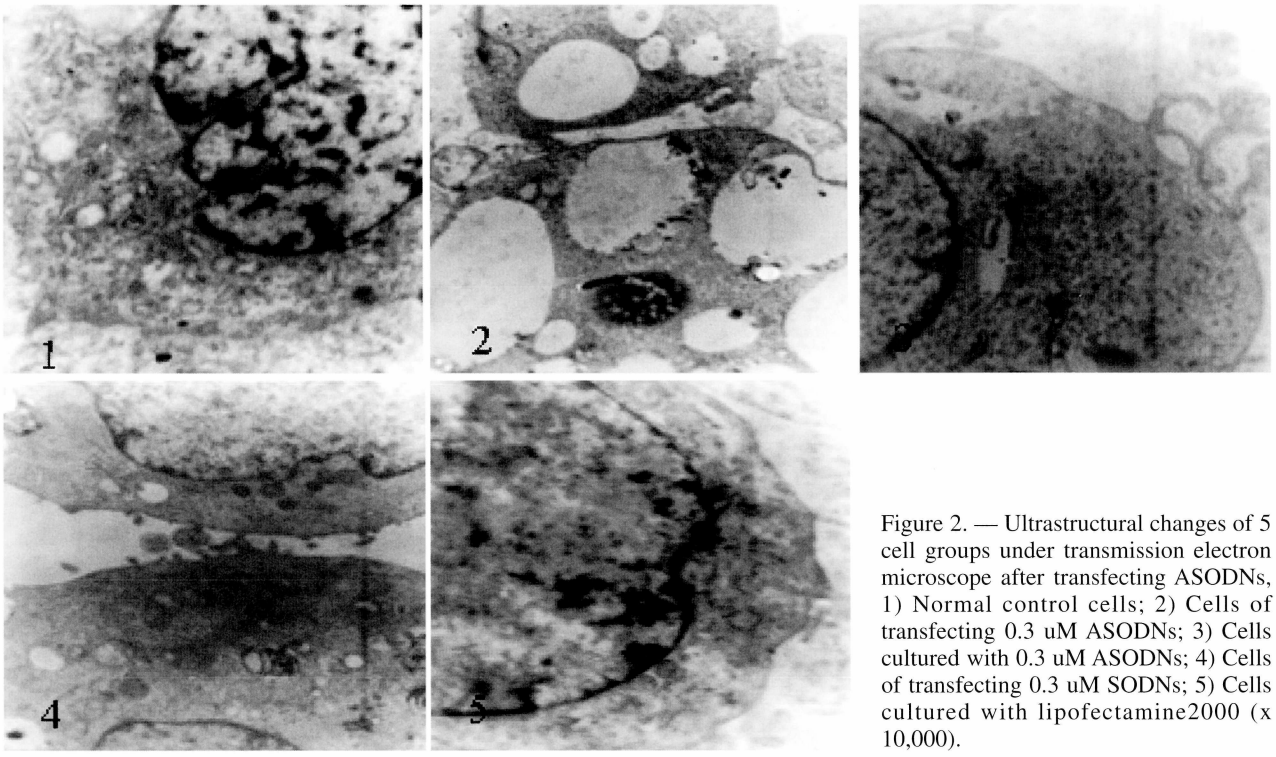

Figure 2. — Ultrastructural changes of 5 cell groups under transmission electron microscope after transfecting ASODNs, 1) Normal control cells; 2) Cells of transfecting 0.3 uM ASODNs; 3) Cells cultured with 0.3 uM ASODNs; 4) Cells of transfecting 0.3 uM SODNs; 5) Cells cultured with lipofectamine2000 (x 10,000).

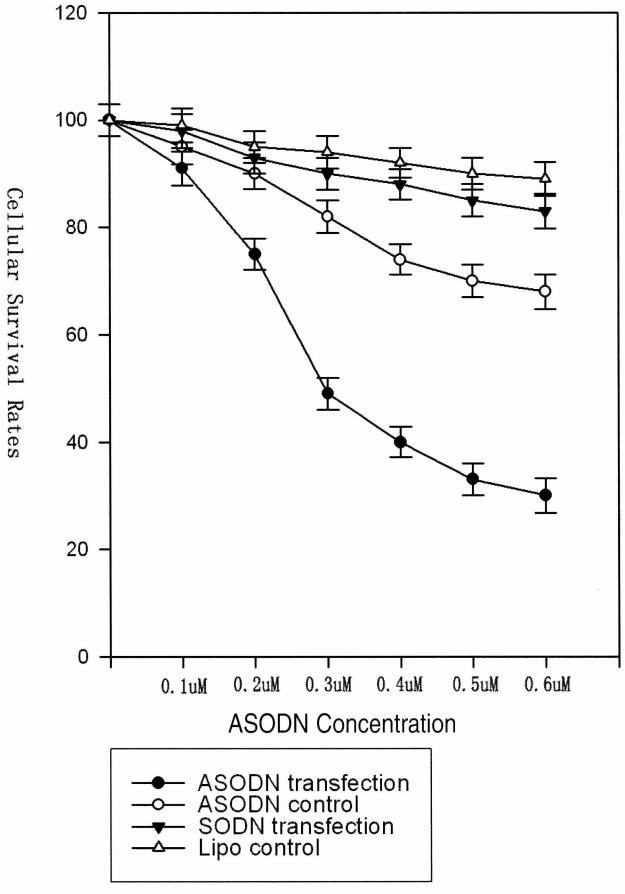

Figure 3. — Cellular growth inhibition rates by MTT.

RT-PCR assay and Western blot analysis

When compared with the normal control cells, the c-erbB-2 mRNA/protein expressions of the cells treated only by 0.3 μM ASODN, lipofectamine2000 and by transfecting 0.3 μM SODN were 81.54%/57.03%, 92.72%/93.80%, and 90.60%/85.82%, respectively (Figures 5 and 6). The mRNA/protein expression of transfecting 0.3 μM ASODN cells was 47.18%/33.60% – much lower than those of other groups.

Discussion

The human proto-oncogene c-erbB-2(HER-2/neu) is located on the long arm of chromosome 17 (17q11-21) and encodes a 185 kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity sharing structural homology with epidermal growth factor receptor (EGFR) suggesting a possible role in cell growth and differentiation. The c-erbB-2(HER-2) gene was originally identified as a viral oncoprotein of c-erbB-2, which is involved in carcinogenic transformation. C-erbB-2 can homodimerize, or heterodimerize with other erbB family members, such as HER-1, HER-3 and HER-4, and leads to phosphorylation of the intracellular region of the receptor, triggering a signal transduction pathways that increases cell growth. Two major downstream signaling pathway of the erbB family include the Ras-Raf-MAPK pathway and the Akt-phosphatidylinositol 3-kinase (PI3k) pathway [6].

Elevated erbB-2 activity is believed to transform cells by transmitting mitogenic and antiapoptotic signals and is also associated with more aggressive tumor growth and poorer patient prognosis [7]. Recently studies on tumor

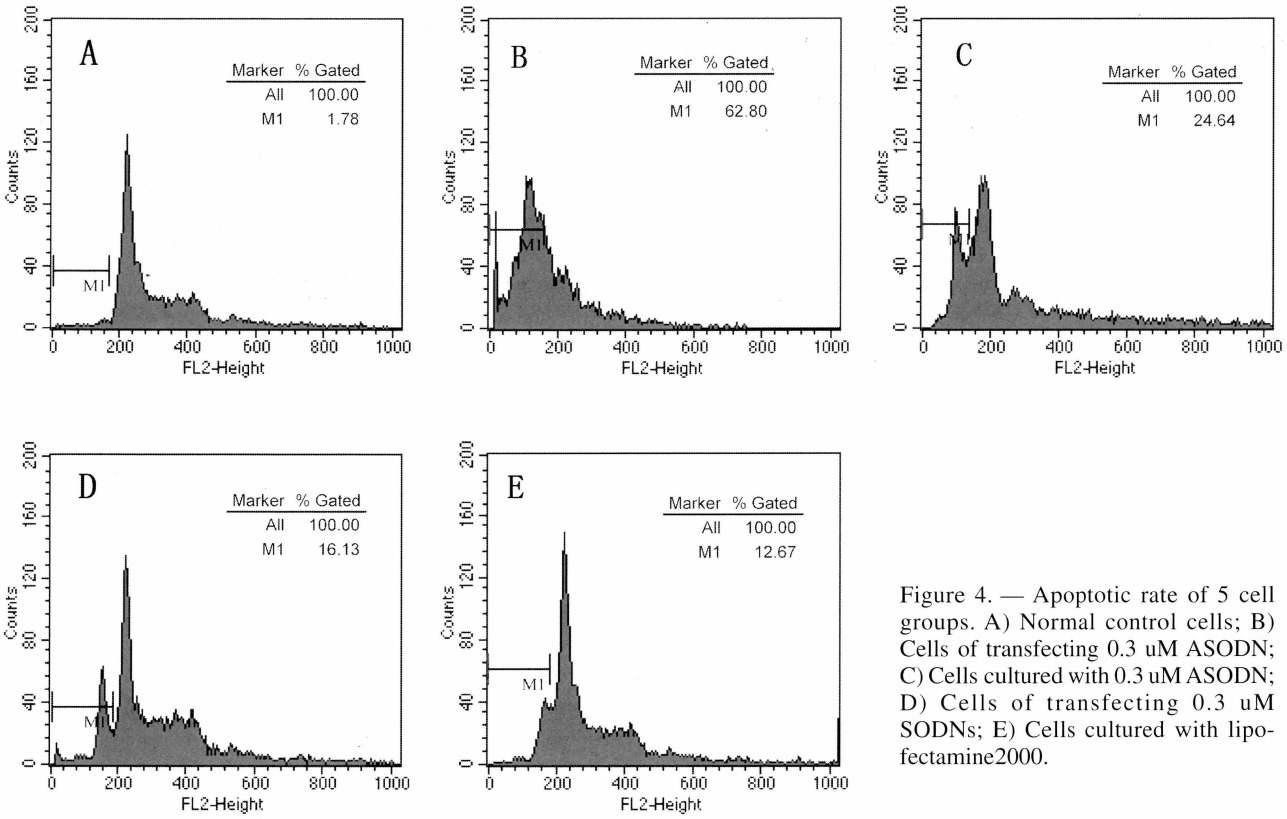

Figure 4. — Apoptotic rate of 5 cell groups. A) Normal control cells; B) Cells of transfecting 0.3 uM ASODN; C) Cells cultured with 0.3 uM ASODN; D) Cells of transfecting 0.3 uM SODNs; E) Cells cultured with lipofectamine2000.

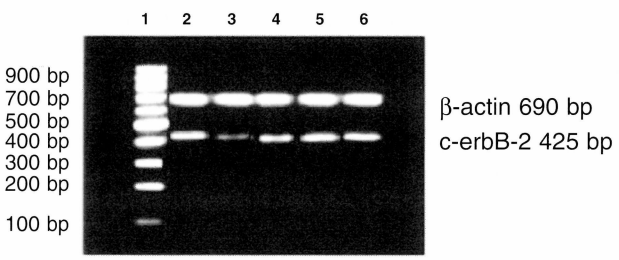

Figure 5. — c-erbB-2 mRNA expression by RT-PCR. 1) 100 bp ladder marker; 2) Normal control (100%); 3) Transfecting 0.3 uM ASODN (47, 18%); 4) ASODN control (81, 54%); 5) Transfecting 0.3 uM SODN (90, 60%); 6) Lipofectamine2000 control (92, 72%).

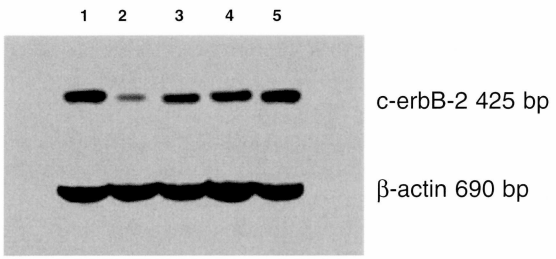

Figure 6. — c-erbB-2 mRNA expression by Western blot. 1) Normal control (100%); 2) Transfecting 0.3 uM ASODN (33.60%); 3) ASODN control (57.03%); 4) Transfecting 0.3 uM SODN (85.82%); 6) Lipofectamine2000 control (93.80%).

gene therapy, through downregulation of c-erbB-2 protein expression by transfecting antisense c-erbB-2 oligonucleotides, have developed rapidly, such as studies on breast cancer [6-9] and ovarian cancer [10-12] which have gained satisfactory results. However such study on endometrial cancer has not been reported up to now. Some studies have shown that c-erbB-2 expression in endometrial cancer ranged from 60-100% [13-15]. Another study showed that c-erbB-2 expressions in endometrial cancer, endometrial hyperplasia and in normal endometrium were 60.90%, 21.90% and 18.80%, respectively [16]. The positive expression of endometrial cancer was significantly higher than that of normal endometrium and endometrial hyperplasia. It suggested that c-erbB-2 is a chief proto-oncogene of endometrial cancer. Its positive expression was associated with clinical stage and histological grade of endometrial cancer. Similarly the results of other authors' studies [13, 17-20] also revealed that positive expression of c-erbB-2 was associated with clinical stage, histological grade and poor prognosis. That is why we chose c-erbB-2 as a target gene, and blocked its mRNA by transfecting ASODN to down-regulate protein expression, and finally to evaluate the therapeutic effects of transfecting c-erbB-2 ASODN. In this study, the immunohistochemistry result showed that the c-erbB-2 protein was positively expressed in HEC-1A cell membranes, thus explaining why we choose the HEC-1A cell line as the target cell of gene therapy.

There are multiple potential mechanisms by which ASODN can inhibit protein synthesis. In the first mecha-

nism, the antisense deoxyribonucleotide anneals to its target mRNA at the most extreme 5'-end of the transcript using Watson-Crick base pairs to form a DNA/RNA double helix. This hybrid prevents ribosomes from recognizing the transcript and inhibits protein synthesis directly by impeding the progress of ribosomes as they attempt to translate the mRNA. Alternatively the antisense oligonucleotide can prevent or modify RNA splicing. Finally the hybrid double helix can be recognized by an enzyme called RNase H. This enzyme can cleave to the RNA in an RNA/DNA double. In this way the target RNA is degraded, thus blocking the production of protein from that message. This latter mechanism has the advantage of being effectively catalytic, as the oligodeoxynucleotide is not destroyed by RNase H and can thus survive to anneal to a new target mRNA transcript. Consequently, one antisense oligodeoxynucleotide can mediate the RNase H-dependent cleavage of many target mRNA molecules [21, 22].

The region spanning the initiation codon of c-erbB-2, two nucleotides before and ten after, was chosen as a target for a phosphorothioate pentadecamer. This sequence was chosen based on stem and loop structures of the c-erbB-2 mRNA followed by in vitro binding studies with various oligonucleotides designed around the initiation codon region. In addition, a series of c-erbB-2 ASODN complementary to the area around and including the initiation codon indicated that this specific oligomer yielded the most efficient inhibition of c-erbB-2 protein synthesis [5].

The sequence of ASODN and the intracellular stability in cells are the keys to using an antisense technique. Unmodified phosphodiester oligomers have been used successfully to modulate expression of certain genes in vitro, but their susceptibility to nucleases makes them unsuitable for therapeutic use. Phosphorothioate oligomers are relatively nuclease-resistant, have the same charge density as phosphodiester oligomers and are relatively easy to synthesize. Phosphorothioate oligomers within cationic liposomes cross the cell membrane and accumulate in the nucleus [23]. The results of Kanamaru *et al.'s* study [21] also suggested that not only the phosphorothioate oligomers' stability in cells was enhanced but the transfecting efficiency was also increased.

Effectively transfecting ASODN into cells is one of the key steps for better complementation of ASODN to the initiation condon. Thus a proper transfecting medium is very important. It has been demonstrated that the lipofectamine 2000 used in this study is a highly efficient transfecting medium, and after transfection through lipofectamine 2000, the stability and integrity of the structure of ASODN can be maintained [22-24].

The receptor tyrosine kinase erbB-2 plays a central role in the development of breast cancer and other epithelial malignancies. Elevated erbB-2 activity is believed to transform cells by transmitting mitogenic and antiapoptotic signals [8]. Therefore, taking c-erbB-2 as a target, blocking its mRNA by transfecting ASODN, and downregulating its protein expression may become the focus of many studies on tumor gene therapy. Yet this kind of study on uterine endometrial cancer has not been reported till now. Our study used lipofectamine2000 as the transfecting medium to transfect c-erbB-2 ASODN into uterine endometrial cancer HEC-1A cells. Then we assayed the c-erbB-2 mRNA and protein expressions, observed cellular ultrastructural changes under TEM and assayed the cellular apoptosis and growth inhibition after transfection. The results of this study have shown: 1) After transfecting 0.3 μM ASODN, the obvious vacuolar degeneration appeared in HEC-1A cell plasma, the nuclear structure and organelles disappeared, and the obvious shrinkage of the nucleus could be observed. It suggested that transfecting c-erbB-2 ASODN could result in damage of the HEC-1A cell ultrastructure; 2) The results of MTT assay showed that with the increase of the transfecting ASODN concentration, HEC-1A cell growth inhibition was also increased. When the transfecting concentration of ASODN was 0.3 μM, the HEC-1A cell growth inhibition rate was 50% while at the concentration of 0.6 μM, cell growth inhibition rate was 75%. Flow cytometric assay showed that the apoptotic rate of the transfecting 0.3 μM ASODN cells was 62.80%, which was significantly higher than that of other groups, suggesting that transfecting c-erbB-2 ASODN may lead to the HEC-1A cell apoptosis and growth inhibition; 3) RT-PCR and Western blot assay showed that compared with normal control cells, c-erbB-2 mRNA/protein expressions of transfecting 0.3 μM ASODN cells was 47.18%/33.60% which was much lower than those of other groups. The above results showed that transfecting c-erbB-2 ASODN into HEC-1A cells can cause damage to the cellular ultrastructure, decrease c-erbB-2 mRNA and protein expressions, elevate cellular apoptosis and lead to obvious inhibition of cell growth. Thus we can conclude that transfecting c-erbB-2 ASODN may have an important role in the antigen therapy of endometrial cancer.

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References

[1] Enomoto T., Inoue M., Perantoni A.O., Buzard G.S., Miki H., Tanizawa O., Rice J.M.: "K-ras activation in premalignant and malignant epithelial lesions of the human uterus". *Cancer Res.*, 1991, *51*, 5308.

[2] Cirisano F.D., Karlan B.Y.: "The role of the HER-2/neu oncongen in gynecologic cancers". *J. Soc. Gynecol. Investing.*, 1996, *3*, 99.

[3] Rubin I., Yarden Y.: "The basic biology of HER-2". *Ann. Oncol.*, 2001, *12* (suppl. 1), S3.

[4] Eccles S.A.: "Cell biology of lymphatic metastasis. The potential role of c-erbB oncogene signaling". *Recent Results Cancer Res.*, 2000, *157*, 41.

[5] Rait A.S., Pirollo K.F., Rait V. *et al.*: "Inhibitory effects of the combination of HER-2 antisense oligonucleotide and chemotherapeutic agents used for the treatment of human breast cancer". *Cancer Gene Ther.*, 2001, *8*, 728.

[6] Kazuaki T., Ryungsa K., Hideki I. *et al.*: "Antisense bcl-2 and HER-2 oligonucleotide treatment of breast cancer cells enhances their sensitivity to anticancer drugs". *Int. J. Oncol.,* 2003, *22,* 875.
[7] Trost T.M., Lausch E.U., Fees S.A. *et al.*: "Premature senescence is a primary fail-safe mechanism of ERBB-2-driven tumorigenesis in breast carcinoma cells". *Cancer Res.,* 2005, *65*, 840.
[8] Yang S.P., Song S.T., Song H.F.: "Advancements of antisense oligonucleotide in treatment of breast cancer". *Acta Pharmocol. Sin.,* 2003, *24,* 289.
[9] Rait A.S., Pirollo K.F., Xiang L. *et al.*: "Tumor targeting, systemically delivered antisense HER-2 chemosensitizes human breast cancer xenografts irrespective of HER-2 level". *Mol. Med.,* 2002, *8,* 475.
[10] Pack S.D., Alper O.M., Stromberg K. *et al.*: "Simultaneous suppression of epidermal growth factor receptor and c-erbB-2 reverses aneuploidy and malignant phenotype of a human ovarian carcinoma cell line". *Cancer Res.,* 2004, *64,* 789.
[11] Wu Y.Z., Ren Q.L., Li S.L.: "Therapeutic effects of c-erbB-2 and c-raf-1 gene combined with antisense oligodeoxynucleotide on the human ovarian carcinoma transplanted subcutaneously in nude mice". *Ai Zheng,* 2003, *22,* 836.
[12] Fei R., Shaoyang L.: "Combination antigene therapy targeting c-myc and c-erbB-2 in the ovarian cancer COC 1) cell line". *Gynecol. Oncol.*, 2002, *85,* 40.
[13] Hetzel D.J., Wilson T.O., Keeney G.L. *et al.*: "HER-2/neu expression: a major prognostic factor in endometrial cancer". *Gynecol. Oncol.,* 1992, *47,* 179.
[14] Bigsby R.M., Li A.X., Bomalaski J. *et al.*: "Immunohistochemical study of HER-2/neu epidermal growth factor receptor and steroid receptor expression in normal and malignant endometrium". *Obstet. Gynecol.*, 1992, *79,* 95.
[15] Berchuck A., Rodriguez G., Kinny R.B. *et al.*: "Overexpression of HER-2/neu in endometrial cancer is associated with advanced stage disease". *Am. J. Obstet. Gynecol.,* 1991, *164,* 15.
[16] Desheng Y., Li L., Bujiang T. *et al.*: "C-erbB-2 oncoprotein expression in endometrial carcinoma and its relationship to clinical course and prognosis". *J. Clin. Oncol.* (China), 1997, *2,* 12.
[17] Saffari B., Jones L.A., el-Naggar A. *et al.*: "Amplification and overexpression of HER-2/neu (c-erbB-2) in endometrial cancers: correlation with overall survival". *Cancer Res.,* 1995, *55,* 5693.
[18] Rolitsky C.D., Theil K.S., Mc Gaughy V.R. *et al.*: "HER-2/neu amplification and overexpression in endometrial carcinoma". *Int. J. Gynecol. Pathol.,* 1999, *18,* 138.
[19] Rasty G., Murray R., Lu L. *et al.*: "Expression of HER-2/neu oncogene in normal, hyperplastic and malignant endometrium". *Ann. Cli. Lab. Sci.*, 1998, *28,* 138.
[20] Kohlberger P., Loesch A., Koelbl H. *et al.*: "Prognostic value of immunohistochemically detected HER-2/neu oncoprotein in endometrial cancer". *Cancer Lett.,* 1996, *98,* 151.
[21] Kunamaru T., Takagi T., Takakura Y. *et al.*: "Biological effects and cellular uptake of c-myc antisense oligonucleotides and their cationic liposome complexes". *J. Drug. Target,* 1998, *5,* 235.
[22] Magin-Lachmann C., Kotzamanis G., D' Aiuto L. *et al.*: "In vitro and in vivo delivery of intact BAC DNA-comparison of different methods". *J. Gen. Med.,* 2004, *6,* 195.
[23] Magin-Lachmann C., Kotzamanis G., D' Aiuto L. *et al.*: "Retrofitting BACs with G418 resistance, luciferase and oriP and EBNA-1-new vectors for in vitro and in vivo delivery". *BMC Biotechnol.,* 2003, *3,* 2.
[24] Ohki E.C., Tilkins M.L., Ciccarone V.C. *et al.*: "Improving the transfection efficiency of post-mitotic neurons". *J. Neurosci. Meth.,* 2001, *112*, 95.

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