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Inhibition of HGF- ASODN in Breast Cancer and its influence on cell proliferation

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Abstract: **Oligodeoxynucleotide (ASODN) was synthesized and added to breast cancer MDA-MB-231 cells to see its influence on cell proliferation. After the transfection of HGF ASODN on the MDA-MB-231 cells, immunocytochemical method, western blot and RT-PCR methods were used to test the HGF, VEGF and PCNA level. Cell proliferation was analyzed by MTT method. The results showed that after transfection, 2-8 uM HGF ASODN. It can inhibit expression of HGF and cell proliferation. The inhibition effect begun at 24 hours and lasted to 96 hours. The inhibition effect was related to the concentration. HGF ASODN could also inhibit protein level of VEGF and PCNA. Besides, HGF ASODN in different concentration could also inhibit tumor growth. HGF promoting autocrine and paracrine of IGF-I is important to breast tumor growth.**

Keywords: Breast cancer; HGF; Proliferation; Tumor growth

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1. Introduction

Breast cancer is the most common type of primary brain tumor with the worst prognosis in humans [1]. Increasing evidence indicates that the rate of tumor cell proliferation, invasion and induction of tumor angiogenesis might be responsible for breast cancer progression. Recent studies have demonstrated that the biological behavior of breast cancer is associated with tumor cell migration ability, increased resistance to apoptosis, and decreased sensitivity to chemotherapy or radiotherapy [2-5]. However, the mechanisms involved in these processes remain to be validated. This study tested influence of HGF ASODN which is synthesized according to HGFcDNA order on breast cancer MDA-MB-231 cells. CAM is an ideal model to clearly evaluate inhibition effect on angiogenesis of drugs.

2. Materials and methods

2.1. Agents and materials

PCR Application reaction kits and Annexin V FITC/PI double marked In Situ Cell Apoptosis Detection kit are made by Jingmei of Shanghai. Trizol is made by Gibco. Second cross-linking sheep anti-mouse Ab marked with FITC is made by Immunotech. LipofectamineTM liposome transfection is made by Invitrogen. The design and synthesization of HGF ASODN are finished by Shanghai Bioengineering and breast cancer MDA-MB-231 cells are kept in our lab.Cell incubator is made by Napco of America.Inverted Microscopic is made by Olympus of Japan. MULTISKANMS Automatic enzyme labelling is made by Finland.

2.2. Synthesization of HGF ASODN

According to HGFcDNA order, ASODN are synthesized:5'-T#C#C TCC GGA GCC AGA C#T#T-3'. # represents base with thiophosphoric acid. Bases with thiophosphoric acid can solve the problem that HGF can be dissolved in vivo and strengthen the stability of HGF. This study synthesized HGF ASODN with 5' and 'bases. The "-OH" between the two bases were replaced by "-SH". HGF was synthesized by Shanghai Bioengineering with automated DNA synthesizer.

2.3. Breast cancer MDA-MB-231 cell culture

Breast cancer MDA-MB-231 cell was cultured in complete medium containing 10% CS DMEM and kept in saturation humidity, 37℃ and 5% CO_2 incubator. Culture fluid was renewed once in 2days. The cells were digested by 0.25% trypsinase and passaged in the logarithm growth period. All the cells were cultured to logarithm growth period,digested and implanted. The cells were cultured in conditioned medium including 0.1% bovine serum albumin, 1% calf serum,1.0μm olgreen copperas and IGF-I 10ng/ml after adherent culture so as to reduce influence of serum on cells and complete experiment successfully.

2.4. RT-PCR method

Cells are regulated commonly and added with 1 ml TRIZOL for schizolysis. After being shaken and stewing for 10 minutes, cells were added with 200ml phenol chloroform and being shaken and centrifugated to give convenience to take supernatant materials. Using isopropanol to settle supernatant materials and 75% ethanol to extract Ribonucleic

Acid(RNA). Determining premier according to real-time quantification. Premier of 18sRNAinternal standard:P1:5c-GTAACCCGTTGAACCCCATT-3cP2: 5c-CCATC-CAATCGGTAGTAGCG-3c. The premier length is 151bp. Premier of HGF: P1: 5c-AGCTCGGTCAGACAGGATGG-3c P2: 5c-TTC-GAAGATTAGTTGGTCCAGC-3c. The premier length is 165bp. 1~5Lg total RNA was added into the reaction pipe. The pipe was heat to 75℃ and lasted for 5 minutes and put on ice quickly. Adding 4L 5×buffer and 2L dNTP and heat the pipe to 95℃ and lasted for 5 minutes, and then 94℃ for 30 seconds, and then 60℃ for 30 seconds, and then72℃ for 30 seconds. Finished the heating circulation for 40 times. The collected pure PCR product was taken as relative quantitative criteria product.HGF and 18sr RNA was taken as a positive model and diluted according to 10-5 and 10-9 and relative copy number was determined. Quantitative reaction was finished and standard cure was achieved. The pipe was heat to 95℃ and lasted for 4 minutes, and then 95℃ for 20 seconds, and then 59℃ for 20 seconds.Finished the heating circulation for 40 times. Lastly, the pipe was heated to 94℃ and lasted for 0 second, and then 60℃ for 15 seconds, and then 95℃ for 0 second. Fluorescence signal was collected and analyzed with solubility curve. Every sample including 3 wells. Standard curve and solubility curve were finished and sample relative quantitative copy number was determined by the data.

2.5. Western blotting methods

Single cell suspension was diluted into 2.5 × 10^5/mL and implanted into 6 well plates, 2mL for each well. The suspends are cultured for 24 hours and cultured in no serum culture medium for 24 hours after cells creep plates. Adding 8μmol/L HGF ASODN. After finishing of the above steps, cell lysate was added for schizolysis and cell total protein was extracted. S-PAGE electrophoresis was finished with Bradford method. Put extracted protein on PVDF membrane and occluded the membrane with 5% skim milk at room temperature for 1 hour. After combined with relative an antibody, the protein was incubated in 4℃ for one night. And then the total protein was combined with HRP marked secondary antibody and kept in table concentrator in 37℃ for one hour. After colouration with ECL, sensitization was finished with X-ray film taken in the darkroom. Developing and fixing X-ray film at room temperature. Integrated optical density (IOD) was detected with scanner and Gelation Image master. β-actin protein bands was taken as internal standard. Expression of Ki-67, VEGF and proliferating cell nuclear antigen (PCNA) were detected by immunohistochemistry.

2.6. MTT testing cell proliferation conditions of breast cancer cells

Cells in logistic period were chosen, digested and made into 1.0×10^6/ml cell suspends. Cell suspends were implanted into 96-wells plates (100μl for each well) and stored for 6hours. Complete medium was suctioned when adhesion of cells and conditioned medium was added. The cells were maintained for 6h and added HGF ASODN with 2, 4, 8μmol/L. The blank control group did not add any nucleic acid. Add 20μl MTT (5mg/L) 24, 46, 72 and 96hs later and cell suspension was kept for 4 hours. The culture fluid was suctioned while 150μl DMSO was added. 6 hours later, absorbency (A) of each well were tested with ELISA (3 wells for each group). Calculated out cell proliferation inhibition rate: (1-A of experimental group/A of blank control group) ×100%.

2.7. Statistical methods

Data in the study were calculated out according to SPSS13.0 software. The chang of HGF were analyzed with t test. It has significance when the value of p is lower than 0.05.

3. Results

3.1. Expression of HGF detected by immunohistochemistry methods before and after 8uM HGF ASODN transfected (Figure 1)

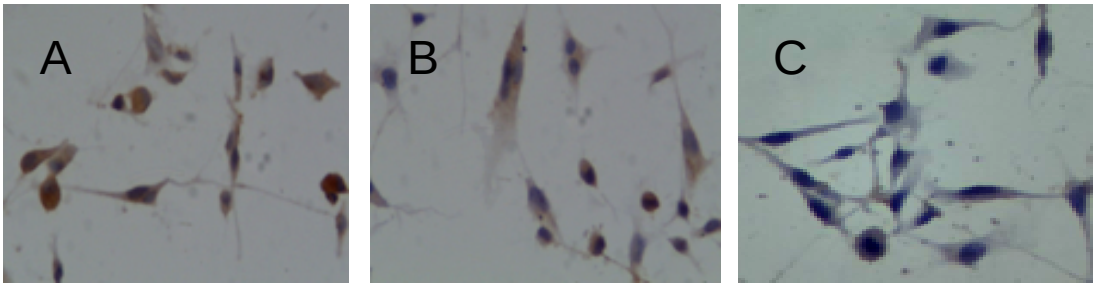

Figure 1 Expression of HGF detected by immunohistochemistry methods before and after 8uM HGF ASODN transfected on MDA-MB-231 breast cancer cells. A:before transfection, HGF is

positively expressed; B: after transfection with no HGF ASODN, HGF is positively expressed; C:after transfection with 8uM HGF ASODN, HGF is negatively expressed.

3.2. Expression of HGF detected by Western blot on MDA-MB-231 breast cancer cells

Before transfection, HGF is highly expressed in MDA-MB-231 breast cancer cells. After transfection, HGF expression is inhibited. It is also true for expression of VEGF and PCNA. (Figure2).

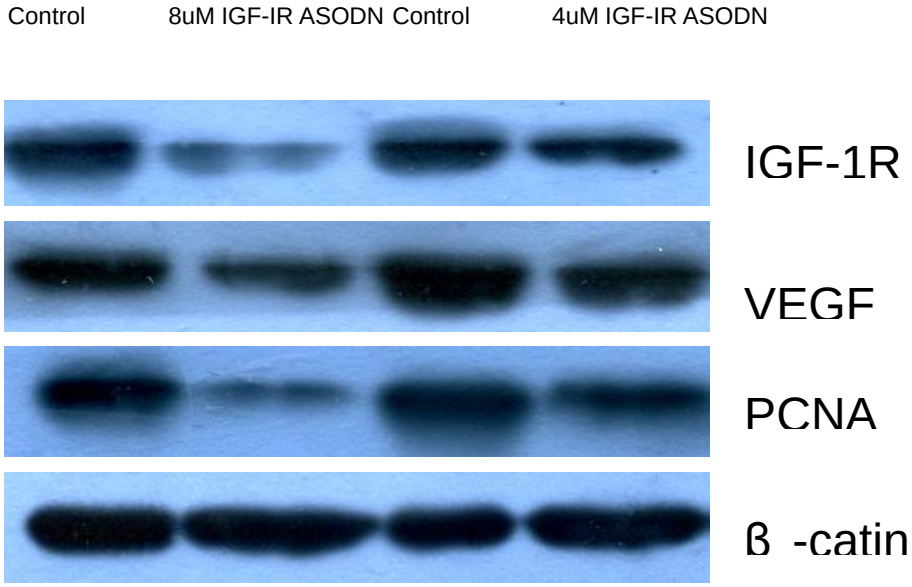

Figure 2. Western blot detecting expression of HGF,VEGF and PCNA after HGF ASODN transfected on MDA-MB-231 breast cancer cells.

3.3. Expression of HGF detected by RT-PCR on MDA-MB-231 breast cancer cells

Expression pf HGF is significantly lowered after 4 uM and 8uM HGF ASODN transfected (Figure3).

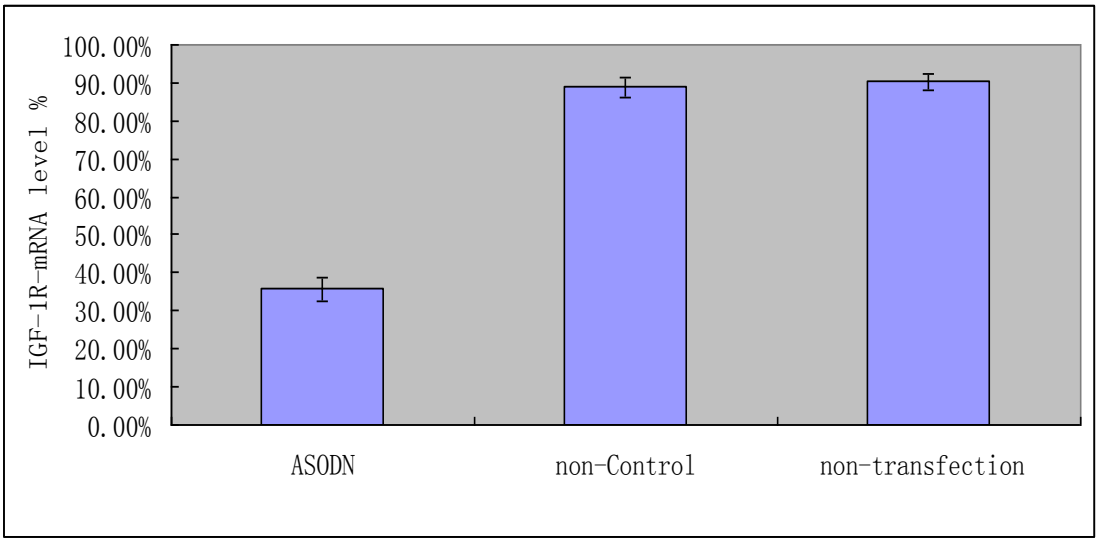

Figure 3. RT-PCR methods detecting expression of HGF mRNA after 8uM HGF ASODN transfected MDA-MB-231 breast cancer cells after transfection, expression rate of HGF mRNA is lowered to 35.77% while he expression rate of HGF mRNA of no transfection patients and blank control group are as high as 88.94% and 90.24% respectively.

3.4. Results of application of MTT in different concentration

After transfected with HGF ASODN, 2-8uM HGF ASODN can inhibit expression of HGF in some way and inhibit breast cancer cell proliferation in vitro. The inhibition effect begins to show at the 24th hours and lasts to the 72th hours. The inhibition effect is related to the concentration. What is more, 2-8uM HGF ASODN can also inhibit expression of VEGF and PCNA (Figure4, Table1).

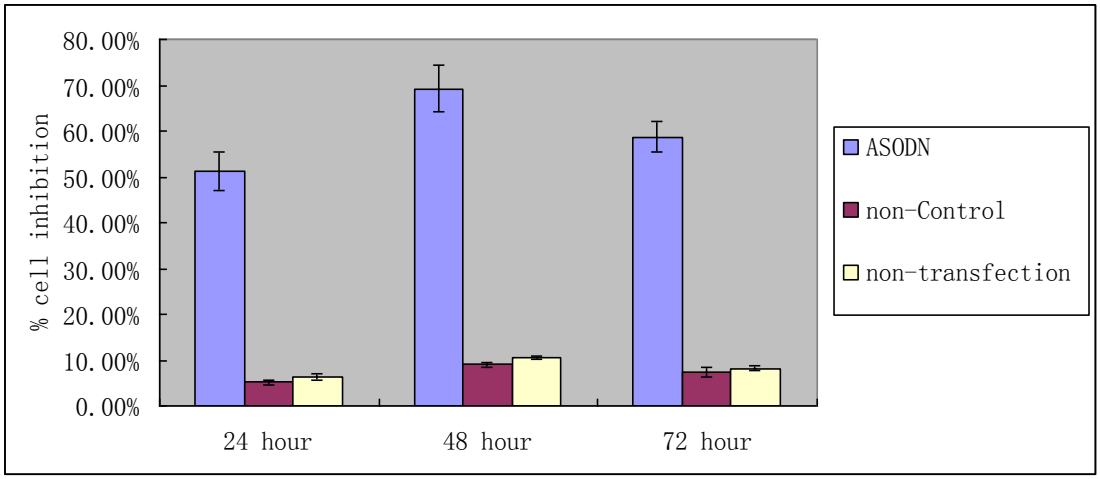

Figure 4 after transfected with 8uM HGF ASODN, cell proliferation inhibition rate is obviously increased. The inhibition effect begins to show at the 24th hours and lasts to the 72th hours.

Table1 comparison of cell proliferation inhibition rate tested by MTT before and after transfection

Group	Inhibition rate(%)		
	24h	48h	72h
2 umol/L	23.54±2.36	32.47±3.02	29.24±0.24
4 umol/L	29.66±3.64	49.32±4.29	33.06±2.74
8 umol/L	51.32±4.21	68.23±3.33	58.64±5.01
Blank control group	5.14±0.47	8.98±1.02	7.24±0.52
No transfection group	6.33±0.55	10.51±0.67	8.23±0.41

4. Discussion

Hepatocyte growth factor (HGF) is a multifunctional cytokine produced by both stromal and parenchymal cellsthat stimulates the motility and invasion of several cancer cell types, and induces angiogenesis [6]. HGF plays an important role in tumor progression and is associated with the prognosis of various human malignancies, including carcinoma of the stomach, liver, lung and nasopharynx [7]. In human gliomas, the expression of HGF and its receptor, c-Met, is associated with tumor grade. Gene transfer of HGF to breast cancer cells enhances their tumor igenicity, tumor growth and tumor-associated angiogenesis. Inhibition of HGF or c-Met expression leads to suppression of in vivo tumor formation and growth [8]. At the cellular level, the HGF and c-Met signaling pathway has been demonstrated to influence tumor formation and malignant progression by inducing cell cycle progression, tumor cell migration, invasion and tumor angiogenesis.

Biological function of HGF has made it an important target gene in tumor treatment and inhibiting and activating expression of HGF with HGF ASODN, micromolecule tyrosine kinase inhibitor, inhibiting antibody, dominant negative mutant and many other ways have been studied by many tumor studies and zoopery. Some of the studies have got a good resolution of inhibiting expression of HGF and have made further clinical studies [9-12].AS a gene therapy method, HGF ASODN have got brilliant result in treating cancers and have been applied in treatment of melanoma, Osteosarcoma, cutaneous cancer, colon cancer, glioma, breast cancer and many other cancers and

IGF-I and HGF ASODN which have obtained inhibition effect both in vivo and vitro. They concluded that it is more useful to treat cancer with focus on HGF receptor than HGF ligand [13-15]. The relationship between HGF and onset and development of breast cancer has been confirmed in the first part in our study, and it is enough to confirm that HGF can be index to diagnose and determine TNM period of breast cancer and target gene for gene therapy and molecule drugs. HGF can be widely developed and used in pharmaceutical area. Our study further researched inhibition of HGF ASODN on inhibiting expression of HGF in breast cancer cells in vitro after transfected in breast cancer cells. This study proves that HGF is highly expressed in breast cancer MDA-MB-231 cells, which confirm that it is right to choose MDA-MB-231 cells to finish the study about HGF. This study uses MTT method to detect influence of HGF ASODN on growth and proliferation of breast cancer MDA-MB-231cells. The result shows that HGF ASODN can markedly inhibit growth and proliferation of cells and inhibition effect increase while concentration of HGF increase. Inhibition effect on breast cancer MDA-MB-231 cells also increases with length of time. With the low expression of HGF, expression of VEGF and PCNA also lowered. Angiogenesis is an important link of tumor growth invasiveness and metastasis. Thus angiogenesis factors are very important in tumor occurrence and metastasis and target factor of inhibiting tumor angiogenesis [16-17]. So far, more than 20 kinds of tumor angiogenic factors and anti tumorangiogenic factors have been found [18-19]. Our research demonstrates that HGF ASODN in all concentration can inhibit formation of CAM vascular. New vascular number on CAM membrane decreased significantly under the influence of HGF ASODN, which proves that HGF ASODN can inhibit the formation of new vascular and inhibiting effect may connect with expression of bFGF, VEGF and other tumor angiogenic factors. IGF-I realize its function on survival and stability of epithelium by transacting vascular epithelium cells by bypass way and adhere and combine with extracellular matrix components in endothelial cell. IGF-I cannot only activate secretion of VEGF, but also induce the formation of vascular by activating metastasis and differentiation of epithelium [20-24]. VEGF is the most effective angiogenic factors and only specific mitogen of vascular endothelial cell which can directly active mitosis and proliferation of cells [25-26]. In this study, HGF ASODN can inhibit expression of VEGF which proves that HGF ASODN can obviously inhibit angiogenesis.

In brief, Treatment with HGF ASODN can effectively inhibit proliferation of breast cancer cells and angiogenesis ability.

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