RESEARCH ARTICLE

Combined EGFR and c-Src Antisense Oligodeoxynucleotides Encapsulated with PAMAM Denderimers Inhibit HT-29 Colon Cancer Cell Proliferation

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Abstract

Colon cancer continues to be one of the most common cancers, and the importance and necessity of new therapies needs to be stressed. The most important proto-oncogen factors for colon cancer appear to be epidermal growth factor receptor, EGFR, and c-Src with high expression and activity leading to tumor growth and ultimately to colon cancer progression. Application of c-Src and EGFR antisense agents simultaneously should theoretically therefore have major benefit. In the present study, anti-EGFR and c-Src specific antisense oligodeoxynucleotides were combined in a formulation using PAMAM dendrimers as a carrier. Nano drug entry into cells was confirmed by flow cytometry and fluorescence microscopy imaging and real time PCR showed gene expression of c-Src and EGFR, as well as downstream STAT5 and MAPK-1 with the tumor suppressor gene P53 to all be downregulated. EGFR and c-Src protein expression was also reduced when assessed by western blotting techniques. The effect of the antisense oligonucleotide on HT29 cell proliferation was determined by MTT assay, reduction beijng observed after 48 hours. In summary, nano-drug, anti-EGFR and c-Src specific antisense oligodeoxynucleotides were effectively transferred into HT-29 cells and inhibited gene expression in target cells. Based on the results of this study it appears that the use of antisense EGFR and c-Src simultaneously might have a significant effect on colon cancer growth by down regulation of EGFR and its downstream genes.

Keywords: Colon cancer - HT-29 - antisense - EGFR - c-Src - PAMAM dendrimers

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Introduction

The blocking of the oncogenic pathways in different malignancies can contribute to molecularly targeted therapeutics and has clinical benefits. c-Src is arguably the oldest known oncogene and most important proto-oncogen in the development and progression of colon cancer. It is often over-expressed and activated in the number of human cancers such as colon, prostate, brain, lung, breast, ovarian, liver and pancreas carcinoma (Talamonti et al., 1993; Summy, 2005; Finn, 2008; Lieu and Kopetz, 2010).Src proto oncogen, has a key role in cellular pathways, such as migration, survival, motility, adhesion, angiogenesis, invasion, immune function and regulation of proliferation (Ellis, 1998; Summy and Gallick, 2003) Furthermore, c-Src has high kinase activity and protein level in the progression stage of colorectal cancer to hepatic metastases (Talamonti et al., 1993). Multiple signaling pathways are correlated with c-Src family kinases activity and human cancers (Mukhopadhyay, 1995; Yamaguchi, 1997). Src level is correlated with over-expression of upstream growth factor receptors such as epidermal growth factor receptor (EGFR), HER2, vascular endothelial growth factor receptor (VEGF), fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR) (Finn, 2008). As EGFR and c-Src are over-expressed in numerous human cancers, their coordination functions are reasonable (Irby and yeatman, 2000). Src and EGFR have interaction in various downstream signaling pathways associated with EGFR-dependent signaling manner such as MAPK (mitogen-activated protein kinase) and STAT (signal transducer and activator of transcription) pathway. Furthermore, it is clear that Src is also able to indirectly activate epidermal growth factor receptor. Epidermal growth factor receptor activation by GPCR agonists to intracellular components depends on protein kinase and Calcium (Sabbah et al., 2008). MAPK signaling pathway plays a crucial role

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in the cell cycling regulation, also its over-expression and activation is essential for progression of colorectal cancer (Chapnick et al., 2011). STAT gene has role in the oncogenesis of various cancers, and also STAT5b involves in progression, cell cycling, migration, proliferation, gene expression and invasion in colon cancer (Yavari et al., 2009). In this study, we have used G5PAMAM denderimers to deliver antisense and investigate the effectiveness of simultaneous use of c-Src and EGFR antisenses in inhibiting the proliferation of HT-29 cells.

Materials and Methods

Cell culture

HT29, human colon cancer cell line was purchased from Pasteur Institute Cell Bank of Iran, These cells were grown as monolayer in 25 cm^2 flask (Orange Scientific) under standard culture medium (RPMI-1640) (Sigma; Chemical Co., St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco-Life technologies.), streptomycin (100 µg/mL), penicillin (100 units/mL) (Sigma) and cultured at 37°C in a 5% humidified CO_2 incubator and cells were grown to density of 50-60% for treatment. The medium was exchange twice a week.

Oligonucleotide transfection

For transfection, cells were grown to density of 50-60%, and then were washed in PBS; the number of live cells was evaluated with trypan blue staining and counted using hemocytometer. After that the cells were seeded into 96-well flat-bottomed culture plates (TPP, Switzerland) for MTT assay(MTT; Sigma) or in 25 cm^2 T-flask for real time PCR and Western blot analysis, then they were incubated at 37°C in 5% a CO_2 incubator. For transfect ion, solutions were prepared as manufacturer's instructions. Briefly, the day of transfection, 24-mer c-Src antisense oligodeoxynucleotide (5'-GGGCTTGCTCTTGCTGCTCCCCAT-3') and EGFR antisense oligodeoxynucleotide (5'-TTTCTTTTCCTCCAGAGCCCG-3') (EurofinsMWGOperon; Germany) incubated with G5 PAMAM solution (QIAGEN; USA) to form complex. Then it was transferred to cells and incubated for 5 h at 37°C, 5% CO_2. Following incubation, the transfection mixture was replaced with fresh cell culture medium and the cells were further incubated for real time PCR and western blot analysis.

Cell proliferation assay

The effect of antisense inhibition on the HT29 cell proliferation was determined by MTT assay. The cells were seeded in 96-well tissue culture plates at a density of 15,000 cells per well and incubated at 37 °C and 5% CO_2 humidified incubator. After 50% confluency, the cells were treated with combined c-Src and EGFR antisense. For MTT assay, 2 mg/ml of MTT solution was added to each well and incubated for 3 h at 37°C. The medium was removed and the blue formazan crystals were dissolved in 200 µl of DMSO and 25 µl Sorenson buffer. The absorbance was read in a microplate reader (Biotek, model Elx808) at 570 nm. Each experiment was repeated in triplicate format, and results were expressed as means ±SEM.

Cell uptake of the nanoparticls

Cellular delivery of ODN by PAMAM denderimer was examined by fluorescence microscopy (Olympus BX61, Olympus Inc.) and FACS flowcytometry (BD.USA).for fluorescence microscopy, HT-29 cells were incubated by FITC-labeled c-Src and EGFR Antisense encapsulating with PAMAM for 5 h at 37˚C under 5% CO_2 Atmosphere at dark. So the medium of each well was removed and washed by PBS.

Flow cytometry

In order to evaluate the transfection performance of antisense after culturing the cells in plates 6 wells, cells were incubated by FITC-labeled c-Src and EGFR Antisense, 5 h after treatment, the cell plate by placing on ice were brought to a temperature of 4 degrees after washing three times with PBS, The cells were separated by 100 ml trypsin, In order to neutralize the trypsin ,medium was added to cells and cells were centrifuged at 850 rpm and 4 °C for 10 minutes .after washing twice with PBS, cells were dispersed in PBS and the fluorescence distribution obtained by BD FACS Calibur flow cytometer and fluorescence input cell populations were analyzed by WinMDI software.

RNA extraction and c-DNA synthesis

48 hours after transfection, medium was removed from monolayer cancer cells and scrapped in 1 ml RNAX-PLUS (Cinagene, Iran). Total RNA was extracted from samples using Cinagene Kit based on the manufacturer's instruction (RNX-Plus Solution, SinaClon, Iran). Genomic DNA contaminant was removed, and then the resulting RNA was selected to DNase treatment using DNase, RNase-free(Fermentas,USA). After purification and quantification, RNA was determined by measuring optical density at 260 and 280 nm by nanodrop (NanoDrop-ND-1000). The cDNA synthesis was performing by cDNA synthase kit (Qiagene).

Real-time PCR

To characterize the EGFR and c-Src antisenses on the related genes expression, we used real-time PCR with iCycler IQ5 Multicolor Real-time PCR Detection System (Bio-Rad, USA). For various mRNA, first-strand cDNA was amplified using P53, STAT5b, MAPK1, c-Src and EGFR primers as described in the Table1. GAPDH was used for housekeeping genes. Each experiment was repeated in triplicate format, and results were expressed as means±SEM.

Preparation of protein lysates

48 hours following treatment with c-Src and EGFR antisense oligonoclutide, the medium was removed and cells were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 1% TXT-100, and 0.1 SDS and protease inhibitor cocktail). For control group cells were treated with medium alone. Total proteins concentrations were determined using Bradford assay as

Table 1. Primers used for Real time- PCR

Genes	Primer sequence (5' to 3')
c-Src	F 5´ ACCACCTTTGTG GCC CTCTATG 3´
	R 5´GCC ACC AGTCTC CCTCTGTGTT 3´
STAT 5	F 5´ GCCAAGCATGGGACTCAGTAG 3'
	R 5´ TGGGTGGCCTTAATGTTCTCC 3´
GAPDH	F 5´AAGCTCATTTCCTGGTATGACAACG3´
	R 5´ TCTTCCTCTTGTGCTCTTGCTGG 3´
MAPK1	F 5´GGATGTGGTGTTATGGAAAGAG 3´
	R 5´ AGCAGAGACGCAGAATGAC 3´
P53	F 5´TCAACAAGATGTTTTGCCAACTG 3´
	R 5´ATGTGCTGTGACTGCTTGTAGATG 3´
EGFR	F 5´GGAGAACTGCCAGAAACTGACC 3´
	R 5´GCCTGCAGCACACTGGTTG 3´

described previously, Bradford (1976).

Western blot analysis

We used Western blot analysis to evaluate the EGFR and c-Src protein expression on the malignant cells, HT29, after antisense therapy. 25 μg total crude proteins were used for each well for SDS-PAGE and Western blot analysis. GAPDH gene was used as control gene in this study. Western blotting for lysed cells performed using Chemiluminescent Immunodetection kit (Western Breeze; Invitrogen). Equal crude protein was loaded in each lane for SDS-PAGE and Western blot analysis. c-Src and EGFR specific antibody (Invitrogen) were used according to the supplier's commendation. Films containing protein bands were scanned and densitometry using Image J software.

Statistics

Statistical analysis was performed with SPSS version 16.0 software and ANOVA test was used to compare between groups. Data are represented Mean±SEM. The differences were considered significant when *P<0.05.

Results

The effect of combined Antisense c-Src and EGFR on cellular proliferation

In order to determine the effect of combined Antisense c-Src and EGFR on the HT29 cell lines proliferation , MTT assay was illustrated at 24, 48, and 72 hours after antisense transfection. The colon cancer cell line (HT29) treated with combined AS-ODN polyfect complex was compared to untreated HT29 cell lines As shown in Figure1, cell growth was inhibited considerably by knock-down of c-Src and EGFR AS-ODN. AS-ODN or polyfect alone have not had significant effect on cell proliferation, however, c-Src and EGFR AS-ODN complex with polyfect was the most effective in the inhibition compared with control groups so that it can be seen in Figure 1, cell proliferation was decreased to 50% (P<0.05) after 48 hours of transfection but this reduction was not significant at 24 and 72 hours.

Intracellular delivery of ODN by PAMAM dendrimer

In order to ensure antisense entry into HT-29 cells by FITC-labeled antisense, Cells treated with antisense labeled by FITC and with Polyfect complex was examined by fluorescent microscopy. As Figure 2 shows ODN is

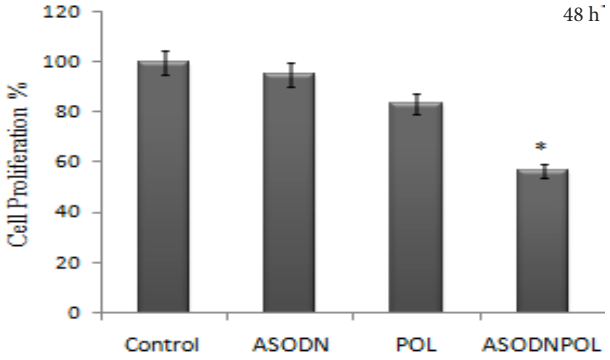

Figure 1. MTT Assay was Assessed the Effect of c-Src and EGFR AS-ODN in the Proliferation of HT29 Colon Cancer Cell Line. Significantly different from transfected cells and controls (P<0.05)*. Data are represented Mean ± SEM. The experiments were repeated as triplicate. ASODN; c-Src and EGFR antisense oligonucleotide without PAMAM; POL, PAMAM dendrimer; ASODN Pol, complex of c-Src and EGFR antisense oligonucleotide with PAMAM dendrimer.

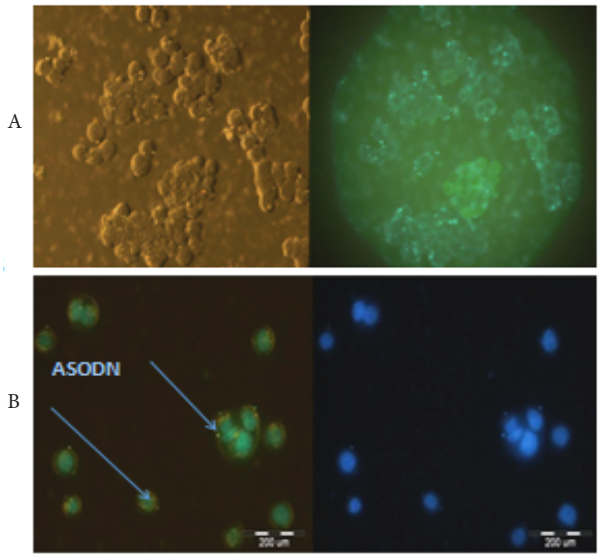

Figure 2. Intracellular Delivery of c-Src and EGFR ODN with PAMAM Denderimer. HT-29 cells were incubated with FITC –labeld ODN for 5 h at 37°C, washed with PBS and was examined by fluorescence microscopy (Olympus BX61, Olympus Inc.) (A) Light microscopy image (left) and Fluorescent microscopy image (right) (B) Fluorescent microscopy image with DAPI Staining shows the location of ASODN in cells.

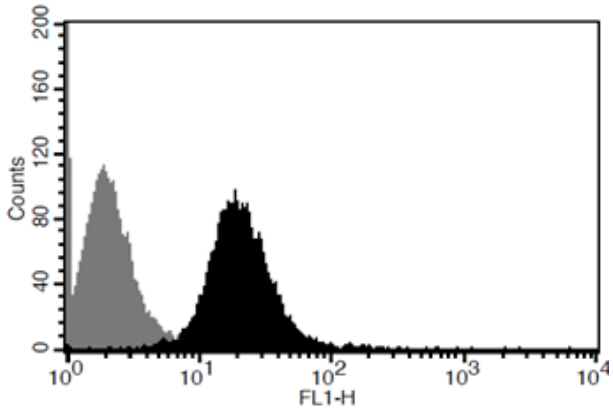

Figure 3. Flow Cytometric of Cells Treated with Antisense- FITC Labeled. Gray: untreated cells and Black: cells treated.

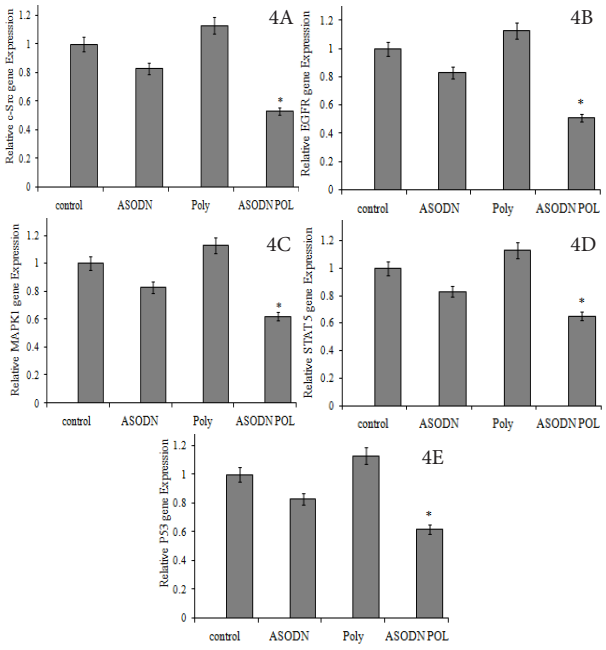

Figure 4. Real Time PCR Analysis. All of data were normalized to GAPDH gene expression, 3A, 3B, 3C, 3D, 3E: results related to decrease in c-Src, EGFR, MAPK1, STAT 5 and P53 gene expression after simultaneous antisense treatment respectively.

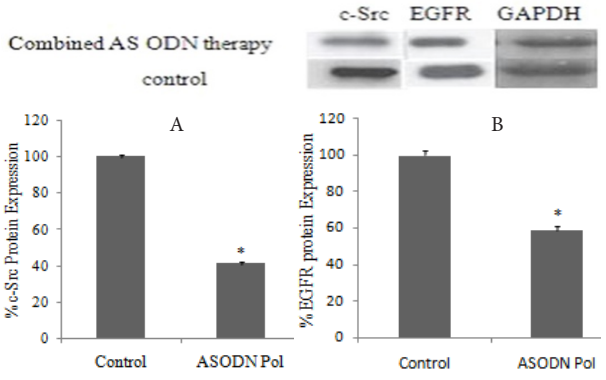

Figure 5. Western Blot Analysis for the c-Src and EGFR Protein Expression in HT29 Cells. A) c-Src protein Expression compared with control group B) EGFR protein Expression compared with control group.

more efficiently delivered into the cells by PAMAM dendrimer.

Flowcytometry

For quantitative determination of transfection rate flow cytometric techniques were used. As Figure 3 shows the complex have entered into more than 75% cells.

Analysis the combined anti-EGFR and c-Src AS-ODN effects on expression of c-Src, EGFR, MAPK1, STAT5 and P53

The down regulation of c-Src and EGFR in HT29 cells were investigated by real time PCR analyzing. After 48 hours of combined anti-EGFR and c-Src Antisense treatment, mRNA levels of EGFR, c-Src, MAPK1, STAT5 and P53 genes were analyzed. The genes CT values were normalized against mRNA level of GAPDH as housekeeping gene and the relative expression for each group was measured.

Figure 4A, 4B, show that, there is significant decrease in level of c-Src and EGFR expression in PAMAM nanoparticle encapsulating c-Src and EGFR antisense (about 60%, 50% respectively $P<0.05$) comparing with other groups. According to Figure 4C and 4D the level of EGFR downstream genes including MAPK1 and STAT5 was decreased considerably (about 40% and 37%, respectively) in transfected HT29 cells with combined anti-EGFR and c-Src AS-ODN ($P<0.05$). This complex has also showed a decrease in P53 expression (Figure 4E).

Inhibitory effect of combined anti-EGFR and c-Src AS-ODN on the level of c-Src and EGFR protein expression

Western blot results show that antisense Oligonucleotide c-Src and EGFR had inhibitory effects in HT29 colon cancer cells (Figure 4). The levels of protein expression of these genes were significantly down-regulated through simultaneous c-Src and EGFR AS-ODN treatment. Comparing with the level of protein expression of control gene (GAPDH gene) and total protein in control group (the HT29 cell without treatment), c-Src and EGFR expression had been decreased about 56% ($P<0.05$).

Discussion

Nowadays it is believed that cancers caused by genetic changes (eliminating, reducing or increasing the expression of a gene) are created, so gene therapy in several Decades as one of the new models in cancer treatment has been mentioned (Eder et al., 2005).

One of the main reasons for the development and progression of various cancers in humans is increased expression of oncogen (Yavari et al., 2009). Abnormal expression of proto oncogen and an external signal generated by the oncogen causing of the proliferation of cancer cells through activation of intracellular signaling pathways. Among oncogenes the role of c-Src and EGFR in colon cancer development and progression has been described in several studies (Talamonti et al., 1993; Kopetz, 2007). increase of c-Src expression cause Transactivation of EGFR and activates some EGFR dependent signaling pathways such as STAT5b (Kopetz, 2007). Inhibitory effect of c-Src antisense alone in decrease of c-src ,EGFR and EGFR downstream genes like MAPK1 and STAT5 has been showed in various studies (Nourazarian et al.,2012). in this study We used combined c-Src and EGFR Antisense oligo nucleotide simultaneously in human colon cancer cells .For Transfection we used G5 PAMAM denderimers (polycationic denderimers). It is noteworthy that the drug formulation in gene carriers PAMAM (polyfect) cause reduce its toxicity also the toxicity of the drug carrier of a gene complex has been shown in other studies (Omidi and Barar, 2009). To confirm entry of AS ODN to the cell Fluorescent microscope and FACS flow cytometry was used. Evaluation of antisense drug transfer into cells using flow cytometry,show the effective AS ODN entry in to cell however the antisense alone is not able to enter cells,that Considering the negative surface charge and Negatively charged cell membranes is also justified (Albertazzi et al., 2010).

To determine whether c-Src and EGFR antisense down-regulation is involved in inhibition of colon cancer proliferation, cell viability assay was used. The result of MTT assay showed that PAMAM nano particle encapsulating both the antisense had significantly decrease the growth rate of HT29 human colon cancer cells. Therefore we investigated the pathway related to c-Src and EGFR signaling pathway by different methods. STAT gene has a major role in most cancers (Xiong et al., 2009). EGFR and STAT5 have an important role in the survival of cancer cells and STAT5b has a role in colon cancer progression, migration, proliferation, invasion, cell cycling and gene expression (Xiong et al., 2009; Nourazarian et al., 2012). Real time PCR results demonstrated that expression of STAT5b gene has down regulated follow of c-src and EGFR inhibition through c-Src and EGFR reduction by PAMAM nano particle encapsulating both antisense. Also we show that simultaneous use of PAMAM nanoparticle encapsulating c-Src and EGFR antisense had more than 50% inhibition effects on the c-Src, EGFR, MAPK1 and STAT5 expression. The important role of MAPK signaling pathway in cell cycle regulating and its role in the development of colon cancer has been determined (Chapnick et al., 2011). Also EGFR supperession cause MAPK inhibition that specified in other investigation (Kumar et al., 2010). Our results showed a significant decrease in the amount of MAPK1 gene expression followed by combined Antisense therapy Compared with single antisense therapy that showed in our previous study (Nourazarian et al., 2012). Decrease in these genes expression can be related to Trans activation of EGFR by c-src. The expression of P53, tumor suppressor gene, was decreased. It is show that apoptosis inhibition either involves in the other pathways such as AKT-1. AKT-1 pathway have a Key roles in the initiation and progression of cancer Including stops apoptosis, cell survival, cell cycle progression, angiogenesis and tissue growth (Bowles et al., 2009). PAMAM dendrimer alone had increasable effects on expression of explained gene. Mentioned phenomenon could explain the unexpected effects of, gene drugs carriers Is considered by many researchers (Beale et al., 2003). Similar this data have been shown on A549 cell lines by Nakhlband and her colleagues (Nakhlband et al., 2010). Decrease in c-Src and EGFR protein expression represents the effects of combined Antisense therapy in the level of transcription and translation of proteins.

We suggested further studies should be performed about simultaneous antisense therapy for the Inhibition of genes involved in colon cancer and also in vivo experiments on laboratory animals to investigate the efficacy of combined antisense therapy in complex biological environments should be done.

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