RESEARCH COMMUNICATION

c-Src Antisense Complexed with PAMAM Denderimes Decreases of c-Src Expression and EGFR-Dependent Downstream Genes in the Human HT-29 Colon Cancer Cell Line

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Abstract

c-Src is one member of non-receptor tyrosine kinase protein family that has over expression and activation in many human cancer cells. It has been shown that c-Src is implicated in various downstream signaling pathways associated with EGFR-dependent signaling such as MAPK and STAT5 pathways. Transactivation of EGFR by c-Src is more effective than EGFR ligands. To inhibit the c-Src expression, we used c-Src antisense oligonucleotide complexed with PAMAM Denderimes. The effect of c-Src antisense oligonucleotide on HT29 cell proliferation was determined by MTT assay. Then, the expression of c-Src, EGFR and the genes related to EGFR-depended signaling with P53 was applied by real time PCR. We used western blot analysis to elucidate the effect of antisense on the level of c-Src protein expression. The results showed, c-Src antisense complexed with PAMAM denderimers has an effective role in decrease of c-Src expression and EGFR-dependent downstream genes.

Keywords: c-Src - antisense - PAMAM dendrimer - human colon cancer

Asian Pacific J Cancer Prev, **13**, 2235-2240

Introduction

Colorectal cancer as the second leading cause of cancer mortality in the Western Societies represents the third most common type of cancer in all over the world (Midgley & Kerr, 1999; Greenlee et al., 2000; Siegel et al., 2012). In spite of advances in the current colorectal cancer therapies; such as surgical resection, chemotherapy and radiotherapy, the mortality rate is still remained high with local tumors develop metastases or local recurrence during treatment (Lieu & Kopetz, 2010; Chua et al., 2011). Therefore the novel strategies for colorectal cancer treatment need to be investigated.

One of the novel methods for treatment of colorectal cancer is gene therapy. In cancer diseases, there are abnormal gene expressions due to gene deletion, replacement or suppression which gene therapy holds promise for treatment through correcting the abnormal gene (Rubenstein et al., 2008). The first gene therapy was used in 1990 and during the time it has been developed. Antisense oligonucleotides (oligos) are common gene therapy technique for silencing the abnormal gene to prevent the cancer development (Raymond, 2007;Rubenstein et al., 2008) and block specifically the over expression of gene causing disease inside the genome.

The most important abnormal gene in the development and progression of colon cancer is c-Src. Src proto oncogen, c-Src, has been shown as one of the abnormal genes that over expressed and activated in the number of human cancers including colon cancer, breast, brain, lung, ovarian, liver, prostate and pancreas carcinoma (Talamonti et al., 1993; Summy et al., 2005; Lieu et al., 2010). c-Src (EC 2.7.10.2), a 60-kD protein, is one of non-receptor tyrosine kinases protein family which has a key role in migration, adhesion, angiogenesis, invasion, immune function and regulation of proliferation (Ellis et al., 1998; Windham et al., 2002). Different mechanisms are correlated with c-Src family kinases activation and regulation of human cancer through the multiple signaling pathways (Yamaguchi et al., 1997; Summy & Gallick, 2003). Vascular endothelial growth factor (VEGF) as an intracellular signaling protein in the stimulation of vasculogenesis and angiogenesis in cancer cells is downstream target for c-Src (Mukhopadhyay et al., 1995). c-Src has implicated in various downstream signaling pathways related to EGFR-dependent signaling pathways (Epidermal growth factor receptor), such as MAPK (mitogen-activated protein kinase) signaling. In colorectal cancer cell lines, EGFR over expression is associated with Src activation which activates signaling pathways such as

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STAT5b (signal transducer and activator of transcription). In addition, Src has a key role in the invasive behavior of colonic carcinoma cells due to increase in Src kinase activity through EGF which causes carcinoma cells move into basement membranes (Kopetz., 2007).

c-Src and have interaction and they are co over expressed in many human carcinomas. The stimulation of MAP kinase activity in human leukemia cells by c-Src (Yamaguchi et al., 1997) and EGFR in colorectal carcinoma is crucial in cancer development. Moreover, the protein level and kinase activity of c-Src are significantly increased in the progression stage of colorectal cancer to hepatic metastases (Talamonti et al., 1993). In this stage, migration, invasion, growth and survival of cells are raised and expressions of proangiogenic molecules are deregulated. Therefore, inhibitors of Src have a key role in cancer treatment (Summy et al., 2005) and Src family kinases can become important targets for anti-cancer therapy in the future (Summy & Gallick, 2003). In addition, c-Src is over-expressed in a HT29 cell line of colon cancer and plays an important role in the development of metastatic stage of primary human colorectal cancer (Talamonti et al, 1993; Termuhlen et al., 1993; Mao et al., 1997). Previous study has shown that c-Src antisense oligonucleotides can silent the c-Src expression in the cancer cells (Charles et al., 1997; Irby & Yeatman, 2000).

Since antisense oligonucleotides have anionic charge and unable to pass into the cells, various delivery systems have been investigated. Polyamidoamin polymers, PAMAM nanoparticals have been developed as a novel non viral DNA delivery vector in recent years. They are highly effective cationic delivery vehicles used and form PAMAM-antisense complex by electrostatic interaction, and carry the antisense into nucleus of target cell (Eichman et al., 2000; Baker et al., 2004; Orava et al., 2010). In addition, PAMAM polymer's ability in protection of antisense from digestion in cytoplasm by restriction enzymes can be investigated (Baker et al., 2004; Orava et al., 2010; Wang et al., 2010).

In this study, we have used nanoparticles G5PAMAM to deliver antisense oligo c-Src into colon carcinoma cell line (HT29) to investigate the role of knocking-down c-Src in inhibiting the proliferation of HT-29 cells. Moreover, we explored the Mechanism and regulation of c-Src in suppression which may establish candidate status of c-Src-mediated signaling for studies in targeted cancer therapy.

Materials and Methods

Cell culture

HT29 cells (the human colorectal carcinoma cell line) was purchased from Pasteur Institute Cell Bank of Iran (Tehran, Iran). The cells were grown in minimal essential tissue culture medium RPMI-1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS: Gibco-Life technologies) penicillin (100 units/mL), streptomycin (100 µg/mL : Sigma) at 37 °C in a 5% CO_2 humidified atmosphere and the medium was exchanged twice a week.

Transfection of oligonucleotides

For transfection, cells were grown to density of 70–80% in 25 cm^2 flask (Orange Scientific), then washed in PBS solution and harvested from tissue culture plate by treatment with trypsin/ EDTA (Sigma). Cells then were seeded into 96-well culture plates(TPP, Switzerland) for MTT (MTT; Sigma Chemical Co., St. Louis, MO, USA) assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) or in T-flask for real time PCR and Western blot analysis,. When the cells reached to 50–60% confluency, the solutions were prepared as manufacturer's instructions. Briefly, the day of transfection for each 25 cm^2 flask, 4 μg/μl of 24-mer c-Src antisense oligodeoxynucleotide (5´ GGG CTT GCT CTT GCT GCT CCC CAT 3') (Eurofins MWG Operon; Germany) were diluted with appreciate amount of RPMI-1640 (Sigma), mixed with 20 μl of PAMAM G5 solution (QIAGEN) and incubated for 5-10 min at room temperature to form antisens PAMAM complex. During the complex formation, the medium was removed from the cells and cells were washed in 2 ml PBS. Then 1000 μl of growth culture medium was added to reaction tube and transferred to each well 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 – hours for real time PCR and western blot analysis.

Cell proliferation assay

MTT assay was used for determination of the effect of antisense oligonucleotide used. The day before transfection, the number of live cells was evaluated with trypan blue staining and counted using hemocytometer. The cells were seeded in 96-well flat-bottomed tissue culture plates at an initial cell density of 15,000 cells per well in 200 µl of growth medium and incubated at 37°C, 5% CO_2 and humidified atmosphere and let to reach 50% confluency. The day of transfection, the antisense solution was prepared according to QIAGEN (QIAGEN, USA) instruction. Briefly, for each well 0.2 μg/μl c-Src antisense was diluted with 30 μl of RPMI-1640 and then 1:20 volume dilution of PAMAM G5 in RPMI-1640 was added to antisense solution to form complex and incubated 5-10 min at room temperature.

During the complex formation, the medium was removed from the cells and washed in 100 µl PBS. Then 100 μl of culture medium was added to reaction, and transformed to each well and incubated for 5 h at 37 °C and 5% CO_2. After exposure times, all the treatments were changed with growth culture medium and the plates were incubated for 24 h, 48 h and 72 h at 37 °C with 5% CO_2. For determination of cell proliferation 50 µl/well (2 mg/ml in PBS) of MTT solution was added to each well and incubated for 3 h at 37 °C. The medium was then removed and the blue formazan crystals were dissolved in 200 µl of DMSO and Sorenson buffer. The absorbance was finally recorded in a micro plate reader (Biotek, model Elx808) at 570 nm. Each experiment was repeated in triplicate format, and pooled results were expressed as means ± SEM.

Cell uptake of nanoparticles

Cellular delivery of ODN by PAMAM denderimers was examined by fluorescence microscopy (Olympus BX61, Olympus Inc.). HT-29 cells were incubated by FITC-labeled c-Src Antisense encapsulating with PAMAM for 4 h at 37 ˚C under 5% CO_2 Atmosphere at dark. Therefore the medium of each well was removed and washed by PBS.

RNA extraction and c-DNA synthesis

After 24 h of transfection, medium was removed and cells were scrapped into 1 ml RNAX-PLUS (Cinagene, Iran) and total RNA was extracted from samples using manufacturer's instruction (RNX-Plus Solution, SinaClon, Iran). To remove contaminating genomic DNA, the resulting RNA was selected to DNase treatment using DNase, RNase-free (Fermentas). After purification, RNA concentration was determined by measuring optical density at 260 and 280 nm by nanodrop (NanoDrop-ND-1000). The cDNA synthase was perform by cDNA synthase kit (Qiagene).

Real-time PCR

To characterize the antisense c-Src effects on the related genes expression, real-time PCR was performed using an IQ5 Multicolor Real-time PCR Detection System (Bio-Rad). GAPDH was used for housekeeping amplification. For various mRNA, first-strand cDNA was amplified using the following primers as described in the Table 1.

Western blot analysis

Western blot analysis was used to evaluate the amount of c-Src expression on the malignant cells, HT29, after antisense therapy. For this, HT29 cells were treated with antisense oligonoclutide solution as above described. After 24 h treatment, the medium was removed and cells were lysed. Western blotting was performed using Chemiluminescent Immunodetection kit (Western Breeze; Invitrogen). Equal cell lysates were loaded in lanes for SDS-PAGE and Western blot analysis. C-Src-specific antibody (Invitrogen) was used according to the supplier's recommendation. Briefly, after transfection, Cells was resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 1% TXT-100, 0.1 SDS and protease inhibitor cocktail), and total proteins concentration were determined using Bradford assay as described previously (Bradford, 1976). 25 μg total crude proteins were used for each well for SDS-PAGE and Western blot analysis. GAPDH gene was used as internal control in this study.

Statistics

Statistical analysis was performed with SPSS version 16.0 software. Statistically comparisons were between the control group and treatment. Differences between mean values were carried out using one-way analysis of variance. Data are represented Mean ± SEM. The differences were considered significant when *P < 0.05.

Results

The effect of ASODN c-Src on cellular proliferation

In order to determine the effect of the c-Src AS-ODN 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 AS-ODN polyfect complex was compared to untreated HT29 cell lines (Figure 1). As shown in Figure. 1A, B and C, cell growth was inhibited considerably by knock-down of c-Src AS-ODN after appreciated times. c-Src AS-ODN or polyfect alone have not had significant effect on cell proliferation, however, c-Src AS-ODN complex with polyfect was the most effective in the inhibition compared with control groups. Figure 1A illustrated cell proliferation was decreased to 40 % (P<0.05) after 24 hours of transfection. Also, c-Src AS-ODN complex was decreased cell proliferation to almost

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´

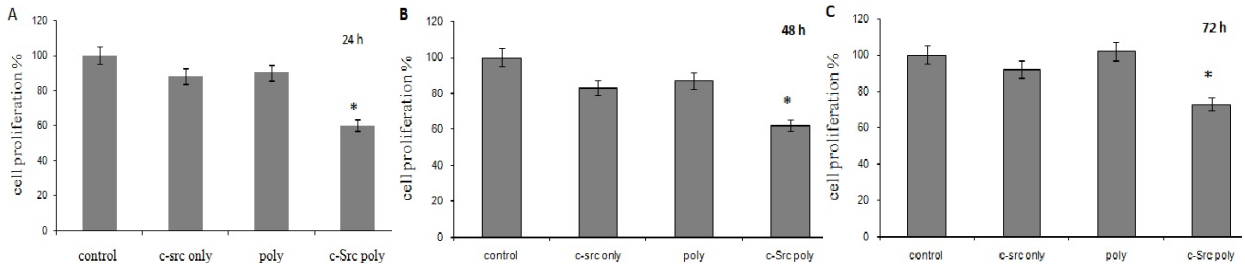

Figure 1. MTT Assay Was Assessed the Effect of c-Src AS-ODN in the Proliferation of HT29 Colon Cancer Cell line. HT29 cells were placed in a 96 well microplate 1A: the effect of three experiments in cell proliferation of HT29 after 24, 1B: 48, 1C: 72 hours transfected with c-Src AS-ODN complex and polyfect respectively. All the data was compared with control groups through ANOVA tests. Note: significantly different from transfected cells and controls (P<0.05)*. Data are represented Mean ± SEM. The experiments were repeated as triplicate. Abbreviations: c-Src only,c-Src antisense oligonucleotide without PAMAM; POL, PAMAM dendrimer; c-Src Poly, complex of antisense oligonucleotide with PAMAM dendrimer

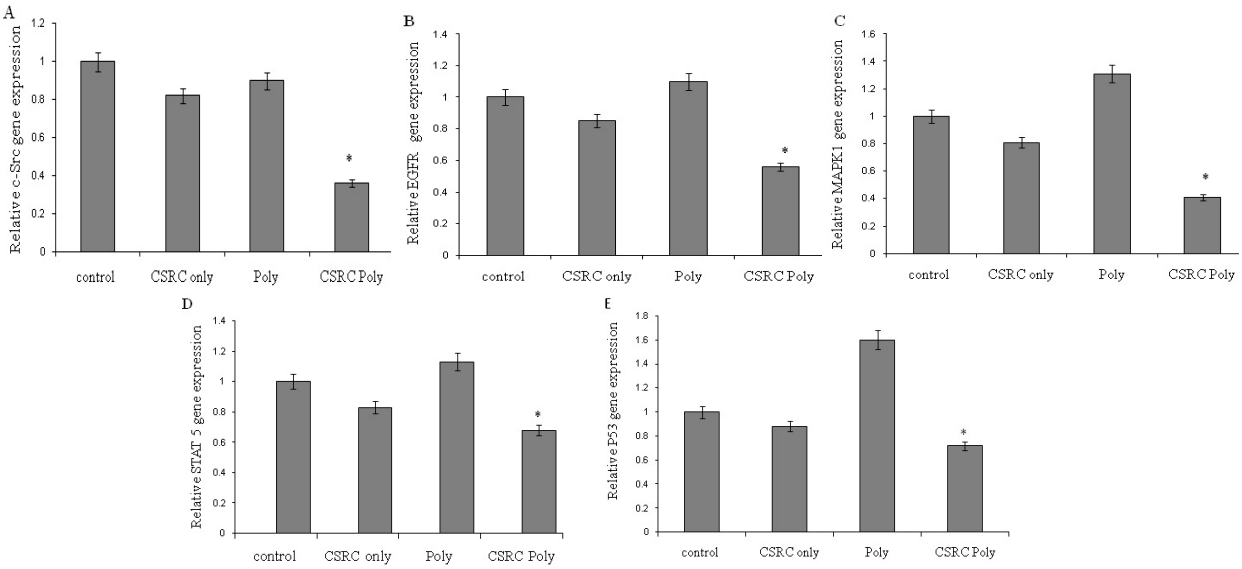

Figure 2. Real Time PCR Analysis of c-Src, EGFR and EGFR Dependent Downstream Genes. Expression in HT29 cells, all of data were normalized to GAPDH gene expression, 2A, 2B, 2C, 2D, 2E: results related to decrease in c-Src, EGFR, MAPK1, STAT 5 and P53 gene expression after antisense treatment respectively

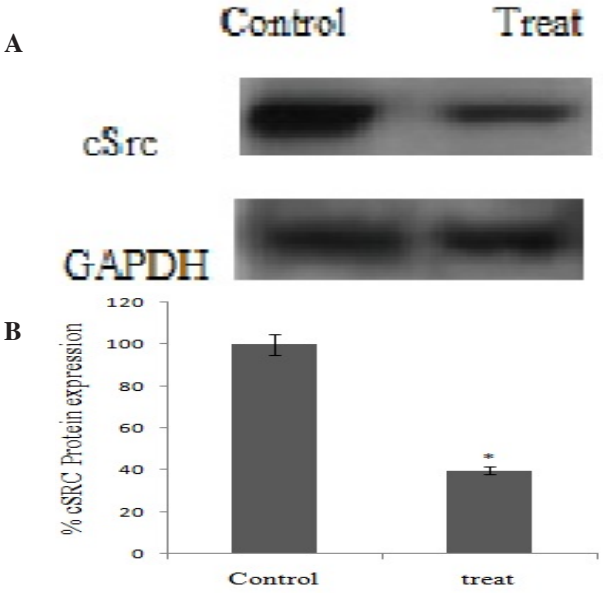

Figure 3. Western Blot Analysis for the c-Src Protein Expression in HT29 Cells Transfected with Antisense Oligonucleotides. Equal amount (22 mg/lane) of total protein extraction was determined by a 10% SDS-PAGE investigated with anti-human c-Src and GAPDH was used as a housekeeping gene for control. (A) c-Src protein expression was compared with control group (cells without treatment) and housekeeping gene. Comparing with control groups, antisense oligonucleotid of c-Src had significant inhibitory effect in HT29 cells. (B) The protein level of c-Src was determined using the image analysis, as figure shows, c-Src protein expression was reduced significantly (% 60)

38 % after 48 hours of transfection and to 31 % after 72 hours (P<0.05). According to the results, inhibition was decreased slightly after 48 and 72 h but differences were not statistically significant (P > 0.05).

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

The down regulation of c-Src in HT29 cells were investigated by real time PCR analyzing (Figure 2). Following 24 hours of antisense treatment, c-Src mRNA

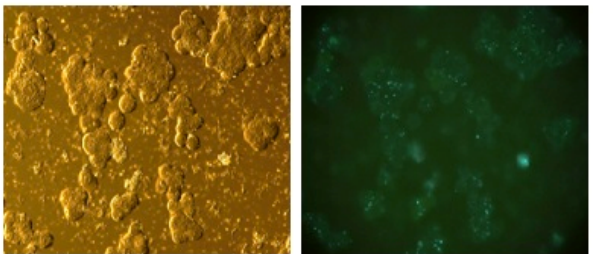

Figure 4. Intracellular Delivery of c-Src ODN with PAMAM Denderimers. HT-29 cells were incubated with FITC –labeld ODN for 4 h at 3 7˚C, washed with PBS and was examined by fluorescence microscopy (Olympus BX61, Olympus Inc.) (A) Light microscopy image (B) Fluorescent microscopy image

levels with EGFR, 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. As shown in Figure 2A, there is significant decrease in level of c-Src expression in PAMAM nanoparticle encapsulating c-Src antisense (about 32%, P<0.05) comparing with other groups. According to Figure 2B, 2C and 2D the level of EGFR and its downstream genes including MAPK1 and STAT5 was decreased considerably (about 50%, 45% and 38%, respectively) in transfected HT29 cells with PAMAM nanoparticle encapsulating c-Src antisense (P<0.05). However, c-Src complex with PAMAM has also showed a reduction in P53 expression (Figure 2E).

Inhibitory effect of ASODN on the level of c-Src protein expression

Western blot analysis shows antisense oligonucleiod c-Src had inhibitory effect in HT29 cells in colon cancer (Figure 3). The level of c-Src protein expression was significantly down-regulated through AS-ODN transfection in to HT29 cells. 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 expression had been decreased about 60 percent ($P<0.05$).

Intracellular delivery of ODN by PAMAM dendrimer

Figure 4 demonstrates that ODN is more efficiently delivered into the cells by PAMAM dendrimer.

Discussion

Oncogene over expression is an important reason for the development and progression of different cancers in human (Yavari et al., 2009). The abnormal expression of proto-oncogene and external signals created by oncogenes induces proliferation of cancer cells through activation of signaling pathway. Among the oncogenes, the role of c-Src in development and progression of colon cancer was reported in several articles (Talamonti et al., 1993; Summy et al., 2005; Lieu & Kopetz, 2010). To elucidate the effect of c-Src expression in human colon cancer cells, we used antisense oligo nucleotide c-Src. However, antisense oligonucleotides have anionic charge and pass the cell difficulty, so various delivery systems have been investigated. Polyamidoamin polymers, PAMAM nanoparticals, have been developed as a novel nonviral DNA delivery vector in the recent years. They are highly effective cationic delivery vehicles used and formed PAMAM-antisense complex by electrostatic interaction, as well as they carry the antisense into nucleus of target cells (Eichman et al., 2000; Baker et al., 2004; Orava et al., 2010; Wang et al., 2010). In addition, PAMAM polymer's ability in protection of antisense from digestion in cytoplasm had been investigated using restriction enzymes (Baker et al., 2004; Orava et al., 2010; Wang et al., 2010), so in this work we used G5 PAMAM polymer as delivery system.

To determine whether c-Src antisense down-regulation is involved in inhibition of colon cancer proliferation, we applied cell viability assay. The result of MTT assay showed that PAMAM nanoparticle encapsulating c-Src antisense had significant reduction effect on the growth rate of HT29 human colon cancer cells. Since the cell proliferation may include different and complex mechanisms; therefore we investigated the pathway related to c-Src and EGFR signaling pathway by different methods.

It has shown that c-Src is implicated in various downstream signaling pathways associated with EGFR-dependent signaling such as MAPK pathway. Transactivation of by c-Src is more effective than EGFR ligands. In colorectal cancer cell lines, EGFR over-expression is associated with Src activation which activates signaling pathways such as STAT5b (Kopetz, 2007). STAT gene is involved in the oncogenesis of numerous cancers (Xiong et al., 2009). STAT5 has two forms including a and b which have different roles in various biological pathways. STAT5b has a role in progression of various tumors. Low level of STAT5 expression is related to G1 phase of cell cycle. In addition, there is a crosstalk relation between MAPK and STAT5 in colon cancer (Mao et al., 1997). EGFR and STAT5 are correlated in cancer cell survival and STAT5b has a role in colon cancer progression, migration, proliferation, invasion, cell cycling and gene expression (Xiong et al., 2009). Our real time PCR results demonstrated that expression of STAT5b gene has down-regulated due to EGFR inhibition through c-Src reduction by PAMAM nanoparticle encapsulating c-Src antisense. In addition, we illustrated that PAMAM nanoparticle encapsulating c-Src antisense had almost 50% inhibition effect on the c-Src, EGFR, MAPK1 and STAT5 expression. According to c-Src mRNA inhibition, we observed that reduction in EGFR and EGFR are related to genes expression including STAT5 and MAPK1.

MAPK signaling pathway has an important role in the cell cycling regulation. MAPK activation and its over-expression play a crucial role in the progression of colorectal cancer (Chapnick et al., 2011). EGFR down regulation causes MAPK inhibition (Kumar et al., 2010), also MAPK has a key role in drug tolerance radiotherapy, our results indicated MAPK1 gene expression was decreased by c-Src inhibition; it seems c-Src down-regulated MAPK1 expression by inhibiting the EGFR-dependent manner.

In contrast to our prediction, the expression of P53, tumor suppresser gene, was decreased by c-Src inhibition. It is advocated that apoptosis suppression either involve in the other pathways such as AKT-1 or increase the survivin gene expressions.

PAMAM denderimers and c-Src antisense oligonucleotide alone didn't reduce genes expression but PAMAM dendrimer alone had increasable effects on expression of explained gene which suggested PAMAM Denderimes have unexpected effects which needs to be investigated Similar this data Nakhlband and her colleges have been shown on A549 cell lines (Nakhlband et al., 2010). Moreover, our results illustrated that PAMAM nanoparticle encapsulating c-Src antisense in HT29 cells decreased the protein level of c-Src in targeted cells.

In sum up, it is suggested that combined effect of gene therapy targeting c-Src or EGFR and radiotherapy can be more effective in colon cancer therapy. Also animal modeling is recommended for more investigation

Acknowledgements

This research has been supported by office of vice chancellor for research of Kerman University of Medical Sciences and Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences.

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