

## RESEARCH ARTICLE

# EGFR Antisense Oligonucleotides Encapsulated with Nanoparticles Decrease EGFR, MAPK1 and STAT5 Expression in a Human Colon Cancer Cell Line

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### Abstract

Epidermal growth factor receptor (EGFR) is over-expressed in several human cancers. This would suggest that inhibition of EGFR is a reasonable approach for cancer treatment. In this study we investigated EGFR blocking and its effects on the mediated signaling such as MAPK and STATb in HT29 cells. For this aim we used FITC-labeled EGFR antisense oligonucleotides encapsulated with PAMAM nanoparticles to inhibit EGFR expression. Cellular uptake of antisense was investigated by fluorescence microscopy and flow cytometry analysis. The effect of EGFR antisense on the expression of EGFR in HT29 cells was examined by real time PCR and Western blots, which showed that antisense encapsulated with PAMAM decreased the level of EGFR mRNA and protein. In addition, real time PCR results confirmed that EGFR inhibition had an effective role in the reduction of EGFR dependent downstream genes. In conclusion, EGFR antisense encapsulated with PAMAM nanoparticles down regulated EGFR and EGFR-mediated genes.

**Keywords:** EGFR - antisense - human colon cancer - nanoparticles

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### Introduction

EGFR (Epidermal Growth Factor Receptor) is a 170-kDa transmembrane glycoprotein tyrosine kinase (TK) receptor on the cell surface which has interaction with EGF growth factors (Jiang and Li, 1997; Yong et al., 2000). In normal cells, the EGFR activation leads to initiation of several signal transduction pathways such as MAPK (mitogen activated protein kinase) STAST (signal transducer and activator of transcription) proteins, SRC kinase family and AKT protein (Protein Kinase B). This activation is correlated with stimulation of the transcription of genes associated with some cellular processes such as cell survival and division (Testa et al., 2000; Bowman et al., 2001; Krasinskas., 2011; Spano et al., 2011).

EGFR level is related to various human tumors such as head, neck, colon, brain glioblastomas, lung, breast, renal and ovarian (Dassonville et al., 1993; Kunkel et al., 1996; Zhang et al., 1998; Etienne et al., 1999). This suggested that blocking EGFR activation would be a logical target in treatment of different cancers. It has been shown that antisense oligonucleotides against growth factors or proto-oncogenes are able to inhibit human cancer proliferation

(Normanno et al., 1996; Witters et al., 1999; Mailik and Roy, 2011; Nourazarian et al., 2012a; 2012b). Antisense oligonucleic acid can bind to specific complementary mRNA and inhibit or decrease protein expression (Witters et al., 1999).

Colorectal cancer is the second leading cause of cancer mortality in the Western Societies and represents the third most common type of cancer in worldwide (Greenlee et al., 2000; Siegel et al., 2012). Metastatic in colorectal cancer has poor response to conservative chemotherapeutics in clinical trials (Prewett et al., 2002). Thus antisense therapy offers one approach to colon cancer treatment. EGFR has high expression in colon cancer, associated with Src activation and multiple signaling pathways such STAT5b Kopetz (2007). Also, there is a crosstalk association between STAT5 signaling and MAPK pathway in colorectal cancer (Mao et al., 1997).

We previously demonstrated that the EGFR and c-Src antisense oligonucleotide nanoparticles combination decrease HT29 cell lines proliferation (Nourazarian et al., 2012b). In this report, we have studied the in vitro activity of EGFR with nanoparticles encapsulating antisense related with its dependent downstream genes using human colon cancer (HT29).

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## Materials and Methods

### Cell culture and oligonucleotide nanoparticles transfection

The human colon cancer cell line HT29 (Pasteur Institute Cell Bank of Iran) was grown as monolayer in 25 cm<sup>2</sup> flask (Orange Scientific) in RPMI-1640 (Roswell Park Memorial Institute 1640) medium (Gibco, invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco, invitrogen), penicilline (100 Unit/ml) (Gibco, invitrogen) and streptomycin (100 µg/ml) (Gibco, invitrogen) and placed in incubator at 37°C and 5%CO<sub>2</sub> in a humidified environment. Antisense oligonucleotide nanoparticles transfected to cells as described previously (Nourazarian et al., 2012a; 2012b). Briefly, FITC-labeled EGFR antisense oligodeoxynucleotide (5'-TTTCTTTTCCCTCCAGAGCCCG-3') (Eurofins MWG Operon; Germany) incubated with PAMAM nanoparticles solution (QIAGEN; USA) to form complex. Then the solution was transferred to cell wells and incubated for 5 hours at 37°C, 5%CO<sub>2</sub> in incubator.

### MTT assay

To determine the effect of EGFR antisense oligonucleotide nanoparticles on the proliferation of the human colon cancer cells (HT29) MTT assay was performed as described previously (Nourazarian et al., 2012a). Briefly HT29 cells were trypsinized from T-flask and 15,000 viable cells seeded per well in 96-well plate and incubated. After exposure time, the cells were treated with EGFR antisense polymers or polymer and antisense alone for control for 5 hours. Then the media was removed and the cells incubated more for 24, 48 and 72 hours for MTT assay. Each experiment was repeated in triplicate format, and results were expressed as means±SEM.

### Cell uptake of the EGFR antisense nanoparticles

To evaluate the cellular FITC-labeled EGFR antisense transfection delivery, fluorescence microscopy (Olympus BX61, Olympus Inc.) was performed. For this aim HT29 cells were incubated with EGFR antisense FITC-labeled encapsulated with PAMAM for 5 hours, then washed with PBS and inspect with fluorescence microscope. Also we used FACS flow cytometry (BD, USA) to confirm data.

### Detection of EGFR antisense nanoparticles effect on the related genes expression

After 48 hours transfection of cells with EGFR nanoparticles, total RNA was extracted by Cinagene Kit based on the manufacturer's instruction (RNX-Plus Solution, SinaClon, Iran). After purification from DNA contaminations, the quality and RNA concentration were determined by detecting light absorbance at 260 (at the ratio of A260/A280) by nanodrop (NanoDrop- ND-1000). cDNA synthase kit (Qiagene) was used for the cDNA synthesis. To characterize the EGFR antisense nanoparticles effect on the related genes expression, real-time PCR with iCycler IQ5 Multicolor Real-time PCR Detection System (Bio-Rad, USA) was used. For various mRNA, first-strand cDNA was amplified using STAT5 (F 5' GCCAAGCATGGGACTCAGTAG3' and R5' TGGGTGGCCTTAATGTTCTCC3', P53 (F

5'-TCAACAAGATGTTTTGCCAACTG-3' and R 5'-ATGTGCTGTGACTGCTTGTAGATG-3'), c-Src (F 5'- ACCACCTTTGTGGCCCTCTATG-3' and R5'-GCCACC AGTCTC CCTCTGTGTT-3'), MAPK1 (F 5'-GGATGTGGTGTATTGGAAAGAG-3' and R5'-AGCAGAGACGCAGAATGAC-3') and EGFR (F5'-GGAGAACTGCCAGAACTGACC-3' and R5'-GCCTGCAGCACACTGGTTG-3'). GAPDH 5' AAGC TCATTTCTGGTATGACAACG3' and R5' TCTTCCTC TTGTGCTCTTGCTGG-3') was used for housekeeping genes. Each experiment was repeated in triplicate format, and results were expressed as means±SEM.

### EGFR protein expression detection

Western blot analysis was used to evaluate the amount of EGFR expression in the HT29 cells, after antisense therapy. For this aim, protein lysates was prepared from HT9 cells after 48 hours transfection as described previously (Nourazarian et al., 2012a; 2012b). For Western blot analysis we performed as Chemiluminescent Immunodetection kit based on the manufacturer's instruction (Western Breeze; Invitrogen).

### Statistical analysis

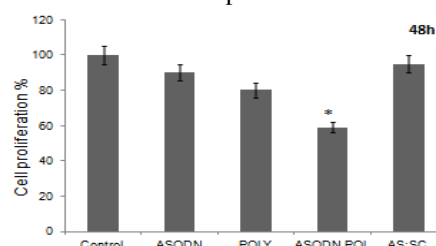
Statistical analysis was performed using by SPSS™ 16.0 software and ANOVA test was used to compare between groups. Data were expressed as Mean±SEM. The results were considered significant in case of P<0.05.

## Results

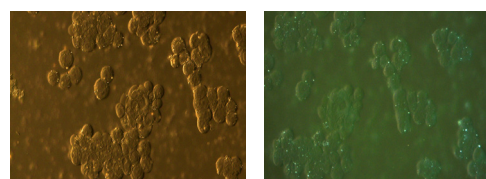
### HT29 cell proliferation

MTT assay was performed to measure the effect of EGFR antisense encapsulating with PAMAM on the human colon cancer (HT29) cells proliferation. As Figure 1 shows, after 48 hours EGFR antisense nanoparticles have most effective rather than control groups on cell growth inhibition (P<0.05). However, antisense or PAMAM nanoparticles alone don't have significant effect on cancer cell lines proliferation (P>0.05).

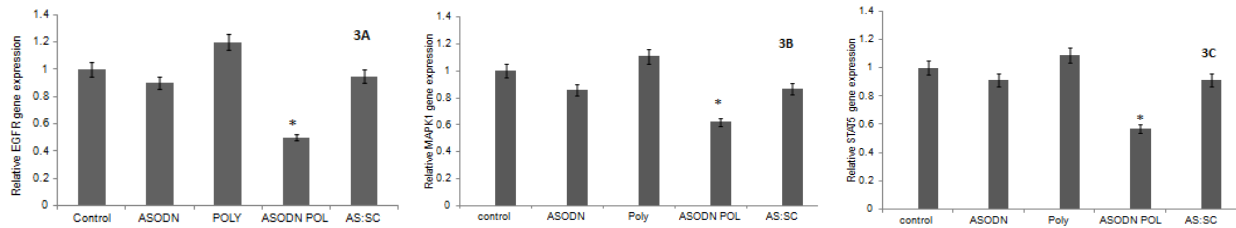
All the data was compared with control groups



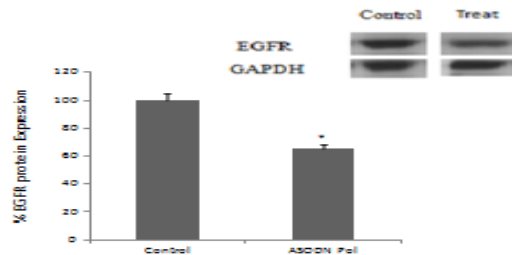
**Figure 1. MTT assay was Performed the Effect of EGFR Antisense Oligonucleotides Encapsulated with PAMAM on the HT29 after 48 Hours Treatment**



**Figure 2. HT29 Cells Treatment with EGFR Antisense FITC-labeled Encapsulated with PAMAM. Light microscopy (left) and fluorescent microscopy image (right)**



**Figure 3. Real Time PCR Analysis.** All of data were normalized to GAPDH gene expression, 3A, 3B, 3C, results related to decrease in EGFR, MAPK1 and STAT 5 after antisense treatment respectively



**Figure 4. Western Blot Analysis for the EGFR Protein Expression in HT29 Cells.** EGFR protein Expression compared with control group

through ANOVA tests. Note: significantly different from transfected cells and controls ( $P < 0.05$ )\*. Data are represented Mean  $\pm$  SEM. The experiments were repeated as triplicate. Abbreviations: EGFR only, EGFR antisense oligonucleotide without PAMAM; POL, PAMAM nanoparticles, EGFR Poly, complex of antisense oligonucleotide with PAMAM. AS:Sc Scrambled antisense oligonucleotide.

#### Intercellular delivery of EGFR encapsulated with PAMAM

Microscopic fluorescence results specified EGFR antisense encapsulated FITC-labeled successfully nanoparticles uptake to HT29 cells (Figure 2) also our data shows the complex entered more than 78% cells.

#### EGFR antisense nanoparticles effects on the related genes expression

We studied the level expression of EGFR and EGFR downstream genes (MAPK1 and STAT5), after antisense therapy on human colon cancer cell lines. The real time PCR results indicate that EGFR antisense encapsulated with PAMAM nanoparticles significantly down-regulated the level of MAPK, EGFR and STAT5 expression in HT29 cell lines ( $P < 0.05$ ) comparing with controls (Figure 3).

#### EGFR protein expression was down regulated

The western blot analyses data show that the level protein expression of EGFR was significantly decreased via EGFR antisense encapsulated with PAMAM treatment compare with housekeeping gene's protein expression as control group ( $P < 0.05$ ) (Figure 4).

## Discussion

The role of EGFR in colon cancer growth and progression has been described in several studies (Kopetz, 2007). Previous studies have been shown that down-regulation of EGFR expression in human cancer cells decrease via EGFR antisense (Rajagopal et al., 1995;

Rubin et al., 1997).

We recently established that the EGFR antisense oligonucleotide encapsulated with PAMAM nanoparticles could reduce human colon cancer cell line (HT29) proliferation and effects on signaling pathway (Nourazarian et al., 2012b). In this study, we have demonstrated that the expression of EGFR, MAPK1 and STAT5 can be significantly reduced in human HT-29 colon cancer cells through EGFR antisense oligonucleotides inhibition. As antisense oligonucleotides have anionic charge and unable to pass into the cells, thus delivery systems should be employed for antisense transfection. For this aim we used polyamidoamin polymers, because they are highly effective cationic delivery vehicles and can form PAMAM-antisense complex by electrostatic interaction, as well as they transfer the antisense into nucleus of target cells and have ability to protect the antisense from digestion in cytoplasm (Eichmanet et al., 2000; Baker et al., 2004; Orava et al., 2010; Wang et al., 2010).

To determine whether EGFR antisense can decrease the HT-29 colon cancer cell line proliferation, we used cell viability assay. The results of MTT assay showed that PAMAM nanoparticle encapsulating EGFR antisense has significantly decreased the growth of HT29 human colon cancer cells. It confirms the studies which showed that EGFR involves in various downstream signaling pathways such as MAPK1 and STAT5 (Xiong et al., 2009; Park et al., 2011). The roles of STAT genes have been identified in many cancers (Xiong et al., 2009). In addition, EGFR and STAT5 have important role in the survival of cancer cells. Moreover, STAT5b has a role in colon cancer progression, migration, invasion, proliferation, cell cycling and gene expression (Xiong et al., 2009). There are several mechanisms that STAT5 can mediate intracellular EGFR signaling by direct activation of STATs by EGFR binding or indirect activation of STATs via Src-mediated EGFR signaling (Kelly et al., 2007).

Our real time PCR results demonstrated that expression of STAT5b gene has decreased following EGFR inhibition through EGFR reduction by antisense encapsulated nanoparticles. Our results demonstrated PAMAM nanoparticle encapsulating EGFR antisense had more than 40-50% inhibition effects on the EGFR, MAPK1 and STAT5 expression.

The MAPK pathways are downstream of many growth factor receptors, including epidermal growth factor. Over expression of this receptor are detected in colorectal cancer (Fang and Richardson, 2005). The important role of this pathway in cell cycle regulation and colon cancer development has been determined (Chapnick et al., 2011).



EGFR inhibition causes reduction of MAPK expression that is specified in other investigation (Kumar et al., 2010). Our results showed a significant decrease in the amount of MAPK1 gene expression after real time PCR trail.

PAMAM nanoparticles alone have increasable effects on the above genes expression that suggested PAMAM nanoparticles have an unexpected effect which needs more investigation (Nakhlband et al., 2010).

We suggest that further studies should attempt to colon cancer antisense therapy in vivo experiments on laboratory animals to investigate the efficacy of antisense therapy in biological environments. That this time, we will approach to clinical trials.

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