EGFR antisense RNA blocks expression of the epidermal growth factor receptor and partially reverse the malignant phenotype of human breast cancer MDA-MB-231 cells

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ABSTRACT

The effects of human EGFR to the malignant phenotype of human breast cancer cell line MDA-MB-231 were investigated experimentally. A retroviral vector containing a 5'1350bp fragment of the human EGFR cDNA in the antisense orientation was transfected into targeted cells by lipofectamine. The effects on cell proliferation, cell cycle and adherent ability to extracellular matrix (ECM) components were studied after the expression of antisense transcripts to EGFR 5'1350bp fragment in target cells. *In vitro* studies showed that the growth ability of the transfected cells was partially inhibited in comparison to parental cells and to cells transfected with the plasmid containing the neomycin resistance gene only. It was found that EGF (10ng/ml) had an augmentation effect on the growth of transfected MDA-AS10 cells but not MDA-MB-231 cells. Flow cytometric analysis showed that the cell cycle of the transfected cells was abnormal with a decrease of cells in G₂/M and S phases and an increase of cells in G₁ phase, indicating a blockage in phase G₁. Immunofluorescence of EGFR expression in transfectants stained with an anti-EGFR antibody was decreased and their growth in soft agarose was also severely impaired. The transfected cells showed less adherence to laminin (LN) and fibronectin (FN). In short, EGFR antisense RNA decreases the expression of EGFR on MDA-MB-231 cells and partially reverses their malignant phenotype as well.

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Key words: *EGFR, antisense RNA, human breast cancer cells, gene transfection.*

INTRODUCTION

Epidermal growth factor is a potent mitogen controlling the growth of various types of cells through their specific receptor[1]. The epidermal growth factor receptor (EGFR) has an intrinsic tyrosine kinase activity and is able to, upon ligand activation, phosphorylate itself and cellular substrates on tyrosine[2]. In addition to serving a normal physiological role, the EGFR has been consistently linked to cell transformation, both in experimental models and in human tumors. The EGFR gene is usually amplified and overexpressed in human tumors of ectodermal origin, suggesting an important role of EGFR in the development of these neoplasia[3]. Furthermore, EGFR overexpression is positively related with poor prognosis and high metastasis in human breast cancer[4]. This made EGFR a suitable target of tumor gene therapy. Studies of EGFR on human colon cancer cells, human rhabdomyosarcoma cells, human transfected KB cells and human hepatocarcinoma cells have been reported[5-8]. To directly investigate the role of EGFR overexpression in human breast tumor, we tried to decrease expression of EGFR in human breast cancer MDA-MB-231 cells. This paper reports that expression of antisense EGFR RNA results in a suppression of the transformed phenotype of MDA-MB-231 cells and the degree of suppression is positively correlated to the decrease in expression of EGFR. In addition, expression of EGFR antisense RNA enables EGF to stimulate the growth of transfected MDA-MB-231 cells.

MATERIALS AND METHODS

Materials and cell culture

The human breast cancer MDA-MB-231 cells were maintained in 25 cm² culture flasks at 37°C in a humidified atmosphere of 5% CO₂ in RPMI1640 medium supplemented with 15% new born bovine serum. Retroviral packaging cell line PA317 was cultured in DMEM medium supplemented with 15% new born calf serum.

Construction of antisense EGFR expression vector

Cleaved from pXEGFR (XbaI, HindIII), 4kb EGFR cDNA was ligated into XbaI and HindIII cloning sites of the vector pUC18 and designated as pUC18-EGFR. A 1350bp EGFR cDNA fragment was cleaved from pUC18-EGFR (BamHI) and ligated to retrovirus vector pLXSN. The recombinant vector pLXSN-AE5' was used to transform *E.coli* DH5 α , which were subsequently plated onto ampicillin-containing agar plates. Resistant colonies were selected and then expanded for plasmid DNA minipreparations. The orientation of the inserts was determined by restriction mapping.

Antisense transfection

Exponentially growing cells were washed with serum-free medium. The mixture of 200 μ l medium containing 10 μ l of lipofectamine (2 mg/ml GIBCO-BRL) and 4 μ g of antisense EGFR

expression vector DNA were added to the cells. Eight hours later, equal volume of culture medium was supplemented to cells. Twenty hours later, the cells were washed with serum-free medium and fed with fresh medium containing 15% new born calf serum. Forty-eight hours later, 400 $\mu\text{g/ml}$ G418 was added to the medium for selection. After 2 ~ 3 wk, the G418 resistant clones were propagated in tissue culture. Antisense expressing vector pLXSN-AE5' transfected cells were designated as MDA-AS8 and MDA-AS10. Retrovirus expression vector pLXSN transfected cells were designated as MDA-pL.

PCR reaction

PCR was performed on genomic DNA extracted from parental and transfected cells. DNA concentration was determined by means of A260, and 0.5 μg was used for each PCR reaction in 50 μl final volume. The following primers were used to detect 433bp neo gene fragment: direct 5' -TCC ATC ATG GCT GAT GCA ATG CGG C-3' and reverse 5' -GAT AGA AGG CGA TGC GCT GCG AAT CG-3'. PCR reaction was performed with a 56 °C annealing temperature and 30 amplification cycles.

RNA isolation and reverse transcription-PCR

Total RNA was isolated from control cells and antisense clone cells according to method in "Molecular Cloning" [9]. RNA concentration was determined by means of A260, and 5 μg RNA were reversely transcribed using 200 ng reverse primers, 30 units of AMV reverse transcriptase, 20 units Rnasin (Promega) and 2.5 mM dNTP in a volume of 20 μl . The mixture was incubated at 42 °C water bath for 1 h, 2 μl reverse transcription products were used for following PCR reaction with the two primers: direct 5'-TTT CTG GCA GTT CTC CTC TC-3' and reverse 5'-CGC AGT TGG GCA CTT T-3'.

Cell proliferation assay

The cell count method described previously[10] was used to examine the proliferation ability.

Flow cytometric study

Cells from subconfluent culture were resuspended, washed with PBS supplemented with 1% BSA (PBS-BSA), and incubated for 30 min on ice with a 1:40 dilution of anti-EGFR monoclonal antibody clone 528 (Oncogene Science, Uniondale, NY) in PBS-BSA. After washing with cold PBS-BSA, cells were incubated for 30 min on ice with a 1:50 dilution of FITC-conjugated goat antimouse immunoglobulin antiserum (Bonding, Beijing). Cells were again washed and resuspended in 1ml PBS, followed by analysis with a FACScan flow cytometer (Becton Dickinson). Each data shown was from 3 parallel experiments.

Growth in soft agarose

To examine anchorage-independent growth, 5×10^3 (MDA-MB-231, MDA-pLSXN, MDA-AS8 and MDA-AS10) cells were plated in triplicate in 24 well culture plates with 1 ml of culture medium, supplemented with 0.35% agarose. The cells in the dishes were incubated for 21 days at 37°C and then colonies were counted under microscope.

Cell cycle analysis

Cells from subconfluent culture were resuspended, washed with PBS and fixed in 70% ethanol for 30 min. Cells were washed, resuspended in PBS with RNase A (100 $\mu\text{g/ml}$) and incubated in 30°C for 30 min. Five microlitre propidium iodide (50 $\mu\text{g/ml}$, Sigma) were added to 10^6 cells for 20 min at 4°C without light. The total DNA content per cell was assessed by analysis of fluorescence using a FACScan flow cytometer (Becton Dickinson).

Adherence to different extracellular matrix proteins

Cells (2×10^5 /well) were seeded on 24-well plates precoated with either laminin (10 mg/ml in PBS) or fibronectin (10 mg/ml in PBS) for 1 h at 37°C and 1% BSA for 2 h at room temperature. After 20 min, 40 min and 1 h incubation respectively at 37°C, the number of attached and unattached cells were counted.

RESULTS

Construction of the expression vector

The sketch map of EGFR 5' 1350bp fragment inserted into pLXSN in antisense orientation was shown in Fig 1. Digested by EcoRI, the sense recombined plasmid was cleaved into 495bp and 6729bp fragments; the antisense recombined plasmid was cleaved into 887bp and 6337bp fragments (Fig 2).

Fig 1. Sketch map of EGFR 5' 1350bp cDNA inserted into retroviral vector pLXSN in antisense orientation.

Amp and neo are selectable markers of vector in bacterium and mammalian cells respectively. The 1350bp fragment was inserted into BamHI site in antisense orientation. Ψ^+ indicates the extended retroviral packaging signal.

Fig 2. Restriction map of EGFR 5' 1350bp inserted in pLXSN.

1. pLXSN-SE5'/EcoRI;
2. λ DNA/HindIII+EcoRI;
3. pLXSN-AE5'/EcoRI.

Integration and expression of antisense fragment

The presence of neo gene fragment in genomic DNA of different antisense transfectants but not parental MDA-MB-231 cells was shown by PCR (Fig 3). The presence of antisense transcripts in cellular RNA of MDA-AS8 and MDA-AS10 cells was shown by RT-PCR (Fig 4).

Effect of antisense EGFR RNA expression on the expression of cell surface EGFR

Cytofluorimetric analysis of membrane EGFR (Tab 1) showed a decreased ex-

pression in AS transfectants when compared to MDA-MB-231 or MDA-pL cells.

Fig 3. PCR analysis of neo gene fragment

A. λ DNA/HindIII

1. MDA-MB-231 cells;

2. pLXSN transfected cells;

3-7. pLXSN-AE5' transfectants.

Fig 4. RT-PCR analysis of cellular RNA

A. λ DNA/HindIII+EcoRI

1. MDA-MB-231 cells;

2. MDA-AS8 cells;

3. MDA-AS10 cells.

Tab 1. Effect of antisense EGFR RNA on cell surface EGFR expression

Cells	Positive rate (%)	Meanfluorescence
MD A- MB-231	96.3	27.7
MD A-pL	93.5	24.62
MD A-AS8	85.1	18.83*
MD A-AS10	75.5	16.66*

* compared to MDA-MB-231 group, $P < 0.01$

Effect of antisense EGFR RNA expression on cell proliferation

The proliferation ability of AS transfectants (Fig 5) was severely impaired. Plasmid pLXSN transfected cells had a similar growth rate comparable to that of the parental MDA-MB-231 cells. The proliferation ability was positively related to the expression level of membrane EGFR.

Growth effect of EGF on MDA-AS10 cells

MDA-MB-231 is a breast epithelial cell line which possesses a large amount of epidermal growth factor (EGF) receptor on the cell surface but does not respond to EGF. MDA-AS10 is an antisense cell clone transfected with recombined plasmid pLXSN-AE5'. The proliferation of MDA-AS10 was greatly reduced as shown in Fig 5. When cultured with EGF (10 ng/ml), MDA-MB-231 cells had the same proliferative rate as before, but proliferation of MDA-AS10 cells was enhanced rapidly as compared to that without EGF (Fig 6).

Cell cycle analysis

After stable expression of antisense EGFR RNA, the proportion of cells in the

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G_0/G_1 phase increased, and concurrently, the proportion of cells in the S phase decreased. It indicated that the cell cycle was blocked in G_1 phase (Tab 2).

Fig 5. Proliferation curve of AS transfectants, pLXSN transfectants and parental MDA cells.

Fig 6. Growth effect of EGF on MDA-MB-231 and MDA-AS10 cells

Tab 2. Distribution of cells in various phases of cell cycle

Cells	G_0/G_1	S	G_2/M	PI
MDA-MB-231	53	29	18	0.47
MDA-pL	48	24	28	0.52
MDA-AS8	64	19	16	0.35*
MDA-AS10	67	17	12	0.29*

* compared to MDA-MB-231 group, $P < 0.01$

Effect of antisense EGFR RNA expression on growth in soft agarose

Compared with parental MDA-MB-231 cells and neo control MDA-pL cells, antisense EGFR RNA expression inhibited the growth of transfectants in soft agarose (Tab 3).

Tab 3. Effect of antisense EGFR RNA on growth in soft agarose

Cells	Cells/plate	Number of colonies			$\bar{X} \pm SD$
		1	2	3	
MDA-MB-231	5000	161	154	147	154 $\pm$ 7
MDA-pL	5000	176	145	135	152 $\pm$ 21
MDA-AS8	5000	121	96	79	99 $\pm$ 21*
MDA-AS10	5000	68	43	54	55 $\pm$ 12*

* compared to MDA group, $P < 0.01$ *Ability of adherence to LN and FN was inhibited*

At different time point, after the stable expression of antisense EGFR RNA, the ability of adherence of transfectants to LN and FN was respectively reduced, especially in the case of MDA-AS10 cells (Fig 7 and 8).

Fig 7. Effect of antisense EGFR RNA expression on adherence to laminin **Fig 8.** Effect of antisense EGFR RNA expression on adherence to fibronectin

DISCUSSION

EGFR has been implicated in the malignant behavior of many tumor types and its overexpression is positively related with poor prognosis and high metastasis in human breast cancer. Antisense technology is an important method to study the function of a specific gene[11]. Overexpression of a gene can be specifically inhibited by antisense RNA followed by studies on phenotype changes.

This study demonstrated that it is possible to downregulate the expression of EGFR in human breast cancer cells and decrease the proliferation ability of MDA-MB-231 cells by expression of antisense EGFR RNA. Moreover, it could be seen that the proliferation rate of different clone cells is positively related to the expression of EGFR on cell surface. Determined by Flow Cytometric studies, we found that in clone such as AS10 cells which possess low number of EGFR, the proliferation ability was more inhibited. This suggests that EGFR may play an important role in malignant proliferation of MDA-MB-231 cells.

It is generally acknowledged that the uncontrolled proliferation of tumor cells is related to the enhanced ability to synthesize DNA. Cell cycle analysis indicated that the two cell clones transfected with antisense EGFR had more cells in G₀/G₁ phases and less cells in S + G₂/M phases, a result which implies that the malignant proliferation of transfected MDA-MB-231 cells were partially inhibited at G₁ phase.

MDA-MB-231 cells do not response to EGF (10 ng/ml) thought it has high-level EGFR[12]. However, in our studies, after being transfected with antisense EGFR, MDA-AS10 cells which exhibited low level of EGFR expression were able to response to EGF (10ng/ml). In human breast cancer cell line ZR75-1, Valverius et al found that there appears to be a threshold level of EGFR expression for the alteration in EGF responsiveness[13]. In addition, Davidson et al reported a tendency for EGF responsiveness only in those lines with fewer than 7×10^4 EGFR per cell, whereas cell lines with higher receptor numbers were generally unresponsive[14]. The mechanism of the correlation between EGF responsiveness and level of EGFR expression remains to be solved.

The invasion and metastasis of tumor cells is a complex process. Tumor cell invasion has been considered as a three-step process involving tumor cell adhesion, release of extracellular matrix (ECM) degrading enzymes and tumor cell motility[15]. Adhesion of tumor cells to ECM is an important step in the process of invasion. LN and FN are noncollagenous accessory glycoproteins of extracellular matrixes. It has been reported that adhesion of tumor cells to LN and FN had important influence on tumor metastasis[16]. It is interesting to note that after the stable expression of EGFR antisense RNA and the down-expression of EGFR on cell surface, the adhesion of transfected cells to LN and FN had been decreased respectively. This suggests that EGFR on MDA-MB-231 cells may influence cell invasion and tumor metastasis as well.

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