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RNAI MEDIATED GENE SILENCING OF EIF3A: A POSSIBLE SOLUTION TO CONTROL BREAST CANCER

ASIYA PARVIN ALLAUDEEN[a], C. MANJULA[a], N. JOTHI[a], C. VIGNESH KUMAR[a], B. INDRAMANI[a], V. SHUNMUGA PRIYA[a], E. BHAKKIYALAKSHMI[b], K. M. RAMKUMAR[b], P. RAJAGURU[a*]

[a]Department of Biotechnology, Anna University BIT Campus, Tiruchirappalli, Tamil Nadu, India, [b]Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur 603203, Tamil Nadu, India
Email: rajaguru62@gmail.com

ABSTRACT

Objective: The eukaryotic translational initiation factor 3A (eIF3A) is reported to be over expressed in most breast cancer cells. In the present study, our aim is to suppress the over expression of eIF3A in human breast cancer MCF-7 cell line using gene silencing technique (RNA interference (RNAi)).

Methods: The artificial microRNA (amiRNA) targeting eIF3A gene was constructed by incorporating short interference RNA (siRNA) sequences against eIF3A gene into endogenous microRNA30 (miR-30) and cloned into pcDNA3.1 vector. The amiRNA containing plasmid was then transfected into MCF-7 cell line and the expression of eIF3A was examined by RT-PCR. The cytotoxicity of plasmid with amiRNA targeting eIF3A on MCF–7 cells was evaluated by MTT assay.

Results: The amiRNA construct significantly inhibited eIF3A gene expression and reduce the cell viability of MCF-7 cell line.

Conclusion: The usage of modified endogenous amiRNA in vector based expression system with significant gene silencing efficiency suggests that RNAi based gene silencing method can be considered as one of the effective means to control cancer.

Keywords: Breast cancer, eIF3A, MCF-7, RNA interference, miR-30.

INTRODUCTION

Breast cancer is the most common cancer affecting women worldwide, comprising 16% of all female cancers. In 2011, an estimated 230 480 new cases of invasive breast cancers were diagnosed among women, in addition to an estimated 57 650 cases of *in situ* breast cancer and approximately 39 520 women are expected to die from breast cancer [1]. Standard cancer therapy generally combines surgery, multi-therapeutic agents and ionizing radiation [2]. Major issues concerning conventional anticancer chemotherapy are the side effects induced by the non-specific targeting of both normal and cancer cells and the emergence of drug-resistance in cancer cells [3]. Prevention and treatment of breast cancer require a better understanding of the molecular mechanisms underlying the progression of breast cancer. Currently, there has been growing interest in the use of RNAi technology to regulate gene expression at different levels.

One important regulation of gene expression in eukaryotes occurs at the level of mRNA translation, specifically at the levels of initiation of translation. Eukaryotic initiation factors (eIFs) have been reported to play an important role in translation initiation, which controls cell growth and proliferation [4]. It has been well documented that altering the expression level or the function of eIFs influences the synthesis of some proteins and consequently causes abnormal cell growth and malignant transformation [5]. P170, the largest putative subunit of eIF3, has been found in elevated levels in human breast, cervical, esophageal, and lung cancers, suggesting that p170 may have a potential role in malignant transformation and/or cell growth control [6]. The expression of eIF3 subunits has been found altered in various human tumors and their altered expression may cause cancer and affect prognosis [7]. It has been well documented that eIF3 has been over expressed in numerous cancers [8-10]. Normally it binds to S6K1 and remains inactive. Upon stimulation either by mitogens, growth factors or drugs, mTOR/Raptor complex gets activated and in turn binds and phosphorylates S6K1, leading to its dissociation from eIF3. The phosphorylated S6K1 is further phosphorylated by PDK1 which activates the S6K1 kinase which in turn phosphorylates eIF4B, S6 and other protein targets [11]. The 20S Proteosome specifically cleaves eIF3a, the largest subunit of

eIF3, and differentially affects translation of different mRNAs and these findings clearly establish that eIF3 is a pivotal player in translational control [12].

The RNAi technology is an effective gene silencing method [13]. The RNAi pathway is activated by a double-stranded RNA (dsRNA) "trigger," which is then processed into short, 21-to 22-nucleotide dsRNAs, referred to a siRNAs by the cellular enzyme Dicer. The siRNAs become incorporated into the RNA induced silencing complex (RISC), in which the siRNA serves as a guide to identify the homologous mRNA for destruction. In mammalian cells, dsRNA longer than 30 nucleotides triggers the interferon pathway, activating RNA-dependent protein kinase (PKR) and 2'-5'-oligoadenylate synthetase rather than RNAi.

However, shorter siRNAs exogenously introduced into mammalian cells or transcribed endogenously bypass the Dicer step and directly activate homologous mRNA degradation without initiating the interferon response [14]. There is a possibility of knockdown of unknown target gene without RNAi processing mechanism by using siRNA in microRNA (miRNA). The use of miRNA has added advantage that it eliminates an immune response. miR-30 is a small non-coding RNA that regulates gene expression of eukaryotic cells. miRNAs is transcribed as ~70 nucleotide stem-loop precursor and subsequently processed by the Dicer enzyme to give a mature ~22 nucleotide products. The mature miRNA sequences are thought to have regulatory roles through complementarities to target mRNA [15].

In this study, artificial miRNA (amiRNA) expressing-vector targeting eIF3A gene was synthesized and transfected into human breast cancer cell line MCF-7. The levels of eIF3A mRNA expression and the cytotoxic profile in MCF-7 cells were investigated.

MATERIALS AND METHODS

Designing and synthesis of amiRNA

The target eIF3A mRNA sequence was retrieved from NCBI (Reference Sequence: NM_003750.2) and the target siRNA sequences were designed by using online siRNA Target Finder, (Ambion Inc; Dharmacon; Genescript). After verifying for homology

using BLAST, they were incorporated in the stem regions of miR-30 hairpins. The resulting artificial miRNAs was aligned and analyzed for the secondary structure using the web based RNA structure prediction tool. Based on the secondary structures the target sequence was selected coding regions 1408–1426 (5'–CCGGAATTGCAGCAGTATG–3').

To develop amiRNA construct, 91-nucleotide long modified miR-30 hairpins for the target mRNAs, flanked by artificial sequences were chemically synthesized (Sigma-Aldrich, USA). Sense and antisense strands of the target siRNAs were included in the stem region of the artificial miR-30 hairpins. Since the natural miR-30 shape is essential for Drosha recognition and processing, loop sequences of the natural miR-30 and the distal A/C mismatch are included in the artificial miR-30 hairpins.

Two universal primers (32 nt) were designed and synthesized (Sigma-Aldrich, USA). The 5' primer includes sequence of 5' flanking region of miR-30, *BamH*1 and *SpeI* restriction sites and a 13 nt complementary overlap to the artificial miR-30 at its 3' end. The 3' primer contains reverse complement sequence of 3' flanking region of miR-30, *XbaI* restriction site and a 21 complementary overlap to the artificial miR-30 at its 3' end (table 1). The artificial miRNAs were synthesized by PCR using the following PCR program: initial denaturation temperature 94 for 5 min, loop of 30 cycles of 15 sec with annealing at 54 °C, 30 sec and a final extension for 10 min at 72 °C. The PCR products were resolved on a 1% agarose gel, extracted and purified using gel elution kit (Invitrogen, USA).

Prediction of amiRNA secondary structure

The secondary structure of amiRNA sequences was analyzed for its stability using the web based tool (http://www. ncrna. org/centroidfold/).

Construction of amiRNA expressing vector targeting eIF3A gene

The purified PCR products were double restricted with *BamH*1/*XbaI* and cloned into similarly treated pcDNA 3.1 plasmid vector which contains GFP expression sequences, transfected into (*E. coli)* DH5-α cells and stored. After selective culture, recombined plasmid was extracted from DH5-α using Medipreps DNA purification system (Qiagen).

Conformation of transformed clones by colony PCR

Colony PCR was performed to confirm the presence of transformed clones in the selected colonies (GeNet Bio, Bangalore). The PCR program was performed using 5 μg of DNA template and forward

primer 5' GGATCCACTAGTTATTGCTCTCGAGAGTGAGC 3' and reverse primer 5'TCTAGACCCTTGAATTCCTCGGCAGTGGGCAG3' (Sigma-Aldrich, USA) with initial denaturation at 94 °C for 5 min, loop of 30 cycles of 30 sec with annealing at 54 °C for 30 sec and a final extension at 72 °C for 10 min. The program was set in a thermo cycler and the template was amplified. The amplified products were loaded in 1 % agarose gel and positive clones were selected.

Cell culture and transfection of recombinant plasmid

The human breast adenocarcinoma cell line (MCF-7) obtained from NCCS was maintained in DMEM Medium (GIBCO, Invitrogen) supplemented with 10% heat inactivated FBS, 2 mM L-glutamine (GIBCO, Invitrogen) and 100 U/ml penicillin-streptomycin (GIBCO). The vectors containing cloned DNA sequence for amiRNA was maintained in (*E. coli*) DH5-α cells. The plasmid DNA was isolated, gel purified, mixed with lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions and used for the transfection experiments.

Reverse transcriptase polymerase chain reaction (RT-PCR)

To check the silencing efficiency, MCF-7 (1×10^4cells/well) were plated onto 12 well plates and allowed to grow in 2 ml of complete medium for 24 h (until 70–80% confluency). Then the cells were transfected with amiRNA carrying vectors. The cells were harvested after 72 h and mRNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA). Approximately, 5 μg of total RNA from each sample was converted into complementary DNA using a commercial RT-PCR kit (Invitrogen, USA).

The resultant cDNAs were used in PCR reactions with gene specific primers and the products were gel electrophoresed. The PCR primers used to amplify eIF3A: forward 5'GTTGGATGATGATCGAGGAC3', and reverse 5'GTCCCTGTCGTCTC TTAGAT3', GAPDH: forward 5-GACCTGCCGTCTAGAAAAAC-3 and reverse 5-TTGAAGTCAGAGGAGACCAC-3.

The optimum numbers of cycles were determined. Initial denaturation was performed at 94 °C for 1 min. The subsequent cycling programs consisted of denaturation at 95 °C for 30 sec, annealing temperature 59.8 °C for 30 sec, and extension at 72 °C for 30 sec, followed by a final extension at 72 °C for 10 min. The products were separated on a 1.8 % agarose gel, stained with Ethidium bromide, and photographed using a gel imaging system (Bio Rad, Hercules, CA). PCR reactions in which the first strand cDNA were omitted served as negative controls. To avoid technical error, each PCR experiment was repeated three times.

Table 1: amiRNA and PCR templates

Ambion–400	Dharmacon–85	Genescript–10
AGCAGCCTGCTCTGGATGT	GAGAAGGGCAGAAGAACA	
GATACACGAACCAATTATG	GGAGAAACATGGATGATGA	GAGCTACGAGAGTATCAGG
AGGAGGGGTTATACCAGTA	GGATGGAGAGAGAAAGAAA	GAACGAGAACGGCGTAGAG
TATTCAAACTCCTGAGAGT	CGTGAATGGGACAGAGAAA	GAGCCAAACGTTTGGAAGA
ACAATTCTAGAGTAGAGCG	AGAAAGGGACAGAGAGAGA	GTTCTTGGAGACGTGCTGA
TTCCGTAAACTGTGTGACA	GCAGAGAAGGGCAGAAGAA	
ACTGTGTGACAATTTGAGA	CGGCGTGATCTAAGAGAAA	
TCAATCTTAATAATCCAGA	GGAAGATATTCACGGGCTA	
ACCTCAGTTGATGGCAAAT	GCAACAAGAGGAAGAAAGA	
ATTACTATAACAAAGTCTC	GAGCAAAACGCGAGGAAGA	
CAAAGTCTCAACTGTGTTT	GGAAGAGCTACGAGAGTAT	
ATGAGAAAGAATCTCACAC		
TCTCACACAAGATGAGATG		
GATGAGATGCAAAGAATA		
TTGGCCTTATTAATGATAT		
TGATATGGTCAGATTTAAT		
TGTACTACAATATGTTGTC		
TATGTTGTCCCAGAAGTGA		
CCGGAATTGCAGCAGTATG		
GAATTGGAACAGAGGGAG		
AGAGCCAAACGTTTGGAAG		
ATTCCTTTGATAAAGAGCG		
GAGGAGGAGCAGAGAAGGG		
GGGCAGAAGAACAAATGCT		
GAGAGAAGACTTGGCGATA		
GTTCTTGGAGACGTGCTGA		
ACATTGGATACGGTGGGGT		

Cytotoxicity assay

The MCF7 cells (1×10³ cells/well) were grown in a 96-well plate for 24 h and transfected with plasmids (pcDNA 3.1 containing amiRNA) using Lipofectamine 2000 (a total of 50 µl) and incubated for 24 h. The tetrazolium salt MTT solution (5 mg/ml; Sigma, USA) was added into each well and incubated at 37 ˚C for 4 h.

The media were carefully aspirated. The blue formazan product converted from MTT was dissolved by the addition of 150 µl/well of dimethyl sulfoxide (DMSO; Sigma) and optical density (OD) readings were obtained using an auto reader (Biorad) at 540 nm. Each experiment was repeated three times.

Statistical analyses

Results are expressed as mean values±SD for each experimental group. Statistical significance of differences between groups was determined by the Student's t-test with Graph Pad Quick calcs software (online t test calculator).

RESULTS

Designing of amiRNA sequence

In this study, according to the siRNA design guidelines, several siRNA targets were designed using ref|NM_003750.2| EIF3A, mRNA FASTA sequence. eIF3A siRNA targets were designed using online tools such as siRNA target finders such as ambion, dharmacon and gene script. From this 400, 85 and 10 sirna targets were obtained

from ambion, dharmacon, gene script and microsynth and from that only 27, 85 and 4 siRNA targets accordingly have got 40-50% GC content as shown in the table 1.

From these six siRNA targets sequences were selected and screened using BLAST tool for homology detection as shown in the table 2. From this target sequence 1 was selected based on high GC content and homology detection.

5'GGAUCCACUAGUUAUUGCUCUCGAGAGUGAGCGACCGGAAUUGCAG
CAGUAUGUAGUGAAGCCACAGAUGUACAUACUGCUGCAAUUCCGGCU
GCCCACUGC CGAGGAAUUCAAGGGUCUAGA3'

The secondary structure of specific single amiRNA is shown in the (fig. 1). The amiRNA construct was synthesized from SIGMA Pvt Ltd., and amplified by PCR using. Forward primer-*5'GGATCCACTAG TTATTGCTCTCGAGAGTGAGC3'* and Reverse primer–*5'TCTAG ACCCTTGAATTCCTCGGCAGTGGGCAG3'.*

Cloning of amiRNA

The amplified amiRNA was eluted from the agarose gel using the QIAGEN elution protocol. The eluted product was cloned into the pcDNA 3.1 vector. Both the amiRNA and pcDNA 3.1 were restricted by using *BamH1* and *Xba1* restriction enzymes separately and ligated using DNA ligase. Ligated product was transformed into the (*E. coli*) DH5-α on the ampicillin resistant agar plate. The transformed colonies were amplified by PCR and the positive clones were confirmed by colony PCR. The PCR product was found to be about 123 bp length as shown in the (fig. 2).

Table 2: SiRNA sequence in mRNA of eIF3A gene

Target sequence	Sense strand siRNA	Antisense strand siRNA	Position in gene sequence	GC content
1	CCGGAAUUGCAGCAGUAUG	CAUACUGCUGCAAUUCCGG	1408	47.6%
2	CUUUGCGUGGAUCUUCGCA	UGCGAAGAUCCACGCAAAG	313	45.6%
3	AGGAGGGGUUAUACCAGUA	UACUGGUAUAACCCCUCCU	347	42.9%
4	ACCAGCUCAUAUACUGCAA	AUUGCAGUAUAUGAGCUGGU	758	38.1%
5	AGAGCCAAACGUUUGGAAG	CUUCCAAACGUUUGGCUCU	2242	42.9%
6	GAUUACUGCGCAAGUGUGA	UCACACUUGCGCAGUAAUC	4914	42.9%

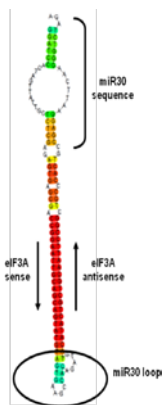Target sequence 1: Position in gene sequence: 1408 GC content: 47.6%

Fig. 1: Centroid fold structure of amiRNA (target 1) carrying target siRNA
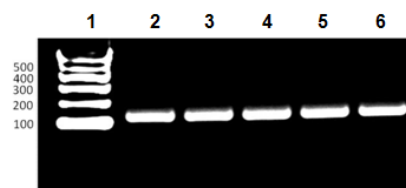

Fig. 2: Colony PCR products showing the positive clones indicating the presence of amiRNA construct. Lane 1: DNA marker; Lane 2-6; positive result shows cloned amiRNA in pCDNA
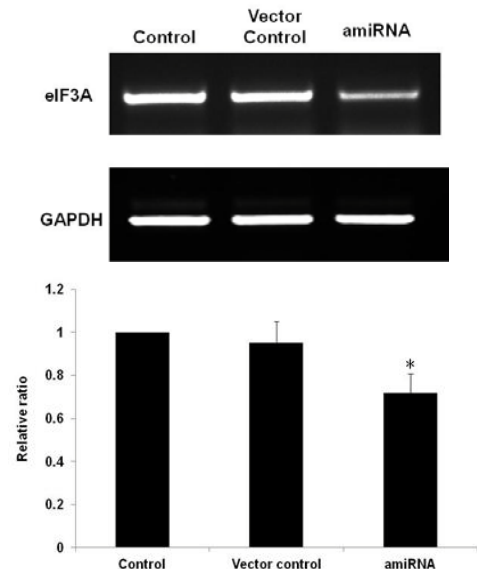

Fig. 3: (a) RT-PCR analysis eIF3A in MCF7 cells with transfected plasmid vector alone and amiRNA carrying plasmids. (b) Relative expression of eIF3A in MCF7 cells transfected with plasmid vector alone and amiRNA carrying plasmids. Results represent the means of three separate experiments, and error bars represent the standard deviation of the means. (*P<0.05, compared with blank control group and negative control group respectively

Transfection

Accordingly, a series of experimental sets harboring control, vector alone and vector with amiRNA against eIF3A gene were prepared and their silencing efficiency was analysed using RT-PCR after transfection. There is perfect base pair amplification in untreated cell and the band intensity decreased in amiRNA treated cells (fig. 3a). The relative ratio of eIF3A in vector alone and vector with amiRNA construct was found to be 0.96 and 0.64 respectively, compared to (non-transfected) control, (fig. 3b). These data illustrate that the designed amiRNA plasmid had successfully been transfected into the cells, and inhibited eIF3A expression.

MTT assay

MCF-7 cells were treated with amiRNA in time points (24 h, 48 h, and 72 h) and cell growth was measured by MTT assay. The inhibitory effect was found to be increased significantly in a time dependent manner (fig. 4). The results revealed that exposure of MCF-7 cells with amiRNA targeting eIF3A caused 50% reduction in cell viability at 72 h when compared to non-transfected cells.
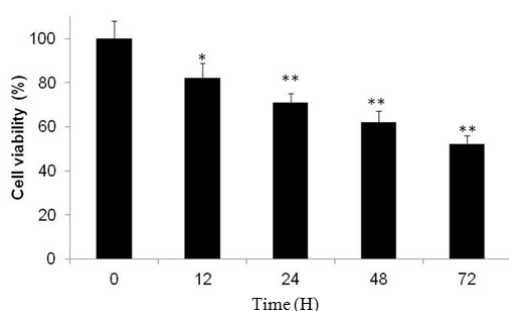

Fig. 4: Time dependent profile of amiRNA transfected MCF7 cells. Results represent the means of three separate experiments, and error bars represent the standard deviation of the means. (*P<0.05, **P<0.001, compared with blank control group and negative control group respectively)

DISCUSSION

The eukaryotic initiation factor eIF3A is becoming an important target in tumor therapy because the largest subunit of the eIF3 complex has been found to be overexpressed in several human cancers, including breast, cervix, oesophagus, stomach and lung [16-19]. The increased eIF3A expression was more frequently observed at the early invasive stage of tumors [12].

Thus alteration of eIF3A expression is reported in the early event of tumorigenesis. Indeed, knocking down eIF3A expression reversed the malignant phenotype of human lung cancer cell lines [4]. Over expression of eIF3A could support cell survival and proliferation and protect tumor from apoptosis [20].

RNAi is expected to have more therapeutic benefit, compared with the other post-transcriptional gene silencing techniques such as antisense and gene knockout technology since it is easier to deliver and requires only small dose of miRNA to produce its silencing effect. Therefore in an effort to target over-expressed eIF3A in breast cancer cells, the RNAi effector molecule was used in our study which inhibited the expression of eIF3A gene. The amiR30 design enhances gene silencing potency [21, 22] and offers more flexibility in gene silencing applications, as these sequences can also be expressed from RNA polymerase II promoters [23]. In the present study, we incorporated our siRNA into miR-30 to enhance the silencimg efficiency.

The inhibition of eIF3A expression in cancer cell may lead to down regulation of Bcl-2 antiapoptotic proteins and induce apoptosis [24]. The result of MTT assay showed that cell proliferation was inhibited remarkably in amiRNA transfected cells when compared to vector control.

Chemotherapeutic resistance is also a common clinical problem for patients with breast cancer. Recent studies have suggested that overexpression of eIF3A could support cell survival and proliferation and protect tumor from chemotherapy induced apoptosis. Hence targeting of eIF3A via RNAi techology is a remarkable strategy to control cancer as it interferes with tumorogenesis; additionally, the sensitivity of breast cancer cells to chemotherapeutic drugs can also be taken care of using this technology.

CONCLUSION

The usage of modified endogenous miRNA in vector based expression system with significant silencing efficiency and remarkable inhibition of cell proliferation suggest that RNAi based gene silencing method may become one of the effective means to control cancer. Thus there is certainty that further focus in eFI3A amiRNA combination therapy with chemotherapeutic drugs will definitely open a new avenue in the treatment of deadly breast cancer.

CONFLICTS OF INTERESTS

All authors have none to declare

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