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Original Article

RNAI MEDIATED SILENCING OF EUKARYOTIC INITIATION FACTOR 4B (EIF4B) TO ENHANCE CHEMO-SENSITIVITY OF BREAST CANCER CELLS

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ABSTRACT

Objective: The eIF4B, a eukoryotic initiation factor 4B plays an important role in binding mRNAs to ribosomes which is over expressed in several breast cancers, thereby causing chemo-resistance. This study aimed to reduce the over expression of eIF4B thereby increasing the chemosensitivity in breast cancer cells.

Methods: eIF4B expression was examined in normal breast tissues and breast cancer cell lines. MCF-7 cell line was established which stably expressed miRNA construct that induced downregulation of eIF4B expression. The possible role of eIF4B in the regulation of chemosensitivity was evaluated using RT-PCR.

Results: eIF4B expression was found to be significantly higher in breast cancer tissues compared with control. High level of eIF4B expression was associated with chemoresistance in cancer cells. The total mRNA was isolated from amiRNA transfected & normal MCF-7 cell lines. In addition, the combined treatment of doxorubicin and miRNA sensitizes tumor cells to the drug and thus enhanced its chemosensitivity.

Conclusions: Our results indicate that miRNA plays an important role in breast cancer by modulating the proliferation of breast cancer cells. Thus, artificial miR-30 based RNAi is a promising prognostic indicator for patients with breast cancer. The changes in the expression of components of translation through signal transduction pathways can lead to more global changes.

Keywords: Breast cancer, Eif4b, RNAi, miRNA, Chemoresistance.

INTRODUCTION

The Eukaryotic protein synthesis initiation factor 4B is a 8000 Da polypeptide, which is essential for binding of mRNA to ribosomes & involves in simulation of eIF4A (Eukaryotic translation Initiation Factor 4A) helicase & eIF4F (Eukaryotic translation Initiation Factor 4F) as ATPase activity [1]. The apparent flexible, elongated shape of eIF4B may allow it to make multiple contacts with several translation factor components (eg.PABP, eIF4G, eIF4A & mRNA) simultaneously during initiation of translation [2]. It contains a canonical RNA recognition motif, RNA binding domain & a DRYG (high content of aspartic acid, arginine, tyrosine & glycine residues) domain that have been implicated in mRNA binding, self association, interaction with eIF3 & simulation of eIF4A helicase activity [3]. eIF4B also have been reported to interact with the eIF3A subunit & 18s rRNA [4].

Breast cancer, the most important cancer types & account for one-third of all cases diagnosed in women of the developing world [5, 6]. About 90% of breast cancers are not due to heredity, but to genetic abnormalities that happen as a result of the aging process and life in general (International Agency for Research on Cancer, IARC). Cancer treatment depends on the types of cancer, the stages of the cancer, age, health status & additional personal characteristics [7]. Chemotherapy is a kind of treatment that uses drugs to attack cancer cells. Once they reach the cancer cells, they act to retard their growth, eventually resulting in their destruction [8]. Patients who initially respond to chemotherapy invariably show a loss of response later on, resulting in tumor regrowth [9]. There are two probable causes for this: (a) the tumor cells may be inherently resistant, due to some genetic characteristic or (b) they may acquire resistance following exposure to the drug [10].

Recently, RNA interference (RNAi) has been introduced as a potent naturally occurring biological strategy for silencing of genes [11]. RNAi is now used to investigate the function of genes, molecular mechanism of tumorogenesis & progression [12]. RNAi mediated by siRNAs are generated from long dsRNAs of exogenous or endogenous origin. siRNAs and miRNAs (Micro RNAs) are essentially similar in terms of molecular structure [13]; however, their different names reflect their different origins. miRNAs originate through endogenous transcription of noncoding genes, commonly found to be arranged in the genome in clusters [14, 15]. The endogenously produced dsRNAs are processed into primary miRNA transcripts, (primiRNAs) that are subsequently cleaved by the RNase III endonuclease Drosha into approximately 70-nucleotide-long hairpin RNA structures (precursor miRNAs, pre-miRNAs or small hairpinRNAs, shRNAs) [16]. Both the exogenous dsRNAs and the endogenous shRNAs are recognized and cleaved by Dicer, a highly conserved RNase III type endonuclease located in the cytoplasm, which generates mature 21–25 dsRNAs [17]. The dsRNAs generated after Dicer cleavage are then bound to the target mRNA by the RNA-induced silencing complex (RISC) [18]. Although a diverse set of gene silencing activities are mediated by RISC including translation suppression, transcriptional silencing and heterochromatic formation [19, 20, 21, 22], it is the induction of sequence-specific mRNA cleavage that has made RNAi a powerful technique for functional genomics.

In cancer several genes are aberrantly over expressed. miRNAs affect the growth of cancer cells *in vitro* & *in vivo* when over expressed or inhibited [23]. Therefore cancer cells growth can be controlled by manipulating miRNAs. Expression or inhibition of miRNA can be combined with the treatment of a drug or other cytotoxic therapy [24]. The silencing of eIF4B by RNAi in mammalian cells leads to polysome depletion & represses translation. Based on the observation on specific mRNA translation, eIF4B knockdown leads to decreased cell survival & proliferation [25]. The expression of the exogenous bcl-2 protein completely prevents DNA fragmentation in eIF4B controlled translational target that plays a crucial role in cell survival. Interestingly, silencing of eIF4B sensitizes cancer cells to low doses to cytotoxic drugs that lower doses. Hence the development of small inhibitor molecules targeting eIF4B would have therapeutic value in cancer treatment [26].

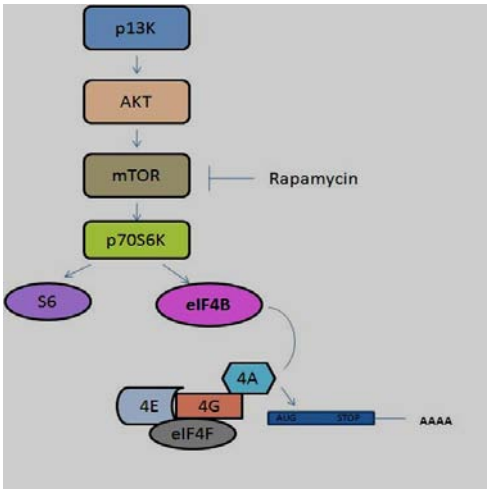

Fig. 1: Mechanism of eIF4B

(Source: The above diagram was drawn based on the reference read from the book, Translational Control in Biology and Medicine. Michael Mathews, Nahum Sonenberg, John W. B. Hershey, CSHL Press)

MATERIALS AND METHODS

Cell line & culture

The MDR human breast cancer cell lines, MCF-7 cell line was kindly provided by IIT Madras, Chennai. They were used in this study as they appear to mirror the heterogenecity of tumor cells *in vivo.* They were cultured in RPMI medium containing at 37°C in a humidified atmosphere containing 5% CO_2. Cells were checked routinely and found to be free of contamination.

Designing of siRNA sequence

There are several methods for preparing siRNA, such as chemical synthesis, in vitro transcription, siRNA expression vectors & PCR expression cassettes.

The mRNA reference sequence (FASTA) for the gene eIF4B was retrieved from the NCBI gene bank (GENE ID: 1975; NM_001417.4). The FASTA sequence was prioritized to increase the chance of finding a functional target sites by the online tools such as Ambion, Dharmacon, Genescript & microsynth. Designed siRNA target sequences with GC content in the range of 30-60% were selected for its stability. The siRNA was selected using the selection parameters and was sorted by rank. Then the BLAST homology search was performed to filter non-specific site (cross - reaction with non-target mRNA) and to avoid off target effects on other genes or sequences. After choosing the database (genome transcript) & the organism (human) it was optimized for the highly similar sequence (megablast).

The siRNA sequence targeting eIF4B corresponded to the coding region 3606 (5' AAAGGTGTGTACCAAGTGAAT 3') relative to the start codon. The siRNA duplex with the following sense and antisense sequences was used: 5'AGGUGUGUACCAAGUGAAUtt (sense) and AUUCACUUGGUACACACCUtt 5' (antisense). The designed siRNA sequence (sense strand and antisense strand) was incorporated into the stem region of miR30 sequence & its secondary structure was analyzed using web based tools such as mFold, sFold & RNA Fold for its stability. The designed sequence was then commercially synthesized from Sigma Pvt. Ltd. & amplified using PCR.

The newly synthesized amiRNA was amplified by PCR using the forward & reverse primers (5-ATATCAGCAGCCCACATCAT-3; 5-GAAGCACTGGGATGTCCGGT-3). Aliquots of PCR products were electrophoresed on 0.8% agarose gels and PCR fragments were visualized by ethidium bromide staining (0.1%). The amplified product was eluted & purified according to the given protocol (QIAGEN gel elution kit).

Construction of Vectors

E.Coli cell having pcDNA3.1 obtained from IIT Madras, Chennai was cultured in LB medium for plasmid isolation by alkaline lysis method. The isolated plasmid was checked by 0.8% ethidium bromide for the observation of bands. The gel containing the band region was cut & purified using the gel elution kit (QIAGEN). To assemble the pCDNA 3.1 vector restriction digestion was performed using the restriction enzymes such as Xba 1 & BamH1. The reaction mixture was incubated at 370C for 2 hrs & inactivated by heating at 650C for 20 mins. The reaction was then ligated using T4DNA ligase at 12-140C. After that it was transformed into E.Coli DH5α. The transformed cells were plated in the ampicillin selective medium. The transformed colonies were patched on the LBamp+ selective medium & incubated at 37oC overnight. Then Colony PCR was performed to screen colonies for the desired primer. Amplification cycles were: 94°C for 5 mins, then 30 cycles at 94°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec, followed by 72°C for 10 min. The amplified product was purified & electrophoresed in 1.7% agarose gel.

Transfection

MCF-7 cells in the exponential phase was plated in six-well plates and grown for 24 hrs and then transfected with synthesized amiRNA using Lipofectamine and OPTI-MEM reduced serum medium (Invitrogen Life Technologies, Inc.,) according to the manufactures protocol. Doxorubicin (Life Technologies) at the concentration of 1mg/ml was used to select cells that was doxorubicin-resistant, indicating that the vector was present in the cells.

Reverse transcriptase – PCR

Total RNA was isolated from the transfected cells using Trizol reagent (Invitrogen Life Technologies, Inc.,) The reverse transcription reaction was performed using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies, Inc.) in a final volume of 20µl containing 10µl of total RNA, 10µl reverse transcription buffer, 2µl MgCl2, 4µl deoxynucleotide triphosphate mixture, 2 µl DTT, RNaseOUT recombinant ribonuclease inhibitor, 50 units of Superscript reverse transcriptase and diethylpyrocarbonate treated water. After incubation at 42°C for 80 min, the reverse transcription reaction was terminated by heating at 70°C for 15 min. The newly synthesized cDNA was amplified by PCR. The primers for PCR were designed using online tools. The cDNA sequence was submitted in the PRIMER-BLAST. The primer sequence was designed considering the length of the bases, Tm value and GC content for its stability. The reaction mixture contained 4 µl of cDNA product, 1 µl of reverse primer, 1 µl of forward primer. Amplification cycles were: 95°C for 30 sec, then 30 cycles at 95°C for 1 min, 58°C for 1 min, 72°C for 30 sec, followed by 72°C for 15min. Aliquots of PCR product was electrophoresed on 1.6% agarose gel and PCR fragments were visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

Designing of siRNA sequence

The siRNA sequence for the target gene eIF4B was designed using bioinformatics tools & selected based on the parameters. The mRNA sequence obtained from the FASTA format was then uploaded in the online tools such as Dharmacon, Microsynth, Genescript & Ambion. The table 1 shows the siRNA sequences obtained from Ambion was selected based on the selection parameters.

RNAfold is used to measure the minimum free energy. The siRNA target sites with minimum free energy lower than -5 kcal/mol were filtered. Sequence complexity, Poly(A) (> 3 mer) and poly(T) (> 3 mer) stretches were removed to avoid premature termination of transcription. Poly(G) (> 2 mer), poly(C) (> 2 mer) and poly(G,C) (> 6 mer) stretches were also removed to decrease the RNA duplex internal stability. Other adjustable parameters included were length of the target sequence, GC content of the target sequence(36-60%), region for target selection, avoid regions within 50-100 bp of the start codon and the termination codon, intron regions, stretches of 4 or more bases such as AAAA, CCCC were avoided [29].

Table 1: sRNA sequence results

Online Tools	Sence strand sequence	Region	Start position	GC%
Dharmacon	GGAAGGACCAGGAAGGAAA	ORF	1763	53
	CCAGAGAGGTTGAAAGGTT	ORF	603	47
	CAGAAATGCACGAAGGAGA	ORF	1508	47
	GGAAGGAGTCAGATAGGAA	ORF	1873	47
	GGGAGAAGATTATGCCGAA	ORF	2021	47
	CCAAAGAAACCTGAGGAAA	ORF	1932	42
	GATGATAGCTTTGGAGACA	ORF	855	42
	CGAAGGAGAGAGAGTGAGA	ORF	1518	53
	CTGATCAAGCACAGGATAA	ORF	718	42
	CCAAAGACCCAAACTGAAT	ORF	1193	42
	CAGAGAGGTTGAAAGGTTT	ORF	604	42
Microsynth	GGAAGGACCAGGAAGGAAA	ORF	545	42
	GGAAUUCUUUCGAGGAUUA	ORF	512	38
	CCUACCCUAUGAUGUUACA	ORF	875	44
	GUAUCGAGAUCGUUAUGAU	ORF	1197	33.1
	AGACCCAAACUGAAUCUAA	ORF	1198	36.4
Genescript	**AAGTTCTTCATGCTACTCTTA**	ORF	2958	33.33
	AAGCAAATCGGTTTCTTGATG	ORF	3074	38.10
	AAGCACCTATGTTTCCAAACC	ORF	298	42.86
	AACATTCGAGAGCAAATCAAA	ORF	2089	33.33
	AATTGCCAATACAGCCAGAGA	ORF	2238	42.86
	AAAGGTGTGTACCAAGTGAAT	ORF	3608	38.10
	AATCTGTAGACTTATTACCTG	ORF	3929	33.33
	AATCACGTGATTGCCTCATCC	ORF	156	47.62
Ambion	**AAGCTTCCCTTGAGAGAATAA**	ORF	3336	38.1
	AAATAATTGGTGTGGATTGGC	ORF	3629	38.1
	AAGTTCTTCATGCTACTCTTA	ORF	2958	33.33
	AAGGTCCTGGTTTCTCTTGCA	ORF	3382	47.6
	AACACAGTAGCTAAACTTGCC	ORF	3402	42.9
	AAAGGTGTGTACCAAGTGAAT	ORF	3606	38.1
	AATTGGTGTGGATTGGCCAGT	ORF	3633	47.6
	AATCTGTAGACTTATTACCTG	ORF	3987	33.3
	AATTTACCCAGTGGACAAAGG	ORF	3590	42.9
	AATCTGTAGACTTATTACCTG	ORF	3927	33.3
	AATGAAACACTCAATAAGGAG	ORF	1548	33.3
	AATAAAGTAGATGGGATGAAT	ORF	1788	28.6

For better efficacy 5' & 3'UTRs region containing binding sequence for regulatory proteins was avoided such that it may affect the accessibility of the RNA target sequence to the RISC complex. BLAST search was used to minimize non-specific effect. Poly A & Poly T were removed to avoid premature termination of transcription. Poly G, Poly C & Poly G, C stretches were also removed to decrease the RNA duplex internal [27, 28].

siRNA target sequence

The siRNA target sequence AAAGGTGTGTACCAAGTGAAT in 3606 ORF region with 38.1% GC content was taken (Sense strand siRNA GUGUGUACCAAGUGAAUtt, Antisense strand siRNA AUUCACUUGGUACACACCUtt)

BLAST Analysis

Homo sapiens eukaryotic translation initiation factor 4B (EIF4B),

mRNA, Length=4041

GENE ID: 1975 EIF4B | eukaryotic translation initiation factor 4B

[Homo sapiens] (Over 10 PubMed links) Score = 42.1 bits (21), Expect = 0.005

Identities = 21/21 (100%), Gaps = 0/21 (0%) Strand=Plus/Plus

Query 1 AAAGGTGTGTACCAAGTGAAT 21

| | | | | | ||||||||||||||||

Sbjct 3606 AAAGGTGTGTACCAAGTGAAT 3626

The above sequence was selected as the target sequence using the BLAST analysis. The siRNA sequence was incorporated into the stem region of miR30 sequence which is more functional in human.

Secondary structure prediction

Sequence1 (Position in gene sequence: 3606, GC content: 38.1%) 5'GGAUCCACUAGUUAUUGCUCUCGAGAGUGAGCGAAAAGGUGUGUAC CAAGUGAAUUAGUGAAGCCACAGAUGUAAUUCACUUGGUACACACCU UUCUGCCCACUGCCGAGGAAUUCAAGGGUCUAGA3'

Sequence2 (Position in gene sequence: 296, GC content: 42.9%)

5'GGAUCCACUAGUUAUUGCUCUCGAGAGUGAGCGAAAGCACCUAUGU UUCCAAACCUAGUGAAGCCACAGAUGUAGGUUUGGAAACAUAGGUGC UUCUGCCCACUGCCGAGGAAUUCAAGGGUCUAGA3'

Sequence3 (Position in gene sequence: 2087, GC content: 33.3%)

5'GGAUCCACUAGUUAUUGCUCUCGAGAGUGAGCGAAACAUUCGAGAG CAAAUCAAAUAGUGAAGCCACAGAUGUAUUUGAUUUGCUCUCGAAUG UUCUGCCCACUGCCGAGGAAUUCAAGGGUCUAGA3'

Sequence 4 (Position in gene sequence: 2956, GC content: 33.3%)

5'GGAUCCACUAGUUAUUGCUCUCGAGAGUGAGCGAAAGUUCUUCAUG CUACUCUUAUAGUGAAGCCACAGAUGUAUAAGAGUAGCAUGAAGAAC UUCUGCCCACUGCCGAGGAAUUCAAGGGUCUAGA3'

The secondary structure of miR30 based construct of the target eIF4B resembles the endogenous miRNA precursor hence it was assumed that their will be minimum off target effects such as interferon response. Among the four **sequence 1** was selected based on the parameters such as siRNA sequence must be positioned in the stem region of miR30 sequence, should not be any mismatch in the siRNA target region, atleast two AC mismatch should be in the secondary structure of miR30 sequence for the recognition of Drosha, loop formation, Stability based on Gibb's free energy. **Sequence 2** almost nearer to the 5' end**,** having 42.1% of GC content was not selected as these regions contain binding sequences for regulatory

proteins that may affect the accessibility of the RNA target sequence to the RISC complex. Also **Sequence 3** and **sequence 4** having low GC% content was not selected. The construct with sequence 1 was synthesized from SIGMA Pvt Ltd., and amplified [30, 31].

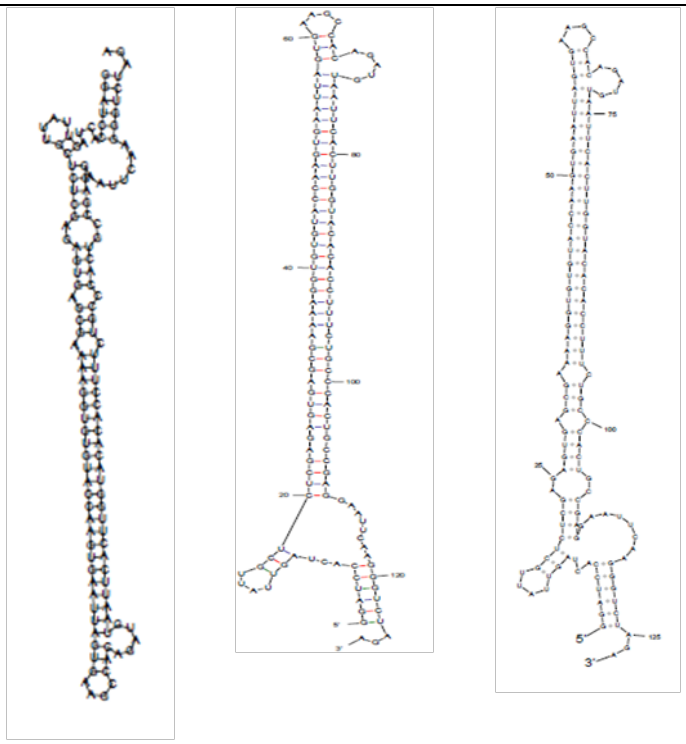

Fig. 2(a): Secondary structure for Sequence 1

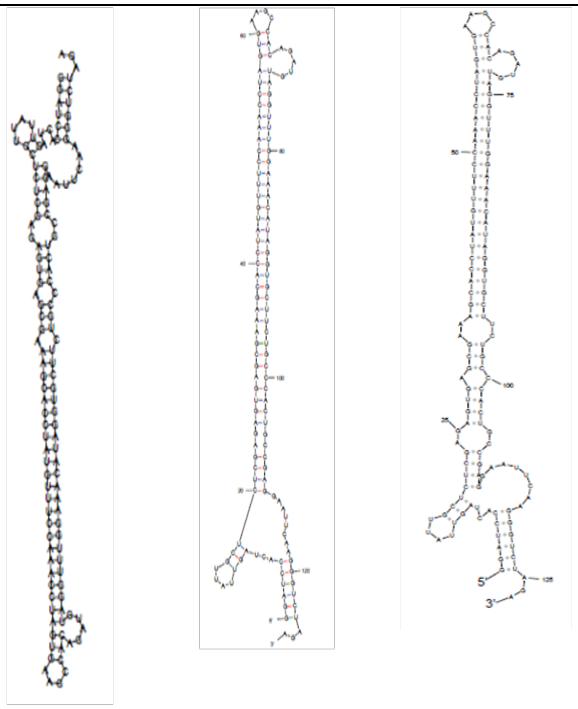

Fig. 2(b): Secondary structure for Sequence 2

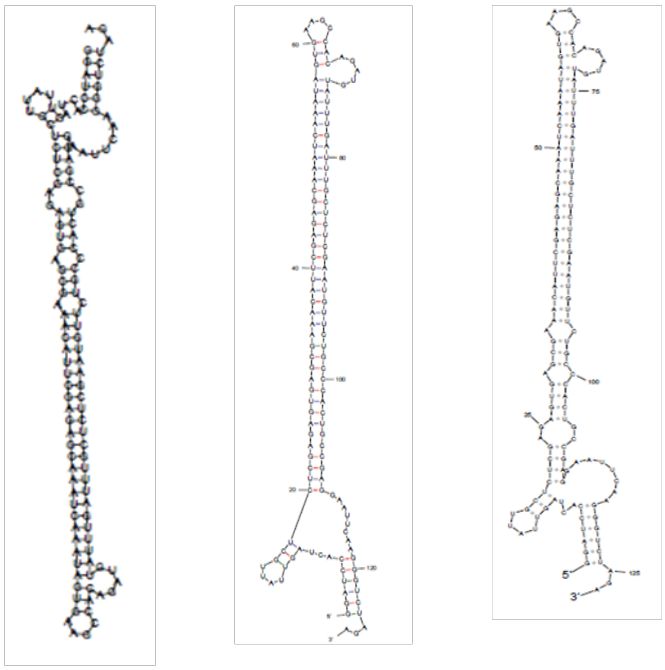

Fig. 2(c): Secondary structure for Sequence 3

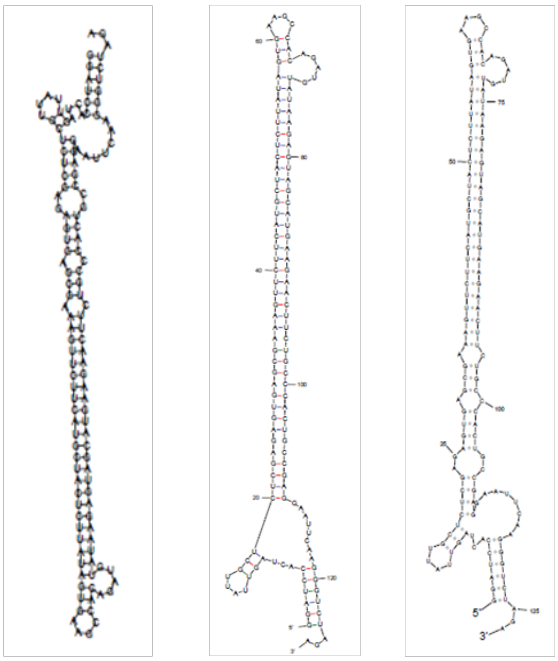

Fig. 2(d): Secondary structure for Sequence 4

Cloning

The amplified amiRNA was eluted from the agarose gel using the QIAGEN elution protocol. The plasmid (pCDNA 3.1 Vector) was isolated from the carrier *E.Coli* cell. The amplified product was inserted into the pcDNA3.1 vector using suitable restriction enzymes BamH1 at 5' end & Xba1 at the 3' end. After it was ligated into the pcDNA 3.1 vector which have the same overhang (BamH1 & Xba1), it was transformed into the *E.Coli* DH5α. The transformed cells plated on the ampicillin selective medium were identified.

The randomly selected patched colonies were amplified by PCR & the products were electrophoresed in agarose gel & the clones were identified. From the figure 3 the clones in the lane 3, 4, 5 shows the positive results. The plasmid (i.e., the vector with insert) was then isolated & quantified.

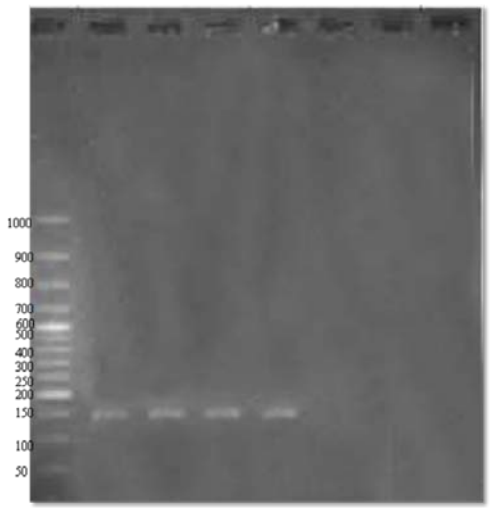

Fig. 3: Identification of transformants

Primers for RT-PCR

The primers were designed to perform RT-PCR using the tool primer blast. The parameters considered were forward & reverse complimentary, self complimentary of primers, TM (Melting temperature) value of forward & reverse primers, GC content, length & size of the amplified product, no of bases & position of the product. The designed forward Primer - 5'GTAGGGAATCAGCTTGGTAGA-3' (Tm-58.3, GC-50%) and reverse primer - 5'CAACCCAAAGGATGGGATTT- 3' (Tm-64.6, GC- 45%) was commercially synthesized from Sigma.

mRNA expression level of eIF4B

The total mRNA was isolated from amiRNA transfected & normal MCF 7 cell line. The RT-PCR shows that the expression level of eIF4B was reduced (Figure 4) when compared with the control (MCF-7) cell line obtained from IIT Madras, Chennai.

Lane 1: 50bp marker Lane 2: amiRNA treated eIF4B Lane 6: Control (MCF-7 cells)

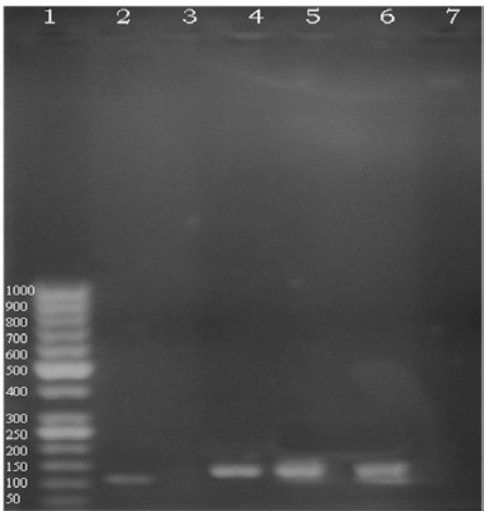

Fig. 4: mRNA expression by RT-PCR

CONCLUSIONS

The effects on translation and survival seen in eIF4B-silenced cells are similar to the consequence of eIF4A inhibition. Hence, phosphorylation of eIF4B regulates the translation of functionally related mRNAs, such as cell cycle regulators and antiapoptotic factors. Overall, these results may contribute to the chemosensitive activity observed with doxorubicin in breast cancer. Further studies on targeting the gene eIF4B, will helps in the prevention and treatment of breast cancer.

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