International Journal of Innovative Technology and Exploring Engineering (IJITEE)
ISSN: 2278-3075 (Online), Volume-9 Issue-3, January 2020

Designing of Short Interfering RNAS (siRNAs) for Tuberculosis

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Abstract*: The control of Tuberculosis is an obstacle that many researchers have been trying to overcome from several decades, as it remains the cause of millions of deaths per year. Though there are several drugs available but due to drug resistance, there is an extensive need of new and effective ways of treatment. Computational study was performed to design siRNA for the genes atpG, dnaK and htpG as all these genes are important for the survival of bacterium Mycobacterium tuberculosis. Multiple sequence alignment was done using Mega X software on the coding sequences (cds) of studied Mtb strains, which were retrieved from the NCBI database. The i-score designer was used for designing of potent siRNA. The SMEpred webserver was used to validate the data collected from the i-score designer, which was further used to calculate the efficacy as well as to predict the chemically modified siRNA (cm-siRNA).

Results show that the studied genes were conserved in all the studied strains. MFE was calculated using RNAfold server and sequences with the lowest MFE was selected, as they are proved to have higher stability. This result can be used to develop novel therapeutic applications.

***Keywords*: Tuberculosis, siRNA, SMEpred, i-score designer, atpG, dnaK, htpG**

I. INTRODUCTION

Tuberculosis (TB) is one of the most dangerous infectious diseases that have caused over a billion of deaths worldwide in the past two hundred years. World Health Organization (WHO) ranked tuberculosis at 10th place responsible for deaths worldwide. Around 10 million incidences were recorded out of which 1.6 million died from the disease, among which 23000 were children [1]. *Mycobacterium tuberculosis* (MTB) is an etiological agent of the devastating disease, tuberculosis. It most often affects the lungs and can spread to any organ of the body.

Revised Manuscript Received on January 30, 2020.
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TB is curable and preventable but MDR (Multi Drug Resistant-TB), XDR (Extensive Drug Resistance) and TDR (Total Drug Resistance) is a major concern [1].

Though there are various armamentariums available for tuberculosis control but due to development of MDR, XDR and TDR there is extensive need

of new and effective ways of treatment. RNA interference (RNAi) is a natural process that is used to silence gene expressions by degrading the mRNA. Gene regulation and innate defence are one of the essential roles of RNAi. MicroRNA (miRNA) and small interfering RNA (siRNA) are the two types of RNA molecules which are vital to RNA interference as they can direct enzyme complexes to decrease the activity of mRNA by degrading it and thus preventing translation, through post-transcriptional gene silencing [2]. Furthermore, transcription can be inhibited via the pre-transcriptional silencing mechanism of RNA interference, through which an enzyme complex catalyzes DNA methylation at genomic positions complementary to complexed siRNA or miRNA. RNA interference also has an important role in defending cells against parasitic nucleotide sequences – viruses and transposons. Hence the merits of RNAi include high efficiency of the gene knockdown and targeting of gene of interest and expression of small noncoding RNAs that can result in stable and long-term silencing [3].

Mechanism of RNAi involves Dicer enzyme, which is a specialized RNase, present in the cytoplasm. It processes dsRNA into smaller dsRNA molecule called siRNA. This siRNA has 21-23 nucleotides which include two nucleotide overhangs at 3' end [4]. RISC (RNA induced silencing complex) is activated when siRNA interacts with it. After RISC interacts with siRNA, its endonuclease argonaute2 (AGO2) component cleaves siRNA's passenger strand, but it does not harm the guide strand which is kept attached with RISC. Consequently, the active RISC is guided towards its target mRNA by the guide strand for cleavage by AGO2. Now the siRNA causes specific gene silencing as it fully complementarily binds to the mRNA [5][6][7].

Target validation and identification is one of the essential roles of siRNA in making and developing the drug, because of its gene silencing effect [8]. Generally, miRNAs are less preferred because they are less specific and can give rise to many diseases like cardiovascular diseases, neurogenerative disorders and even cancer [9]. Traditional small drug molecules can only target some classes of proteins, even monoclonal antibodies, the highly specific protein-based drug can only target circulating proteins of the surface receptors of the cell.

On the contrary siRNA can reduce the expression of almost all genes and their mRNA transcripts. Even the diseases caused by undesired or mutated gene's expression and overexpression of normal genes can be treated by targeting using siRNA [1].

In pathogenic bacteria *Mycobacterium tuberculosis*, the gene atpG, dnaK and htpG play an essential role in its survival within the host cell. The gene of atpG encodes gamma chain of ATP synthase which is a ubiquitous enzyme that is largely conserved across the kingdoms of life. It is required in energy formation at both aerobic and hypoxic dormant stage of its life cycle. The studies suggest atpG as a potential drug target [10]. DnaK is a homologue of Hsp70 (Heat shock protein 70) which belongs to a class of highly conserved proteins found in eukaryotes. Protein folding, degradation, assembly and transport are some of their functions. Under stress conditions Hsp expression tends to increase. Amongst the genes dnaK and htpG (in *Mycobacterium*) coding for heat shock family proteins. Hsp70 is hence important for bacteria's survival which makes it a potential drug target.

Besides being important for bacteria, studies have shown that mycobacterial DnaK has an important role in the proteotoxic network in host [11][12]. DnaK can also increase DNA binding activity of HspR (hspR is the last gene of the dnaKJE operon) in two ways (i) DnaK binds with the denatured HspR and forms a complex which will result in repair of binding activity of HspR. (ii) Thermal instability of HspR can be evaded with the help of DnaK using other methods also [13][14].

The third essential gene, targeted through our research, is htpG also belonging to the class of heat shock proteins a homologue of Hsp90 and working similar to DnaK. Hsp90 is also involved in drawing out immune response. Studies show that it is responsible for T-cell immunity [15]. Chaperone peptide's processing and MHC-1 presentation is shown to be increased with the help of hsp90 and hsp70 (dnaK).

Realising the importance of the three genes, in this research paper potential siRNA for atpG, dnaK and htpG are designed for inhibition of ATPG, DnaK and HtpG production in *Mycobacterium tuberculosis* for obstructing its overall growth and replication process.

II. MATERIAL AND METHODS

Data acquisition

The coding sequences (cds) for the genes atpG, htpG, dnaK of studied *Mycobacterium tuberculosis* strains were retrieved in fasta format from the Nucleotide database available at National Centre for Biotechnological Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The information regarding respective genes and their cds region coding is shown in table no. 1.

Multiple Sequence Alignment

To analyze the relationship between the studied strains, multiple sequence alignment was done using "MEGAX (Molecular Evolutionary Genetics Analysis Version 10.0.4)" developed by "Penn State University" and ClustalW [29].

Table 1 : Information for (atpG, htpG, dnaK) genes

MTB Strains	Gene	CDS size	Length (bp)	Accession no	CDS Coded by region
H37Rv	dnaK	1..1878	1877	AL123456.3	419835..421712
H37Ra	dnaK	1..1878	1877	CP000611.1	421197..423074
CDC1551	dnaK	1..1878	1877	AE000516.2	419895..421772
Erdman = ATCC 35801	dnaK	1..1878	1877	AP012340.1	420203..422080
CAS/NIT R204	dnaK	1..1923	1922	CP005386.1	418738..420660
H37Rv	atpG	1..918	917	AL123456.3	1464884..1465801
H37Ra	atpG	1..918	917	CP000611.1	1466194..1467111
CDC1551	atpG	1..918	917	AE000516.2	1464425..1465342
ERDMAN_ATCC 35801	atpG	1..918	917	AP012340.1	1463458..1464375
CAS/NIT R204	atpG	1..918	917	CP005386.	1460689..1461606
H37Rv	htpG	1..1944	1943	AL123456.3	2570059..2572002
H37Ra	htpG	1..1944	1943	CP000611.1	2579963..2581906
CDC1551	htpG	1..1944	1943	AE000516.2	2565905..2567848
Erdman = ATCC 35801	htpG	1..1944	1943	AP012340.1	2554785..2556728
CAS/NIT R204	htpG	1..1941	1940	CP005386.1	2562502..2564442

Target Identification for siRNA designing

To find out the siRNA target and designing of the potential siRNA for the genes atpG, htpG, and dnaK of all the studied strains of *Mycobacterium tuberculosis*, "SMEpred" webserver and "i-Score Designer" were used. The i-Score Designer is a server that works on a simple algorithm, i-Score (inhibitory-Score), for prediction active siRNAs [18]. The SMEpred webserver (http://bioinfo.imtech.res.in/manojk/smepred/sirna_out_all.php?ran=60558) was used for confirmation of the predicted siRNA sequence, validation of the target and predicting the efficacy of the siRNA [19]. SMEpred webserver also predicts the chemically modified siRNA sequence. "OligoCalc" was used to calculate the melting temperature of the seed region (http://biotools.nubic.northwestern.edu/OligoCalc.html) (eq. 1) [20][21]. "RNAfold" was used to generate secondary structure of siRNA and to calculate its Minimum free energy (MFE)(http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) [22][23][24].

III. RESULTS

The study was conducted on the genes atpG, htpG, dnaK that code for ATPase subunit gamma (ATPG), chaperon protein DnaK (hsp70) and HtpG (hsp90) of *Mycobacterium tuberculosis* respectively. The uniqueness of AtpG (Mtγ165–178) in *Mycobacterium tuberculosis* makes it a potential drug target, as it does not produce any off-target effects [25]. The AtpG is important for the energy production, without which the pathogen will be unable to survive. DnaK is not present in humans so it can be a potential target for *Mycobacterium tuberculosis*.

HtpG and DnaK are essential for cell growth, native protein folding and immune response. Several studies suggested DnaK and HtpG as potential drug target [26][27].

Different strains of *Mycobacterium tuberculosis* show different infection pattern, so comparative studies between the various strains of *Mycobacterium tuberculosis* were done. The CDS sequences of genes atpG, htpG and dnaK were downloaded from NCBI (Table 1) and multiple sequence alignment was done using MEGAX software to see the relationship between the sequences. All the sequences showed 100% similarity. It shows that the gene atpG, htpG and dnaK are conserved among all the studied strains. Strain H37Rv were used for identifying target specific siRNA as it may show least off target effects.

The server i-Score Designer that uses 9 different algorithms (Ui-Tei, Amarzguioui, Hsieh, Takasaki, s-Biopredsi, i-Score, Reynolds, Katoh, and DSIR) were used for analyzing the potential and effective siRNA. Scores calculated by all these algorithms are shown in Table 2. The importance of these algorithms is given below and the rules of algorithms are shown in Table 3.

i. Ui-Tei rule indicates that the functional siRNA has asymmetrical stability in 5' and 3' terminals.

ii. Amarzguioui rule ensures the asymmetry in the stability of the duplex ends.

iii. DSIR is used for scoring threshold, similarity search and computing SCF (Seed complement frequencies).

iv. The s-Biopredsi performs siRNA seed region match in combination with 10 or more bases of additional homology to unintended target genes, which is used for prediction of off-targets. siRNAs predicted to have a large number of off-targets, are rejected.

v. Katoh is used for multiple sequence alignment and Takasaki rule is used for Gene degradation [17].

The oligoCalc tool was used to check the temperature of seed region as studies show that the melting temperature (Tm) of the seed region (2-8 position from 5') of siRNA should be below 21.5 °C. All the desired siRNAs have Tm≤21.5°C. The formula that was used by the oligoCalc to calculate Tm is shown below (1) [20] :

Tm= {(1000 ×· H)/ (A +· S +Rln (CT/4))}- 273.15 + 16.6 log [Na+]…….....(1)

Off-target effect was considered during siRNA designing as it is one of the major problems in siRNA designing. As the siRNA is designed for treating tuberculosis in humans, there should be no homologue with the siRNA other than the target gene. Off-targets were identified through NCBI homology search and no off-target effects were found in all the three studied genes viz. atpG, dnaK and htpG. The siRNA should be specific and effective therefore the chemical modifications were carried out to provide specificity which includes removal of off-target effects. There are many modifications present in siRNAs like sugar modification, phosphate linkage modification, base modification and the modifications to the overhangs and termini. These modifications help in improving serum stability, increasing potency, modulating immunostimulatory activity, reducing hybridization dependent off-target effects and also helps in achieving temporal or spatial control of RNAi induction. [28]. The SMEpred webserver was used to obtain the chemically modified siRNAs, and 7 genes of highest efficacies were selected for each of the three genes. The modified siRNA sequence and the information regarding type of modification with its efficacy are shown in Table 4.

The effectiveness and the specificity are important factors, but the stability is also an important criterion that should be considered during siRNA designing. MFE (Minimum free energy) value was calculated. Lower MFE is more preferred in siRNA because studies revealed that the lower will be the MFE, the higher will be the stability [29]. Nucleotide's number, composition and arrangement affects the RNA's MFE. The longer the sequence is, the more stable it will be. The formation of hydrogen bond results in more stacking of the structure. GC rich RNAs are preferred more over AU rich RNAs.

The RNAfold server was used to calculate the MFE of the siRNAs as well as to predict the structure of the natural siRNA and of cm-siRNA with least MFE (Table 5) (Figure 1 a. - c.) [23].

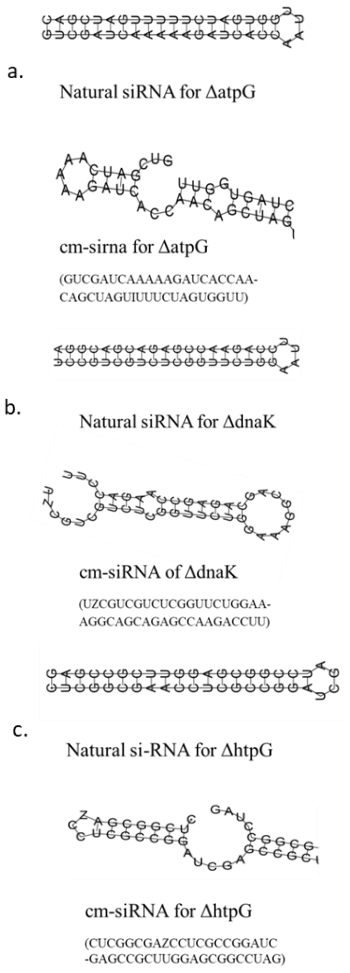

Figure 1 (a-c): Predicted 2° structure of natural siRNA and cm-siRNA with possible folding and minimum free energy.

IV. CONCLUSION

Tuberculosis is a devastating disease that can be controlled and prevented but the drug resistance in tubercular bacteria is becoming a major concern in the treatment. There is an extensive need of new and effective therapeutics and detection techniques.

The siRNA therapeutics provides a great potential through sequence-specific gene-silencing.

The advances in the RNA interference technology allows us to silence the desired gene by degrading the target mRNA sequence with the help of RISC complex mechanism. The siRNA and cm-siRNA were designed through various algorithms. These molecules may prove to be a novel therapy against tuberculosis. The outcome of the study provides a foundation for the researchers to develop antitubercular therapy at genomic level.

We have followed computational approach as it is less time consuming than the conventional methods, more economical and helps in better error analysis.

Table 2: The details of siRNA, designed through i-Score Designer.

Pos	Sense	Antisense	SecdG	5'end	3'end	Whole dG	GC stretch	%GC	Ui-Tei	Amarzguioui	Hsieh	Takasaki	s-Biopredsi	i-Score	Reynolds	Katoh	DSIR
ATPG	CGAUCAAAAGAUCACCAA	UUGGUGAUCUUUUUGAUCGac	-3.8	-2.4	-0.9	-32.6	2	36.8	Ib	4	1	5.4	0.857	82.4	6	92.4	96.4
DnaK	CGUCGUCUCGGUUCUGGAA	UUCCAGAACCGAGACGACGga	0.2	-2.4	-0.9	-40.5	3	57.9	Ib	3	2	2.7	0.837	69.7	5	49.4	90.4
HtpG	CGGCGAACCUCGCCGGAUC	GAUCCGGCGAGGUUCGCCGag	-9.3	-2.4	-2.4	-45.5	6	73.7	II	4	2	2.3	0.670	50.0	2	46.9	70.0

Table 3: Working rule of different algorithms used for designing siRNA

Ui-Tei Rules	Reynold's Rules	Amarzguioui Rules
• A or U at position 1 from 5' terminus (guide strand). • G or C at position 19. • AU richness (AU≥4) in position 19. • No long GC stretch≥ 10.	• GC content (30-52%) • A/U ≥3 at position 1-5. • Absence of internal repeats. • A at position 1. • A at position 17. • U at position 10. • No G/C at position 1.	• G or C at position 19. • A or U at position 1. • A or U at position 14. • No U at position 19. • No G at position 1.
DSIR Rules	**Hsieh Rules**	**Takasaki Rules**
• Avoid 4 or more nucleotide runs. • Avoid immunostimulatory motifs.	• No Cat position 6 • A at position 13 • C/G at position 11. • Gat position 16. • U at position 19. • No G at position 19.	• No A/U at position 1 • G at position 1 • A at position 6 • G at position 7. • No U at position 7. • A at position 8. • No G at position 8and 9. • U at position 9. • U at position 15. • No G at position 19.

Table 4: Designing of siRNA and prediction of cm-siRNA efficacy through SMEpred webserver. [18]

Natural siRNA sequence of atpg			siRNA efficacy
5' --GUCGAUCAAAAAGAUCACCAA --3' 3' --CAGCUAGUUUUUCUAGUGGUU --5'			92.8
cm-siRNAs sequence of atpg	**Nicleic Acid Modifications**	**cm-siRNAs efficacy**	**Efficacy modulation**
GUCGAUCAAAAAGAUCACCAA-CAGCIAGUUUUUCUAGUGGUU	Altritol Nucleic Acid	99.17	6.37
GUCGAUCAAAAAGAUCACCAA-CAGCUAGIUUUUCUAGUGGUU	Altritol Nucleic Acid	99.17	6.37
GUCGAUCAAAAAGAUCACCAA-CAGCUAGUIUUUCUAGUGGUU	Altritol Nucleic Acid	99.17	6.37
GUCGAUCAAAAAGAUCACCAA-CAGCUAGUUIUUCUAGUGGUU	Altritol Nucleic Acid	99.17	6.37
GUCGAUCAAAAAGAUCACCAA-CAGCUAGUUUIUCUAGUGGUU	Altritol Nucleic Acid	99.17	6.37
GUCGAUCAAAAAGAUCACCAA-CAGCUAGUUUUICUAGUGGUU	Altritol Nucleic Acid	99.17	6.37
GUCGAUCAAAAAGAUCACCAA-CAGCUAGUUUUUCIAGUGGUU	Altritol Nucleic Acid	99.17	6.37
Natural siRNA sequence of dnaK			**siRNA efficacy**
5' --UCCGUCGUCUCGGUUCUGGAA --3' 3' --AGGCAGCAGAGCCAAGACCUU --5'			80.2

cm-siRNAs sequence of dnaK	Nucleic Acid Modifications	cm-siRNAs efficacy	Efficacy modulation
UZCGUCGUCUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	2'-Deoxy Nucleic Acid	96.17	15.97
UCZGUCGUCUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	2'-Deoxy Nucleic Acid	96.17	15.97
UCCZUCGUCUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	2'-Deoxy Nucleic Acid	96.17	15.97
UCCGUZGUCUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	2'-Deoxy Nucleic Acid	96.17	15.97
UCCGUCZUCUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	2'-Deoxy Nucleic Acid	96.17	15.97
UCCGUCGUZUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	2'-Deoxy Nucleic Acid	96.17	15.97
UCCGUCGUCUZGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	2'-Deoxy Nucleic Acid	96.17	15.97
Natural siRNA sequence of htpG			siRNA efficacy
5' --CUCGGCGAACCUCGCCGGAUC --3' 3' --GAGCCGCUUGGAGCGGCCUAG --5'			74.8
cm-siRNAs sequence of htpG	**Nicleic Acid Modifications**	**cm-siRNAs efficacy**	**Efficacy modulation**
CUCGGCGZACCUCGCCGGAUC-GAGCCGCUUGGAGCGGCCUAG	2'-Deoxy Nucleic Acid	82.59	7.79
CUCGGCGAZCCUCGCCGGAUC-GAGCCGCUUGGAGCGGCCUAG	2'-Deoxy Nucleic Acid	82.59	7.79
CUCGGCGAACCUCGCCGGZUC-GAGCCGCUUGGAGCGGCCUAG	2'-Deoxy Nucleic Acid	82.59	7.79
CUCGGCGbACCUCGCCGGAUC-GAGCCGCUUGGAGCGGCCUAG	2'-Deoxy-2'-Fluoro Nucleic Acid	81.08	6.28
CUCGGCGAbCCUCGCCGGAUC-GAGCCGCUUGGAGCGGCCUAG	2'-Deoxy-2'-Fluoro Nucleic Acid	81.08	6.28
CUCGGCGAACCUCGCCGGbUC-GAGCCGCUUGGAGCGGCCUAG	2'-Deoxy-2'-Fluoro Nucleic Acid	81.08	6.28
CUCGGCGYACCUCGCCGGAUC-GAGCCGCUUGGAGCGGCCUAG	2'-O-Methyl Nucleic Acid	80.47	5.67

Table 5: MFE (Minimum free energy) calculation for natural siRNA and chemically modified siRNA. [22][23]

Gene	Type	Sequence	MFE
Atpg	Natural siRNA	GUCGAUCAAAAAGAUCACCAA- UUGGUGAUCUUUUUGAUCGAC	-30.10
	cm-siRNAs	GUCGAUCAAAAAGAUCACCAA-CAGCIAGUUUUUCUAGUGGUU	-4.90
		GUCGAUCAAAAAGAUCACCAA-CAGCUAGIUUUUCUAGUGGUU	-7.70
		GUCGAUCAAAAAGAUCACCAA-CAGCUAGUIUUUCUAGUGGUU	-8.80
		GUCGAUCAAAAAGAUCACCAA-CAGCUAGUUIUUCUAGUGGUU	-8.80
		GUCGAUCAAAAAGAUCACCAA-CAGCUAGUUUIUCUAGUGGUU	-8.80
		GUCGAUCAAAAAGAUCACCAA-CAGCUAGUUUUICUAGUGGUU	-8.20
		GUCGAUCAAAAAGAUCACCAA-CAGCUAGUUUUUCIAGUGGUU	-4.10
DnaK	Natural siRNA	UCCGUCGUCUCGGUUCUGGAA- UUCCAGAACCGAGACGACGGA	-38.30
	cm-siRNAs	UZCGUCGUCUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	-15.40
		UCZGUCGUCUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	-15.40
		UCCZUCGUCUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	-15.40
		UCCGUZGUCUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	-15.10
		UCCGUCZUCUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	-12.20
		UCCGUCGUZUCGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	-11.60
		UCCGUCGUCUZGGUUCUGGAA-AGGCAGCAGAGCCAAGACCUU	-15.40
HtpG	Natural siRNA	CUCGGCGAACCUCGCCGGAUC- GAUCCGGCGAGGUUCGCCGAG	-41.00
	cm-siRNAs	CUCGGCGZACCUCGCCGGAUC-GAGCCGCUUGGAGCGGCCUAG	-18.00
		CUCGGCGAZCCUCGCCGGAUC-GAGCCGCUUGGAGCGGCCUAG	-19.70
		CUCGGCGAACCUCGCCGGZUC-GAGCCGCUUGGAGCGGCCUAG	-19.40
		CUCGGCGbACCUCGCCGGAUC-GAGCCGCUUGGAGCGGCCUAG	-18.00
		CUCGGCGAbCCUCGCCGGAUC-GAGCCGCUUGGAGCGGCCUAG	-19.70
		CUCGGCGAACCUCGCCGGbUC-GAGCCGCUUGGAGCGGCCUAG	-19.40
		CUCGGCGYACCUCGCCGGAUC-GAGCCGCUUGGAGCGGCCUAG	-18.00

Conflicts of interest

All authors have none to declare.

ACKNOWLEDGEMENT

The authors express the deep sense of gratitude to the Department of Biotechnology of Graphic Era Deemed to be University for all the support, assistance, and constant encouragements to carry out this work.

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