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Prediction and Depiction of Potential RNA-Based Therapeutics for Oncogenic E6 and E7 Genes of Human Papilloma Virus Types 16 & 18: A New Class of Treatment for Lung Cancer

Moinul Abedin Shuvo[1, *], Sayeedul Alam Prince[2], Arifuzzaman[1]

[1]Department of Biochemistry and Biotechnology, University of Science and Technology Chittagong, Chittagong, Bangladesh
[2]Institute of Nuclear Medicine and Allied Sciences, Bangladesh Atomic Energy Commission, Cox's Bazar, Bangladesh

Email address:

moinulshuvo@yahoo.com (M. A. Shuvo), princenmc@yahoo.com (S. A. Prince), larif67@yahoo.com (Arifuzzaman)

[*]Corresponding author

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Abstract: Unlike almost all the cervical, penile, vulvar, and anal cancers, where Human papilloma virus has long known to play a vital role, a causative link between carcinogenic Human papilloma virus and lung cancer have been found to be highly variable and contradictory. Data also shows geography and race-dependenty. Apart from etiological factors, viral carcinogen can manipulate the cell cycle, hamper cell apoptosis and also interrupt the cell division in host cell which lead to the lung cancer. Molecular studies of carcinogenic Human papilloma virus have found that E6/E7 acts as mitotic mutators which play an important role in pathogenicity and oncogenicity. Analysis of genome sequence of Human papilloma virus revealed that ORF having conserved in early region, E6 and E7 required for viral pathogenicity and oncogenicity can be the suitable target for RNAi technology. RNAi works by silencing or turning off gene expression to control pathogenicity and oncogenicity by blocking its replication processes. Therefore, the work is done on the basis of rational siRNA designing method by targeting viral oncogenic E6 and E7 genes of Human papilloma virus types16 & 18. Searching siRNA target sequences, multiple sequence alignment, forecasting secondary structure and RNA-RNA interaction prediction was done by various computational software tools for designing RNA-based therapeutics (siRNA). In this study, four effective siRNA were predicted rationally for oncogenic E6 and E7 genes of Human papilloma virus types 16 & 18 which might be used as a potential RNA based therapeutics to control the rate of carcinogenesis and degree of oncogenicity. The outcome of this study provides a basis of the researchers towards understating to development of RNA-based therapeutics (siRNA) at genomic level.

Keywords: Lung Cancer, Human Papilloma Virus, RNA-Based Therapeutics, RNAi Technology

1. Introduction

Lung cancer is the key health problem and prevalent cancer for men and women whole over the world. It is the foremost reason of cancer-related mortality worldwide, accounting for 3.1% of the total number of deaths per year worldwide, and 17.6% of cancer-related deaths [1]. Both the certain and relative rate of lung cancer has raised dramatically [2]. In Bangladesh, the prevalence of lung cancer is 16.7% of all cancers and the most frequent cancer (25%) among the male cancer patients where male female cancer patient ratio is 6.1:1 [3].

The causes of lung cancer can be extremely complex. However, the epidemiological studies have found that lung cancer relics partially unresolved since the vast majority of tobacco users do not develop such tumors while at least 10-15% of lung cancers occur in non smokers [4]. Thus, etiological factors such as cooking fumes, work-related factors (asbestos, radon), arsenic, environmental pollution may also have an impact as risk factors for lung cancer [5-9].

Different studies suggested that apart from etiological factors, viruses have been implicated in human lung carcinogenesis to develop lung cancer [10-12]. These viral agents can manipulate the cell cycle, hamper cell apoptosis, and also interrupt the cell division in host cell. Human papilloma virus is one of such agents. Recent research has found that Infection with possible types of Human papilloma virus such as Human papilloma virus 16/18 that leads to lung cancer and also known as the main causative event for almost all the cervical, penile, vaginal, vulvar, and anal cancers [13-16].

Research into the association between Human papilloma virus and lung cancer has been proposed in a number of countries. Many epidemiological studies have reported on the prevalence of carcinogen human papilloma virus in lung cancer are highly variable and contradictory. Prevalence of pulmonary Human papilloma virus infection in Western world and Asia, ranges from 0% to 36% and 9% to 78% respectively [17-19], where the worldwide frequency is 20% to 25% [20-23]. But some data from Japan and Northern European countries, such as Finland and Norway, revealed that prevalence of carcinogen Human papilloma virus infection in lung cancer notably higher frequency of 69% to78.3% [24, 25]. Therefore, it is rationally suggested that, the association between infection of carcinogen or high-risk Human papilloma virus and lung cancer is geography and race dependent.

The first Human papilloma virus was seen in human warts in 1907 and isolated from the rabbit in 1983 [26]. Human papilloma virus (family *Papillomaviridae*, genus *alpha-papilloma virus*) are diverse group of small non-enveloped oncogenic virus (about 55nm in size) that infects the mucosal and cutaneous epithelia of a broad variety of higher vertebrates [27]. Currently, more than 200 different Human papilloma virus types have been identified by DNA sequencing. In addition, these viruses are also grouped into high- and low- risk types based on the risk of the virus to cause squamous cell carcinomas in the uterine cervix. Alpha-papilloma virus types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 have been classified by the WHO as carcinogenic virus or high-risk in humans (carcinogens type 1) due to their high prevalence in cervical cancer samples and other cancer types [28] and non-carcinogenic or low-risk types 6, 11, 40, 42, 43, 44, and 54 are co-related with the development of genital warts [29].

The Human papilloma virus genome consists of a circular double stranded DNA comprising almost 8Kb as their genetic material and closely related to plyoma virus [30]. The viral genome encodes eight open-reading frames that can be divided into three functional regions; the early (E) region that encodes six regulatory proteins (E1, E2, E4, E5, E6, and E7) required for viral replication and transcription, the late (L) region that encodes the viral Capsid proteins (L1 and L2), and a non-coding region (Long control region, LCR) lies between early and late region which contains cis-elements necessary for viral DNA replication and transcription regulation [31]. The six early proteins; E1/E2: protein that control the function of E6 and E7 genes, E4: protein function basically unknown but may control virus release from host cell, E5: hydrophobic protein which enhances immortalization of the cell, E6: oncogenic protein which inhibit negative regulations of the cell cycle and apoptosis by binding to the p53 and E7: viral oncoprotein interferes with the pRB function contributes to the development of malignancy [30]. Therefore, the E6 and E7 act as mitotic mutators which manipulate cell proliferation, senescence and apoptosis [32].

Growing recognition of the significance of RNA is shedding light on diseases and on how it might be treated-particularly throughout a process called RNA interference (RNAi). RNAi technology is a promising research tool for use in functional genomics, and is also shows potential for use in future RNA-based therapeutics. It has become one of the most exciting frontiers in medicine, in such short order that two of its pioneers, Andrew Fire and Craig Mellow, won the 2006 Nobel Prize for Medicine, just eight years after their key work was published [33]. RNAi relies on double-stranded RNA molecules called siRNAs (short interfering RNAs), each about 21 units in length. siRNAs interfere with the activity of genes that generate the same sequence in mRNA, so that lower quantities of proteins produced. These provoke the down regulation of gene expression in a very sequence-specific manner by the aid of different enzymes [34]. It can be introduce into the cell for knockdown of a gene of interest by using various methods [35]. The technique's medical potential lies in its ability to target particular genes and their protein products with great precision. RNAi can therefore be used to switch off rouge genes, of the sort that drive cancer or their disorders, without messing up the chemistry of healthy cells. As a result, siRNA may be a useful RNA-based therapeutic tool, siRNA-mediated transcriptional silencing has to be efficient, specific, and causes decreased tumor growth. So siRNA may also be used for the therapeutic purposes as chemical drugs [36].

In this computational approach, to design a potential RNA-based therapeutics to degrade the E6 and E7 oncogenes of Human papilloma virus types 16 & 18 on the principle of post-transcriptional gene silencing mechanism.

2. Methods and Materials

2.1. Retrieval of Sequences

The nucleotide sequences of E6 and E7 gene of Human papilloma virus types 16 & 18 were collected from the NCBI database [37] with the following accession numbers (gi|310698439, gb|AY262282.1).

2.2. Prediction of siRNA with Target Position

Prediction of functional siRNA with target positions of these nucleotides sequences of E6 and E7 gene of Human papilloma virus were carried out with the help of the siDirect 2.0 [38]. It is followed some rules like Ui-Tei, Amarz-guiout, Renold rules and melting temperature (*T*m) should be below

21.5°C for potential siRNA duplex [39-41].

2.3. Alignment of Target Position

Alignment of the selected siRNA target positions was constructed under default conditions by using Clustal W program [42].

2.4. Calculation of GC Content and Checking of off-Target Sequence

DNA/RNA GC content calculator program was used to calculate the GC content of the selected siRNA [43]. The blast tool program was used for the checking of off-target sequence resemblance in human genome transcript [44]. This program employed against whole Genebank database under default conditions.

2.5. Prediction of siRNA Secondary Structure and RNA-RNA Interaction

The mfold server was used to calculate the free energy of folding of siRNA [45]. Prediction of RNA-RNA interaction like thermodynamics of interaction between the target gene and predicted siRNA with hybridization energy, RNAcofold program was used [46]. This program functions as an extension of McCaskill's partition function algorithm to calculate probabilities of base pairing, rational interaction energies and the equilibrium concentration of duplex structures.

Flow chart showing the complete approaches used for screening of effective siRNA molecules against E6 and E7 gene of Human papilloma virus types 16 & 18 [Figure. 1].

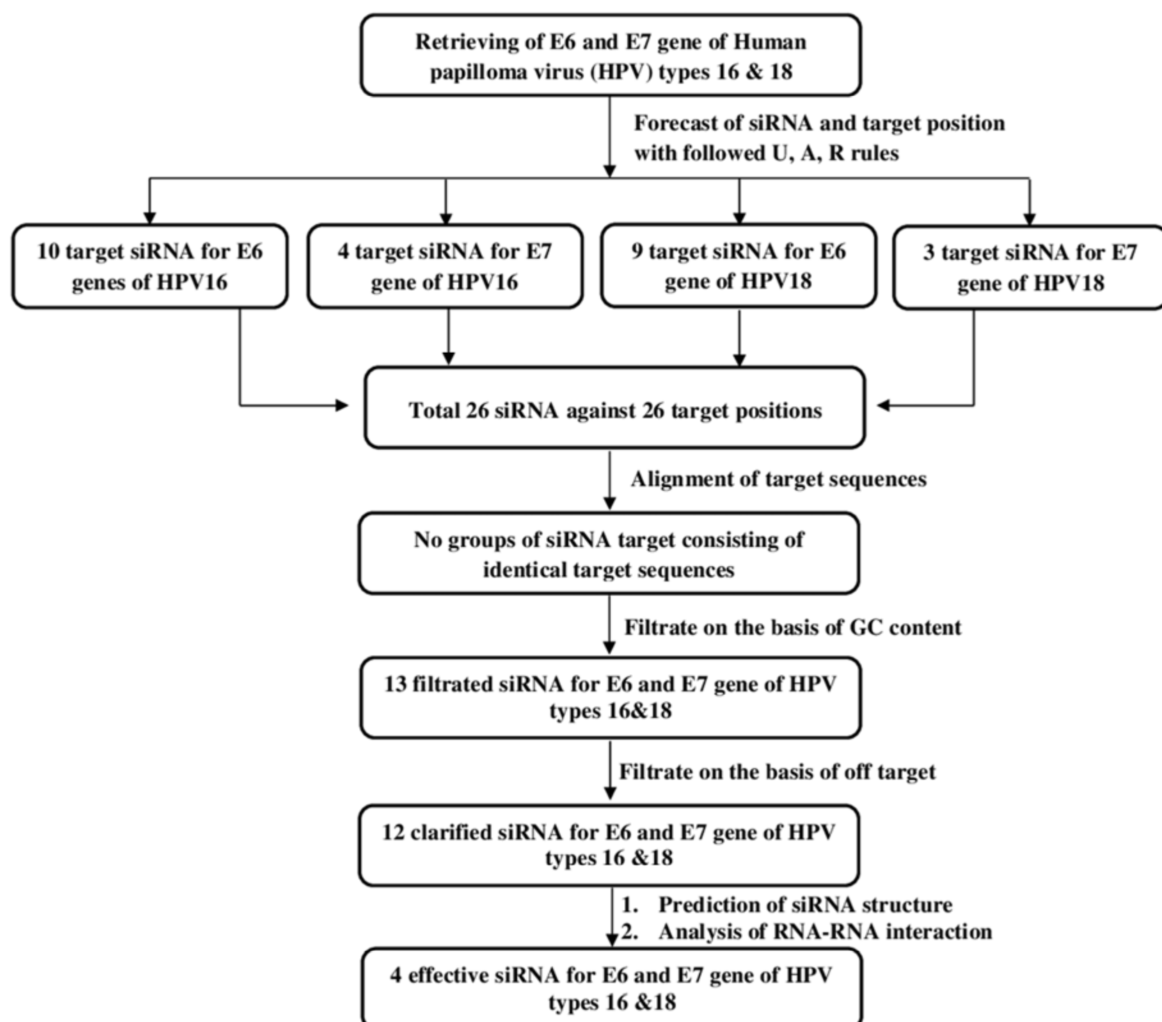

Figure 1. *Flow chart showing the complete approaches used for screening of effective siRNA against oncogenic E6 and E7 genes of Human papillomavirus types 16 & 18 in this study.*

3. Results & Discussion

This present study was conducted with E6 and E7 gene of Human papilloma virus types 16 & 18. Gene sequences available in the viral database from NCBI, were retrieved [37]. All the retrieved nucleotide sequences were used for the construction of target siRNA by siDirect 2.0 program [38]. siDirect 2.0 follow the algorithms of Ui-Tei, Amarzguioui and Reynolds and other criteria to predict and depict the potential target siRNA [47].

In this study, found total 26 siRNA against 26 target

sequences in E6 and E7 gene of Human papilloma virus types 16 & 18. Among them, 10 and 4 target siRNA for E6 gene and E7 gene of Human papilloma virus type 16 respectively, 9 and 3 target siRNA for E6 gene and E7 gene of Human papilloma virus type 18 respectively which fulfilled all the criteria and algorithms of Ui-Tei, Amarguioui and Reynolds Therefore, maximum 10 siRNA target positions were found for E6 and E7 gene of Human papilloma virus types 16 & 18 [Table 2].

```
>gb|AY262282.1 HPV18E6      ---AAGACAGTATTGGAACTTACAGA----------   23
>gb|AY262282.1 HPV18E6      ------------TTGGAACTTACAGAGGTATTTGA-   23
>gb|AY262282.1 HPV18E6      -AGGTATTTGAATTTGCATTTAAA-----------    23
>gb|AY262282.1 HPV18E6      -------TTGAATTTGCATTTAAAGATTTA------   23
>gb|AY262282.1 HPV18E7      ------------TTCTATGTCACGAGCAATTAAGC    23
>gi|310698439  HPV16E7      --GACAACTGATCTCTACTGTTATG-----------   23
>gi|310698439  HPV16E7      ------------CTCTACTGTTATGAGCAATTAAA-   23
>gb|AY262282.1 HPV18E7      GTCACACAATGTTGTGTATGTGT------------    23
>gi|310698439  HPV16E6      -------------GAGTATAGACATTATTGTTATAG   23
>gi|310698439  HPV16E6      ----ATCCATATGCTGTATGTGATAAA---------   23
>gi|310698439  HPV16E6      -------------CTGTATGTGATAAATGTTTAAAG   23
>gi|310698439  HPV16E7      -----TACTTTGGAA------GACCTGTTAATGG--   23
>gi|310698439  HPV16E7      --ACCTACATTGCATGAA-TATATGT----------   23
>gb|AY262282.1HPV18E6       -AACTAACACTGGGTTAT-ACAATT-----------   23
>gb|AY262282.1HPV18E6       ---------CTGGGTTAT-ACAATTTATTAATA---   23
>gb|AY262282.1HPV18E6       -----------GGGTTAT-ACAATTTATTAATAAG-   23
>gb|AY262282.1HPV18E7       -----------GCCAGAA-TTGAGCTAGTAGTAGA-   23
>gb|AY262282.1HPV18E6       ---GTGGTGTATAGAGAC---AGTATACC-------   23
>gb|AY262282.1HPV18E6       -------TAGAAATAACC-TGTGTATATTGC-----   23
>gi|310698439  HPV16E6      --CGGGATTTA----------TGCATAGTATATAG-   23
>gi|310698439  HPV16E6      ----------AAGCAAAG-ATTCCATAATATAAG--   23
>gi|310698439  HPV16E6      -----------AGCAAAG-ATTCCATAATATAAGG-   23
>gi|310698439  HPV16E6      --CTGCAAACAACTATAC-ATGATAT----------   23
>gi|310698439  HPV16E6      ---TGCAAACAACTATAC-ATGATATA---------   23
>gi|310698439  HPV16E6      ------AACAACTATAC-ATGATATAATAT-        23
>gi|310698439  HPV16E6      ----------AACTATAC-ATGATATAATATTAG--   23
```

Figure 2. *Predicted siRNA target sequences by Clustal W.*

Multiple sequence alignment was done to sort out these target siRNA into groups and design a common siRNA against more than one target. Multiple sequence alignment of all 26 targets siRNA produced a result, in which there is no group of siRNA target consisting of identical target sequences. [Figure. 2].

The GC content of siRNA is an important contender for a parameter that represents as the functionality of siRNA. It typically recommended to designing potential siRNA that has low GC content within the range of 31.5%-57.9%, cause of significant negative correlation between GC-content and RNAi activity Here, GC-content of 26 siRNA against 26 target sequence observed within the range of GC-content from 14% to 42%. All the siRNA were filtrated into 13 siRNA on the basis of GC content (between 31.5% to 57.9%) [Table 2].

To reduce off-target effect, T_m should be less then 21.5°C [49]. Based on the nearest neighbor model with the thermodynamic parameter, T_m was calculated for the seed-target duplex. In the siRNA tool, predicted siRNA with minimum T_m value at the seed region and result of siDirect defines no possibilities for off targets silencing. All the filtrated 13 siRNA were clarified by Blast similarity search of whole human genome.

Result from the off-target similarities, found that 12 clarified siRNA target against 12 designed siRNA. This aided the contrition of common siRNA against multiple genes of Human papilloma virus types 16 & 18.

12 clarified siRNA were analyzed with following different parameters to demonstrate their performance. Prediction of thermodynamic of RNA-RNA interaction which is used for siRNA efficiency of these clarified siRNA was subjected to Vienna RNA program tool. This program tool is an abundant collection of program, web services that offer algorithms for RNA folding, assessment and prediction of RNA-RNA interaction. RNA-RNA interaction of these siRNA with their target sequence was predicted using RNAcofold program.

These clarified 12 siRNA with target sequences were sort out into 4 siRNA on the basis of lowest hybridization energy of binding between siRNA (*, **,***,****) with target sequence (a, b, c, d) and also compute the stability of the clarified siRNA (guide strand), the minimum free energy (kcal/mol) of the optimal folding was calculated by using mfold program followed by most used algorithms for the prediction of RNA secondary structure, based on the minimal free energy state for exploring effective folding of siRNA (guide strand) (Table 1). Earlier study has recommend that a guide strand siRNA must have smallest free energy for their stability [50].

Table 1. *Four designed siRNA molecules with GC%, free energy of folding and free energy of binding with target.*

Target sequence	Location of target within mRNA	siRNA target within target	Designed siRNA duplex at 37°C	GC%	Free energy of folding	Free energy of binding with target
a	76-98	CTGCAAACAACTATACATGATAT	AUCAUGUAUAGUUGUUUGCAG * GCAAACAACUAUACAUGAUAU	33%	1.09	-31.80
b	231-253	TACTTTGGAAGACCTGTTAATGG	AUUAACAGGUCUUCCAAAGUA ** CUUUGGAAGACCUGUUAAUGG	33%	-0.64	-32.30
c	279-301	AACTAACACTGGGTTATACAATT	UUGUAUAACCCAGUGUUAGUU *** CUAACACUGGGUUAUACAAUU	33%	-0.88	-32.20
d	208-230	ACGGATCATAGGTGATGAAGAGA	UACUACUAGCUCAAUUCUGGC **** CAGAAUUGAGCUAGUAGUAGA	42%	-5.6	0.58

Here, four designed consensus siRNA molecules: * siRNA A, ** siRNA B, *** siRNA C and **** siRNA D for oncogenic E6 and E7 genes of Human papillomavirus type 16 & 18.

Table 2. *Predicted siRNA target sequences with GC% for oncogenic E6 and E7 genes of Human papillomavirus types 16 & 18.*

S/N	accession number	Oncogenes	Target	Location of target position	siRNA target sequence	Designed siRNA	GC%
1			1	76-98	CTGCAAACAACTATACATGATAT [d]	AUCAUGUAUAGUUGUUUGCAG GCAAACAACUAUACAUGAUAU	33%
2			2	77-99	TGCAAACAACTATACATGATATA [d]	UAUCAUGUAUAGUUGUUUGCA CAAACAACUAUACAUGAUAUA	28%
3			3	81-103	AACAACTATACATGATATAATAT [d]	AUUAUAUCAUGUAUAGUUGUU CAACUAUACAUGAUAUAAUAU	19%
4			4	84-106	AACTATACATGATATAATATTAG [d]	AAUAUUAUAUCAUGUAUAGUU CUAUACAUGAUAUAAUAUUAG	14%
5			5	163-185	CGGGATTTATGCATAGTATATAG [d]	AUAUACUAUGCAUAAAUCCCG GGAUUUAUGCAUAGUAUAUAG	33%
6	gi|310698439	E6	6	194-216	ATCCATATGCTGTATGTGATAAA [d]	UAUCACAUACAGCAUAUGGAU CCAUAUGCUGUAUGUGAUAAA	33%
7			7	203-225	CTGTATGTGATAAATGTTTAAAG [d]	UUAAACAUUUAUCACAUACAG GUAUGUGAUAAAUGUUUAAAG	23%
8			8	244-266	GAGTATAGACATTATTGTTATAG [d]	AUAACAAUAAUGUCUAUACUC GUAUAGACAUUAUUGUUAUAG	23%
9			9	385-407	AAGCAAAGATTCCATAATATAAG [d]	UAUAUUAUGGAAUCUUUGCUU GCAAAGAUUCCAUAAUAUAAG	23%
10			10	386-408	AGCAAAGATTCCATAATATAAGG [d]	UUAUAUUAUGGAAUCUUUGCU CAAAGAUUCCAUAAUAUAAGG	23%
11			1	15-37	ACCTACATTGCATGAATATATGT [b]	AUAUAUUCAUGCAAUGUAGGU CUACAUUGCAUGAAUAUAUGU	28%
12	gi|310698439	E7	2	54-76	GACAACTGATCTCTACTGTTATG [b]	UAACAGUAGAGAUCAGUUGUC CAACUGAUCUCUACUGUUAUG	38%
13			3	64-86	CTCTACTGTTATGAGCAATTAAA [b]	UAAUUGCUCAUAACAGUAGAG CUACUGUUAUGAGCAAUUAAA	33%
14			4	231-253	TACTTTGGAAGACCTGTTAATGG [b]	AUUAACAGGUCUUCCAAAGUA CUUUGGAAGACCUGUUAAUGG	33%
15			1	83-105	TAGAAATAACCTGTGTATATTGC [c]	AAUAUACACAGGUUAUUUCUA GAAAUAACCUGUGUAUAUUGC	28%
16			2	106-128	AAGACAGTATTGGAACTTACAGA [c]	UGUAAGUUCCAAUACUGUCUU GACAGUAUUGGAACUUACAGA	33%
17			3	115-137	TTGGAACTTACAGAGGTATTTGA [c]	AAAUACCUCUGUAAGUUCCAA GGAACUUACAGAGGUAUUUGA	33%
18			4	128-150	AGGTATTTGAATTTGCATTTAAA [c]	UAAAUGCAAAUUCAAAUACCU GUAUUUGAAUUUGCAUUUAAA	23%
19	gb|AY262282.1	E6	5	134-156	TTGAATTTGCATTTAAAGATTTA [c]	AAUCUUUAAAUGCAAAUUCAA GAAUUUGCAUUUAAAGAUUUA	19%
20			6	160-182	GTGGTGTATAGAGACAGTATACC [c]	UAUACUGUCUCUAUACACCAC GGUGUAUAGAGACAGUAUACC	38%
21			7	279-301	AACTAACACTGGGTTATACAATT [c]	UUGUAUAACCCAGUGUUAGUU CUAACACUGGGUUAUACAAUU	33%
22			8	287-309	CTGGGTTATACAATTTATTAATA [c]	UUAAUAAAUUGUAUAACCCAG GGGUUAUACAAUUUAUUAAUA	23%
23			9	289-311	GGGTTATACAATTTATTAATAAG [c]	UAUUAAUAAAUUGUAUAACCC GUUAUACAAUUUAUUAAUAAG	19%
24	gb|AY262282.1	E7	1	74-96	TTCTATGTCACGAGCAATTAAGC [a]	UUAAUUGCUCGUGACAUAGAA CUAUGUCACGAGCAAUUAAGC	33%

S/N	accession number	Oncogenes	Target	Location of target position	siRNA target sequence	Designed siRNA	GC%
25			2	173-195	GTCACACAATGTTGTGTATGTGT [a]	ACAUACACAACAUUGUGUGAC CACACAAUGUUGUGUAUGUGU	38%
26			3	208-230	UACUACUAGCUCAAUUCUGGC [a]	UACUACUAGCUCAAUUCUGGC CAGAAUUGAGCUAGUAGUAGA	42%

Here, Predicted maximum 10 siRNA target position of E6 and E7 oncogenes of Human papillomavirus types 16 and 18: 3 target position, 4 target positions, 9 target positions and 10 target positions were represented by 4 alphabet: a, b, c and d character respectively.

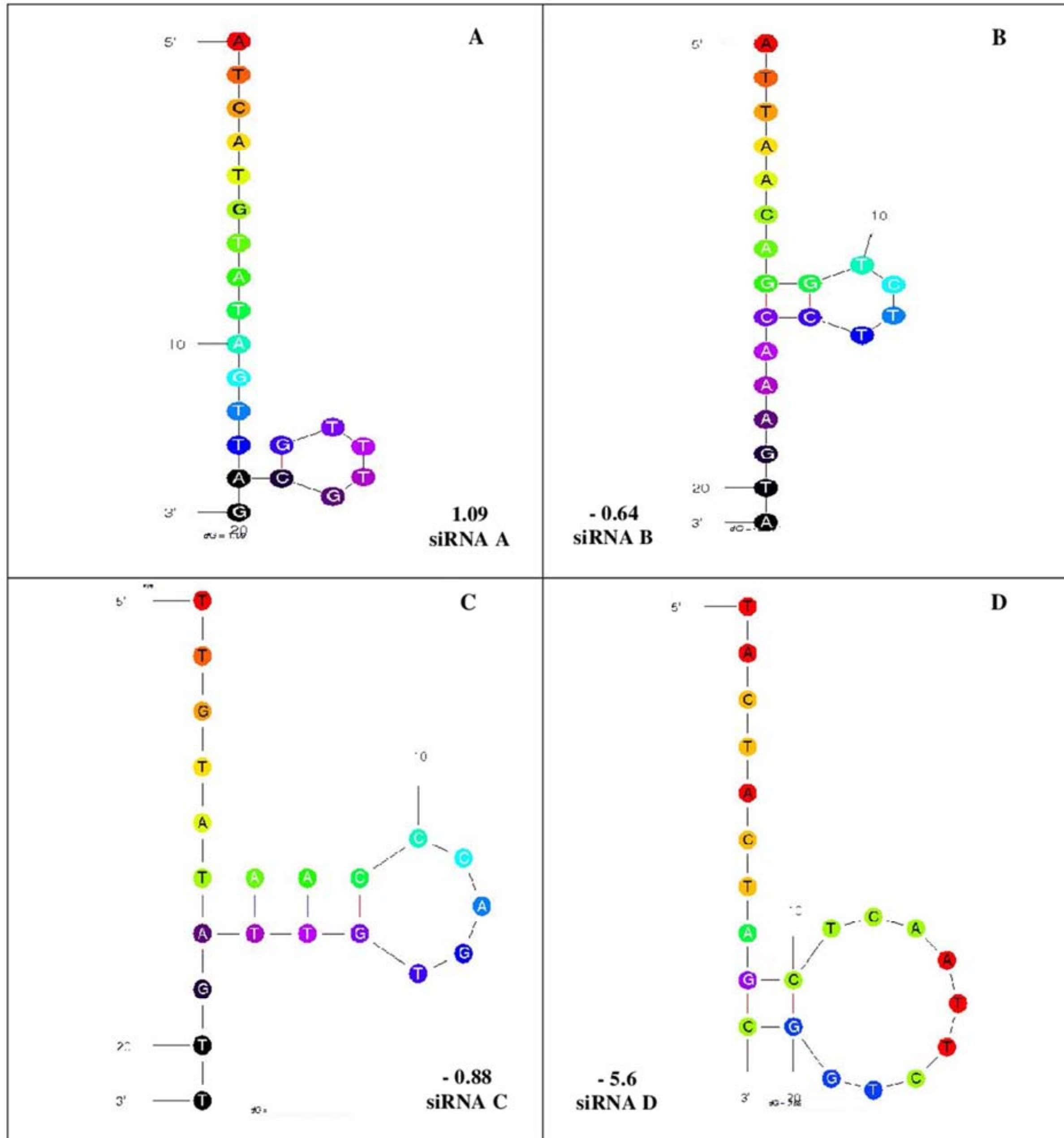

Figure 3. *Predicted 4 siRNA (A, B, C and D) secondary structures with possible folding and minimum free energy.*

In this study free energy of folding (Kcal/mol) of 4 siRNA (A, B, C and D) were 1.09 Kcal/mol,-0.64 Kcal/mol, -0.88 Kcal/mol, -5.6 Kcal/mol respectively [Figure. 3] and on the other hand, hybridization energy of binding of these siRNA with target sequence was found within the range from 0.58 Kcal/mol to -32.20 Kcal/mol [Table 1].

In conclusion, these observations support the findings for the efficiency of siRNA against their target by used all parameter. This study successfully designed 4 siRNA (A, B, C and D) against 4 target (a, b, c, and d) which fulfill all the criteria of a siRNA as antiviral therapeutic agent (Table 1). So, these potential Antiviral siRNA might be used as

potential contender within the advanced RNAi treatment of E6 and E7 gene of Human papilloma virus 16/18.

4. Conclusion

This work supports the hypothesis that rate of infection and degree of oncogenicity can be reduced by our designed siRNA through RNAi technology. Apart from this, investigation will help to make effective rational siRNA against E6 and E7 oncogene of Human papilloma virus types 16 & 18 for therapeutic application. However, experimental approaches and validation will be required for establishing this hypothesis. The outcome of this study provides a basis to the researchers towards understanding the development of an antiviral RNA as a therapeutic at genomic level.

Conflict of Interest

All the authors do not have any possible conflicts of interest.

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