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# Bioinformatics analysis of Exonic Splicing Enhancers (ESEs) for predicting potential regulatory elements of hTERT mRNA Splicing

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**Abstract. – OBJECTIVES: Alternative splicing of human telomerase reverse transcriptase (hTERT) has an important effect on regulating telomerase activity. Exonic splicing enhancers (ESEs) are a family of conserved splicing factors that participate in multiple steps of the splicing pathway. Our aim is to analyze the ESEs for predicting the potential regulatory elements of hTERT mRNA splicing.**

**MATERIALS AND METHODS: Enter the FASTA format of hTERT total sequences or individual exon as the input data in the main interface of ESEfinder3.0 and ESEfinder2.0 program. Analyze the data of output results and compare the differences between ESEfinder3.0 and ESEfinder2.0 program.**

**RESULTS: Five ESEs were predicted in exon 5 to exon 9 of hTERT. They were at position 108 located in hTERT exon 5, at position 92 located in exon 6, at position 22 located in exon 7, at position 73 located in exon 8 and at position 5 located in exon 9. There were no differences between ESEfinder 3.0 and ESEfinder 2.0 in our case.**

**CONCLUSIONS: The identification of these potential ESEs of hTERT might be helpful for the design of antisense oligonucleotides, which could modulate hTERT alternative splicing and inhibit telomerase activity.**

*Key Words:*
Exonic splicing enhancer, Telomerase, hTERT, Alternative splicing, Transcription regulation.

## Abbreviations

hTERT = human telomerase reverse transcriptase; hTR = human telomerase RNA component; ASVs = alternatively spliced variants; ESEs = Exonic splicing enhancers; ORF = open reading frame; RRM = RNA-recognition motif.

## Introduction

Telomeres are specialized structures located at the ends of eukaryotic chromosomes, that protect linear chromosome ends from unwanted repair or recombination. Progressive telomere shortening occurs in somatic cells due to incomplete replication of chromosome ends. Telomerase, a ribonucleoprotein complex, is capable of adding telomere repeats to the 3' end, which is essential for the telomere length maintenance in germ cells and stem cells[1]. Human telomerase holoenzyme consists of three components: a RNA component (human telomerase RNA component, hTR), which serves as a template for DNA replication., a catalytic subunit of telomerase with reverse transcriptase activity (human telomerase reverse transcriptase, hTERT) and the telomerase-associated proteins[2,3]. Most of the human tissues that lack detectable telomerase activity also lack the expression of hTERT but not hTR and telomerase-associated proteins. This suggests hTERT acts as a rate-limiting factor for telomerase activity[4,5].

Telomerase activity is under precise control at the transcriptional, translational, and post-translational level. hTERT gene pre-mRNA alternative splicing is thought be one of the most important mechanisms for regulation of telomerase activity. Alternative splicing is a process by which the exons of the pre-mRNA produced by transcription of a eukaryotic gene are reconnected in multiple ways to form alternatively spliced variants (ASVs) during RNA splicing. ASVs may be translated into different protein isoforms. Alternative splicing is a major method of gene regulation for many proteins[6,7].



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The hTERT gene consists of 16 exons and 15 introns and full length of hTERT mRNA is about 4.0 kb long[8]. hTERT protein contains a telomerase-specific motif and seven reverse transcriptase motifs. To date, seven alternatively spliced sites in the hTERT mRNA have been identified, including three deletion sites (α, β, γ) and four insertion sites. Therefore, the alternate splicing sites produce a large number of possible combinations, resulting in different hTERT ASVs. Currently, a few hTERT ASVs have been identified in mammalian cells. However, only the full-length hTERT mRNA without deletion or insertion sites has telomerase activity *in vivo*.

Most studies on hTERT ASVs involve deletions at the two main splicing sites. Deletion of the α splicing site leads to a deletion of 36 nucleotides in exon 6, which removes most of the reverse transcriptase (RT) motif without disrupting the open reading frame (ORF). Splicing at the β site (deletion of exons 7 and 8) results in a 182-nucleotide deletion that causes a premature termination codon, generating a truncated protein lacking the C-terminal and RT motifs B to E. Splicing at either site, occurring alone or in combination, produces a number of ASVs at different levels and proportions. So far, none of the ASVs identified retains the telomerase catalytic activity of the full-length product. Nevertheless, some recent data suggests that splicing patterns of hTERT may play a role in physiological and pathological regulation of telomerase activity, and that ASVs are also possibly involved in other cellular functions[9,10].

Exonic splicing enhancers (ESEs) are the regulators of pre-mRNA alternative splicing[4] and participate in the splicing of constitutive exons[11-13]. ESEs bind to the members of the serine/arginine-rich (SR) protein family, which function both as general splicing factors and as regulators of alternative splicing[14-17]. SR proteins have a motif consisting of one or two copies of an RNA-recognition motif (RRM) followed by a C-terminal domain highly enriched in argine/serine dipeptides (RS domain)[18,19]. ESE-bound SR proteins recruit the splicing machinery through their RS domains, and/or antagonize the splicing silencer elements[20,21].

hTERT, the rate-limiting subunit, plays a crucial role in regulating telomerase activity. Therefore, the alternative splicing of the hTERT gene may make significant influence on telomerase activity at the transcriptional level. In this article, we will analyze the ESEs of hTERT to predict the potential regulatory elements of telomerase activity, which might become attractive gene therapy targets.

## Materials and Methods

### Principle of ESEfinder

ESEfinder is a web-based program applied in rapid analysis of exon sequences to identify putative exonic splicing enhancers (ESE) responsive to the human SR proteins SF2/ASF, SC35, SRp40 and SRp55, and to predict whether exonic mutations disrupt such elements. This program was released by Cold Spring Harbor Laboratory (Krainer's Lab and Zhang's Lab) and has been updated to Version 3.0 since 2007. The homepage for ESEfinder3.0 is http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home and the homepage for ESEfinder2.0 is http://rulai.cshl.edu/tools/ESE2/ (Figure 1). The ESEfinder 2.0 program


**Figure 1.** The main operating interface of ESEfinder 3.0 (*left*) and ESEfinder 2.0 (*right*).

searches for sequences that act as binding sites for four members of the SR splicing enhancer proteins. Input sequences are screened for consensus binding sequences for the SR proteins SF2/ASF, SRp40, SRp55 and SC35, using the SELEX (systematic evolution of ligands by exponential enrichment) method. The program scores the input sequences according to fit with the loose consensus sequences. The scores above a default threshold value are predicted to act as binding sites with SR protein and thus function as ESEs. Increased threshold values of ESEfinder 2.0 program for SF2/ASF (from 1.956) and ESEfinder 3.0 for SRp40 (from 2.670), SRp55 (from 2.676) and SC35 (from 2.383) were used in order to minimize false-positive results. In ESEfinder 2.0, only the high-score values (the values above the selected threshold) are represented. There are two options for output information in ESEfinder 3.0, one is "Report only the best hit in each sequence (instead of hits above the thresholds)" and the other is "Report all scores in each sequence (instead of hits above the thresholds)"[22,24].

### Methods and Procedure

Find nucleic acid sequence of hTERT (GenBank: AH007699.1) from NCBI (http://www.ncbi.nlm.nih.gov/) and save the information in FASTA format. Open the homepage of ESEfinder2.0 and ESEfinder3.0. The α splicing site is located in exon 6 and deletion of the β site is located in exon 7 and exon 8, therefore, the sequences of each exon between exon 5 and exon 9 were chosen as the input sequences. The sequences of hTERT exon 5 to exon 9 were shown in Table I. They were predicted both by ESEfinder 3.0 and by ESEfinder 2.0. In ESEfinder 3.0, "Report only the best hit in each sequence (instead of hits above the thresholds)" was checked and the "Send" button was clicked to generate output data. In ESEfinder 2.0 "Send" button was clicked to generate output data directly.

## Results

### Prediction of Each Exon Between Exon 5 and Exon 9 of hTERT by ESEfinder3.0 (Table II)

Predicted by ESEfinder 3.0, the highest scored position for each SR protein in each exon between exon 5 and exon 9 is presented here. Successfully, we predicted the ESEs at position 108 (from 5' end), which got the highest score in exon 5 of hTERT, the SR protein was SRSF5 (SRp40) and the motif was CCACAGG. In exon 6, the ESEs was at position 92, the SR protein was SRSF6 (SRp55) and the motif was TGCGTC. In exon 7, the ESEs was at position 22, the SR protein was SRSF1 (SF2/ASF) and the motif was CAGCCGT. In exon 8, the ESEs was at position 73, the SR protein was SRSF6 (SRp55) and the motif was CGCATC. In exon 9, the ESEs was at position 5, the SR protein was SRSF6 (SRp55) and the motif was TACGTC (Table II, Figure 2 to 6).

### Prediction of Individual Exon 5 to Exon 9 of hTERT by ESEfinder2.0

Only the high-score values (above the selected threshold) are mapped on the output graph. In the color-coded bars, the height of the bars represents the motif scores, the width of the bars indicates the length of the motif (6, 7 or 8 nucleotides), and the color of the bars indicates var-

**Table I.** The sequences of hTERT exon 5 to exon 9.

| Exon of hTERT | Sequence |
|---|---|
| Exon 5 (180 nt) | GCCGAGCGTCTCACCTCGAGGGTGAAGGCACTGTTCAGCGTGCTCAACTACG-AGCGGGCGCGGCGCCCCGGCCTCCTGGGCGCCTCTGTGCTGGGCCTGGACGATATC-CACAGGGCCTGGCGCACCTTCGTGCTGCGTGTGCGGGCCCAGGACCCGCCGCCT-GAGCTGTACTTTGTCAAG |
| Exon 6 (156 nt) | GTGGATGTGACGGGCGCGTACGACACCATCCCCCAGGACAGGCTCACGGAGGTC-ATCGCCAGCATCATCAAACCCCAGAACACGTACTGCGTGCGTCGGTATGCCGTG-GTCCAGAAGGCCGCCCATGGGCACGTCCGCAAGGCCTTCAAGAGCCAC |
| Exon 7 (96 nt) | GTCTCTACCTTGACAGACCTCCAGCCGTACATGCGACAGTTCGTGGCTCACCTG-CAGGAGACCAGCCCGCTGAGGGATGCCGTCGTCATCGAGCAG |
| Exon 8 (86 nt) | AGCTCCTCCCTGAATGAGGCCAGCAGTGGCCTCTTCGACGTCTTCCTACGCTTCATG-TGCCACCACGCCGTGCGCATCAGGGGCAA |
| Exon 9 (114 nt) | GTCCTACGTCCAGTGCCAGGGGATCCCGCAGGGCTCCATCCTCTCCACGCTGCTCTG-CAGCCTGTGCTACGGCGACATGGAGAACAAGCTGTTTGCGGGGATTCGGCGGGACGG |

**Table II.** Prediction of individual exons by ESEfinder3.0.

| Exon of hTERT | SR Protein | Position# | Motif | Score |
|---|---|---|---|---|
| Exon 5 | SRSF1 (SF2/ASF) | 109 (-72) | CACAGGG | 5.25819 |
| | SRSF1 (SF2/ASF, IgM-BRCA1) | 109 (-72) | CACAGGG | 4.75761 |
| | SRSF2 (SC35) | 71 (-110) | GCCTCCTG | 4.18474 |
| | SRSF5 (SRp40) | 108 (-73) | CCACAGG | 5.96771* |
| | SRSF6 (SRp55) | 59 (-122) | CGCGGC | 4.49126 |
| Exon 6 | SRSF1 (SF2/ASF) | 47 (-110) | CGGAGGT | 4.93828 |
| | SRSF1 (SF2/ASF, IgM-BRCA1) | 47 (-110) | CGGAGGT | 4.88224 |
| | SRSF2 (SC35) | 41 (-116) | GGCTCACG | 4.29958 |
| | SRSF5 (SRp40) | 144 (-13) | CTTCAAG | 4.04055 |
| | SRSF6 (SRp55) | 92 (-65) | TGCGTC | 6.13588* |
| Exon 7 | SRSF1 (SF2/ASF) | 22 (-75) | CAGCCGT | 4.99607* |
| | SRSF1 (SF2/ASF, IgM-BRCA1) | 22 (-75) | CAGCCGT | 4.38164 |
| | SRSF2 (SC35) | 16 (-81) | GACCTCCA | 4.23723 |
| | SRSF5 (SRp40) | 2 (-95) | TCTCTAC | 3.62094 |
| | SRSF6 (SRp55) | 32 (-65) | TGCGAC | 3.53067 |
| Exon 8 | SRSF1 (SF2/ASF) | 21 (-66) | CAGCAGT | 2.47234 |
| | SRSF1 (SF2/ASF, IgM-BRCA1) | 66 (-21) | CGCCGTG | 3.10673 |
| | SRSF2 (SC35) | 18 (-69) | GGCCAGCA | 3.96960 |
| | SRSF5 (SRp40) | 60 (-27) | CCACCAC | 2.82027 |
| | SRSF6 (SRp55) | 73 (-14) | CGCATC | 4.69647* |
| Exon 9 | SRSF1 (SF2/ASF) | 27 (-88) | CACAGGG | 4.81097 |
| | SRSF1 (SF2/ASF, IgM-BRCA1) | 27 (-88) | CACAGGG | 5.05584 |
| | SRSF2 (SC35) | 21 (-94) | GGATCCCG | 4.56569 |
| | SRSF5 (SRp40) | 40 (-75) | CCTCTCC | 3.52645 |
| | SRSF6 (SRp55) | 5 (-110) | TACGTC | 5.52722* |

#Positions from 5'end (through 1) and 3'end (through -1) are given; *The highest score (the best hit) in prediction of each exon of hTERT by ESEfinder 3.0.

ious SR protein, other motifs or the splicing factor predicted. The score value is the most intuitive and the highest bar represents the motif of the maximum score value, where the probability of the position to become an ESE is the highest (Figure 7 to 11) The summarized results and

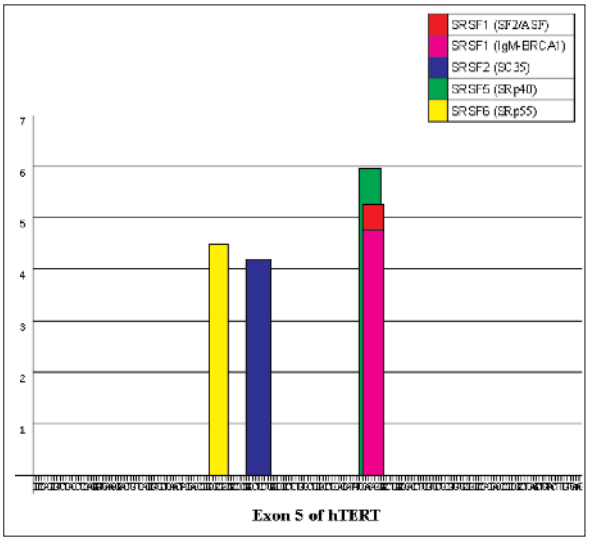


**Figure 2.** Graph output of hTERT exon 5 by ESEfinder3.0 (*Indicates SRp40 protein gets the highest score of 5.967709 in position 108, green bar).

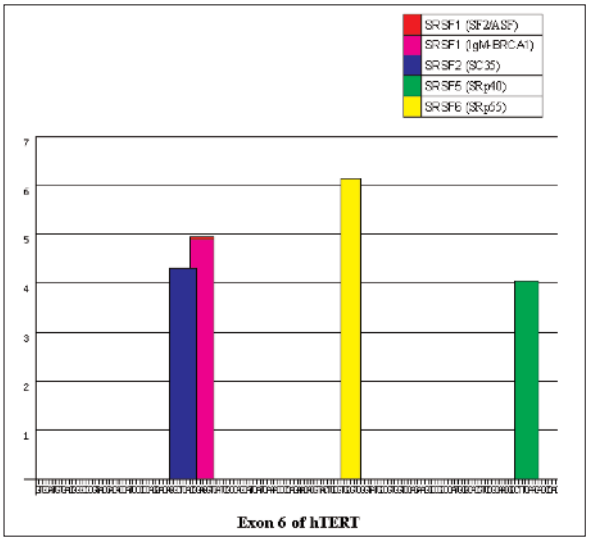


**Figure 3.** Graph output of hTERT exon 6 by ESEfinder3.0. (*Indicates SRp55 protein gets the highest score of 6.135878 in position 92, yellow bar).

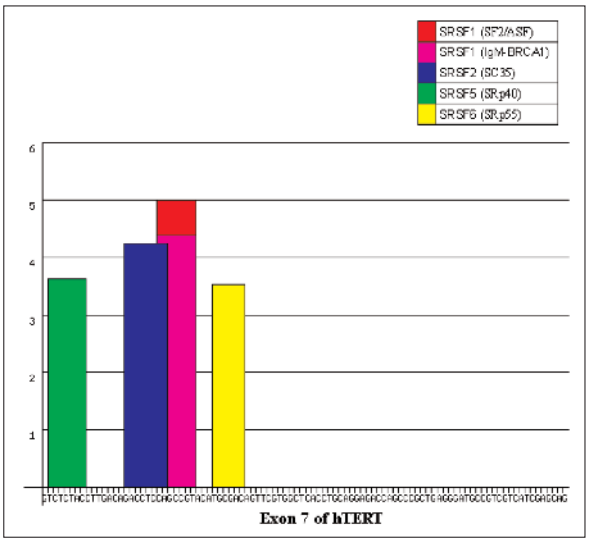


**Figure 4.** Graph output of hTERT exon 7 by ESEfinder3.0. (*Indicates SF2/ASF gets the highest score of 4.996070 in position 22, red bar).

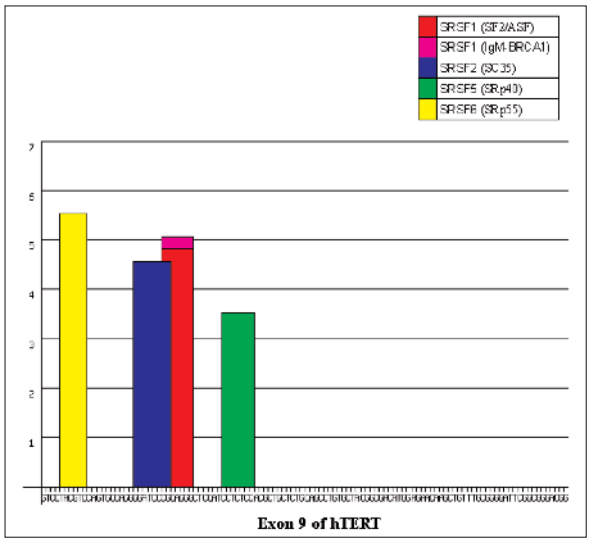


**Figure 6.** Graph output of hTERT exon 9 by ESEfinder3.0. (*Indicates SRp55 protein gets the highest score of 5.527218 in position 5, yellow bar).

comparisons by ESEfinder 3.0 and ESEfinder 2.0 are shown in Table III. There is no difference observed from different versions of this ESEfinder program in our present case.

## Discussion

Processing of pre-mRNA is a very important aspect of gene regulation. It has been estimated that more than 15% of point mutations that give rise to human genetic diseases cause mRNA splicing defects[25]. Most of the eukaryotic genes are composed of several relatively short exons that are interrupted by much longer introns. The exons must be joined together before generating the mature mRNAs and this process requires the coordinated operation of five small nuclear (sn) RNAs (U1, U2, U4, U5 and U6) and some polypeptides[26,27]. In higher eukaryotes, the requirement for accurate splicing is met by exon-intron junctions, which are intronic cis-elements including the 5' splice site, 3' splice site and branch site[28].

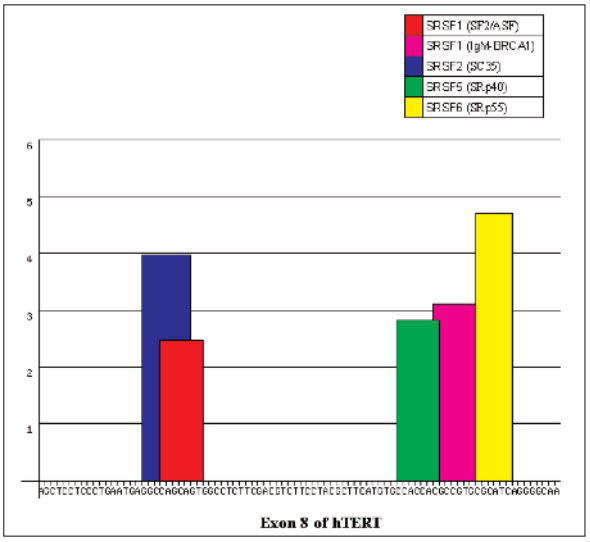


**Figure 5.** Graph output of hTERT exon 8 by ESEfinder3.0. (*Indicates SRp55 protein gets the highest score of 4.696469 in position 73, yellow bar).

Several cis-elements that are important for accurate splicing site recognition and are distinct from the classical splicing signals have been identified. These cis-elements, including exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and Intronic splicing silencers (ISSs), can act either by enhancing or by silencing splicing, thus modulating alternative splicing. In particular, ESEs appear to be very prevalent and may be present in most exons. ESEs participate in both alternative and constitutive splicing, and act as binding sites for members of SR proteins. SR proteins are a family of structurally related proteins that share a conserved structural feature called the RS domain, which is highly enriched in RS dipeptides and one or two RNA-recognition motifs (RRM)[29-32]. The RRMs mediate sequence-specific RNA binding and determine substrate specificity, whereas the RS domain is

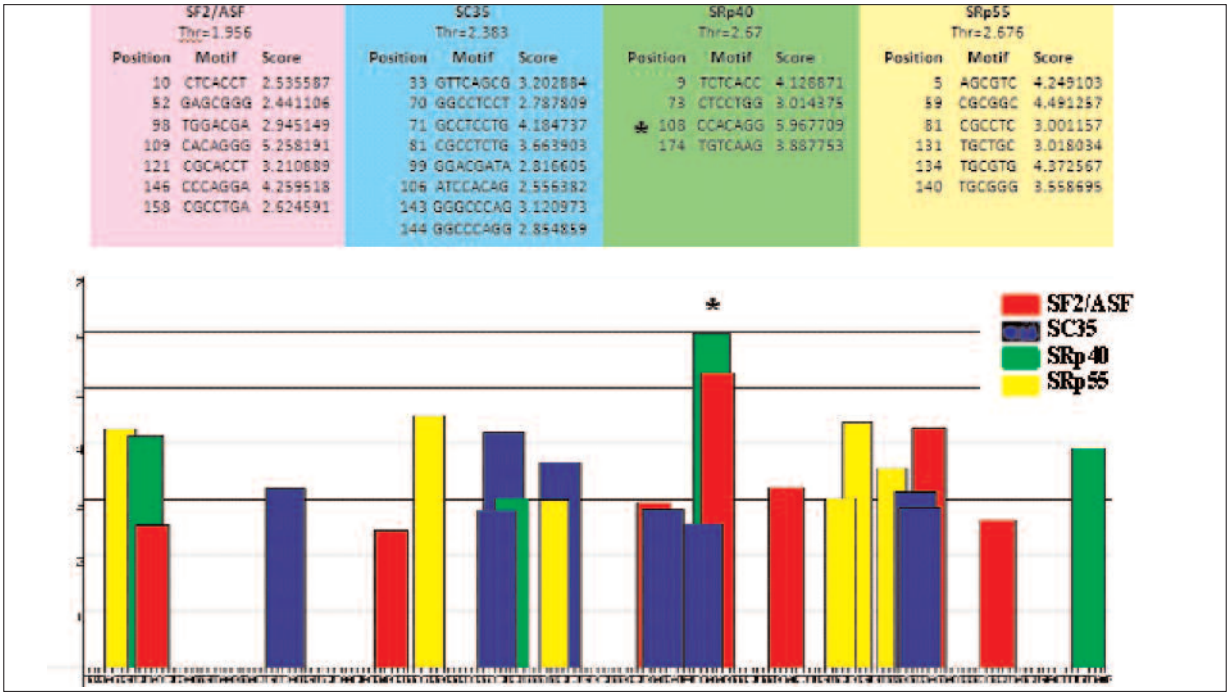


**Figure 7.** Graph output of hTERT exon 5 by ESEfinder2.0. (*Indicates SRp40 protein gets the highest score of 5.967709 in position 108, green bar).

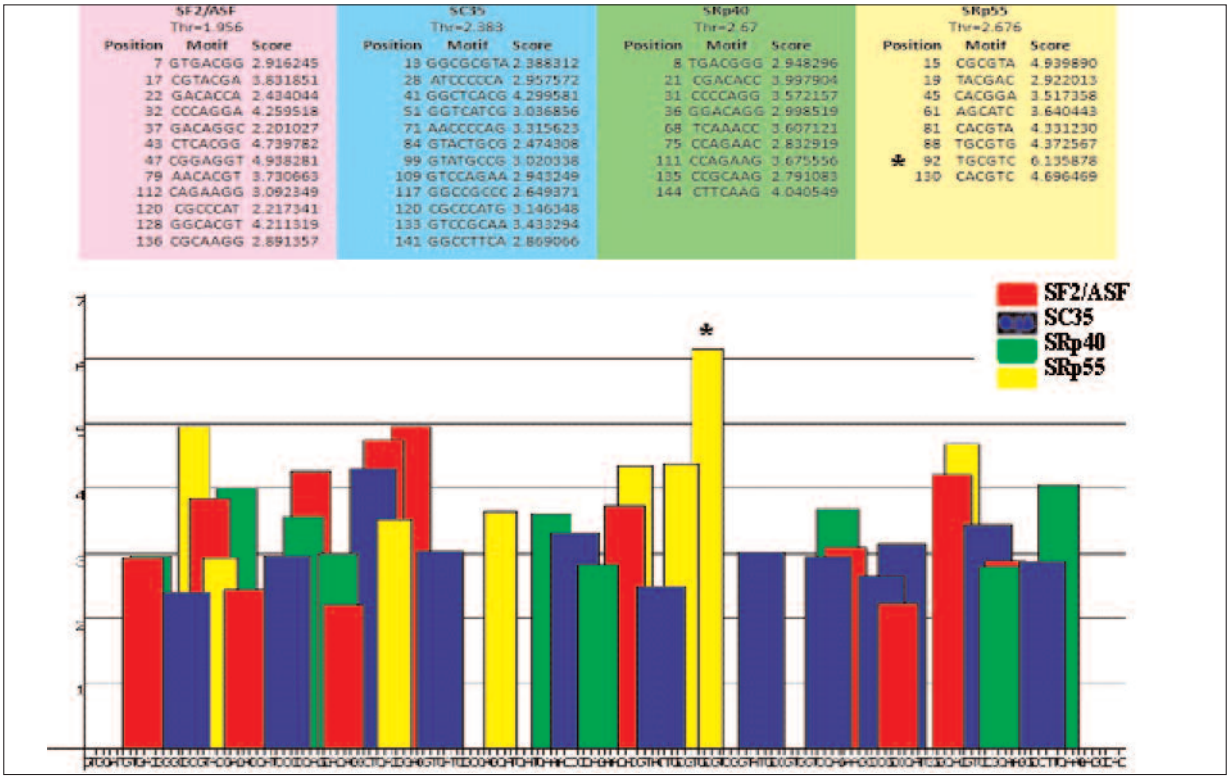


**Figure 8.** Graph output of hTERT exon 6 by ESEfinder2.0. (*Indicates SRp55 protein gets the highest score of 6.135878 in position 92, yellow bar).

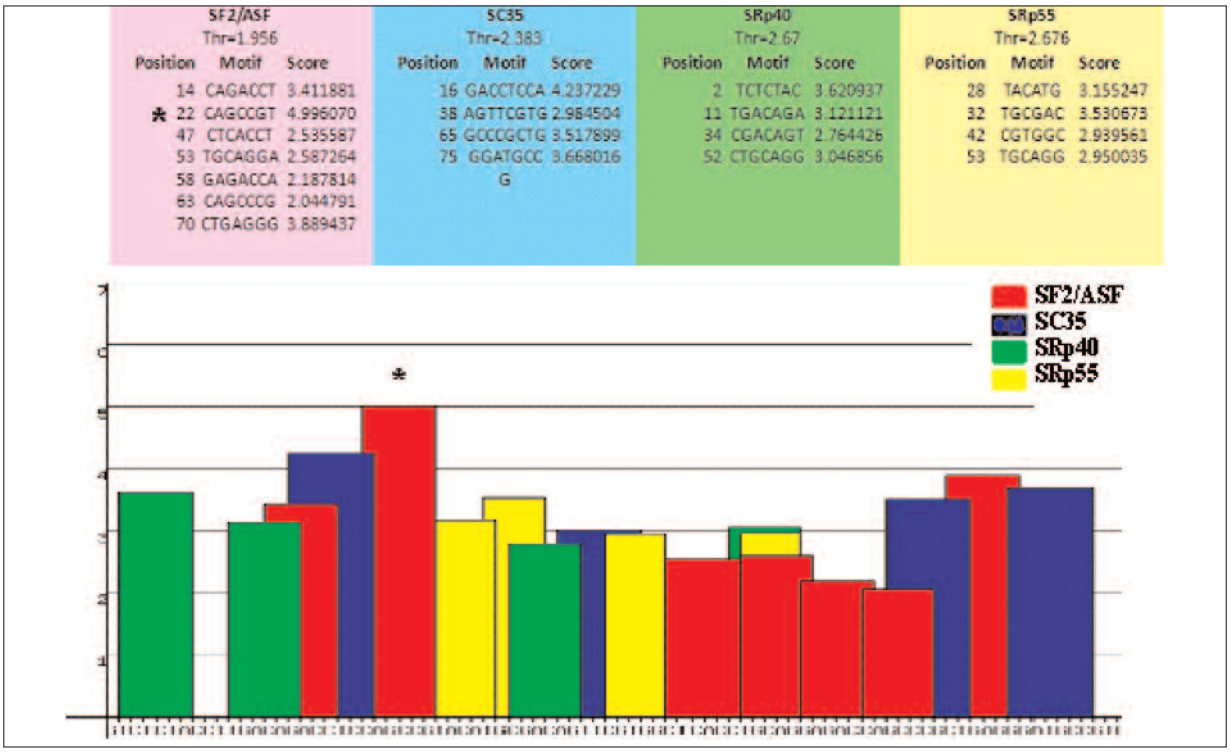


**Figure 9.** Graph output of hTERT exon 7 by ESEfinder2.0. (*Indicates SF2/ASF gets the highest score of 4.996070 in position 22, red bar).

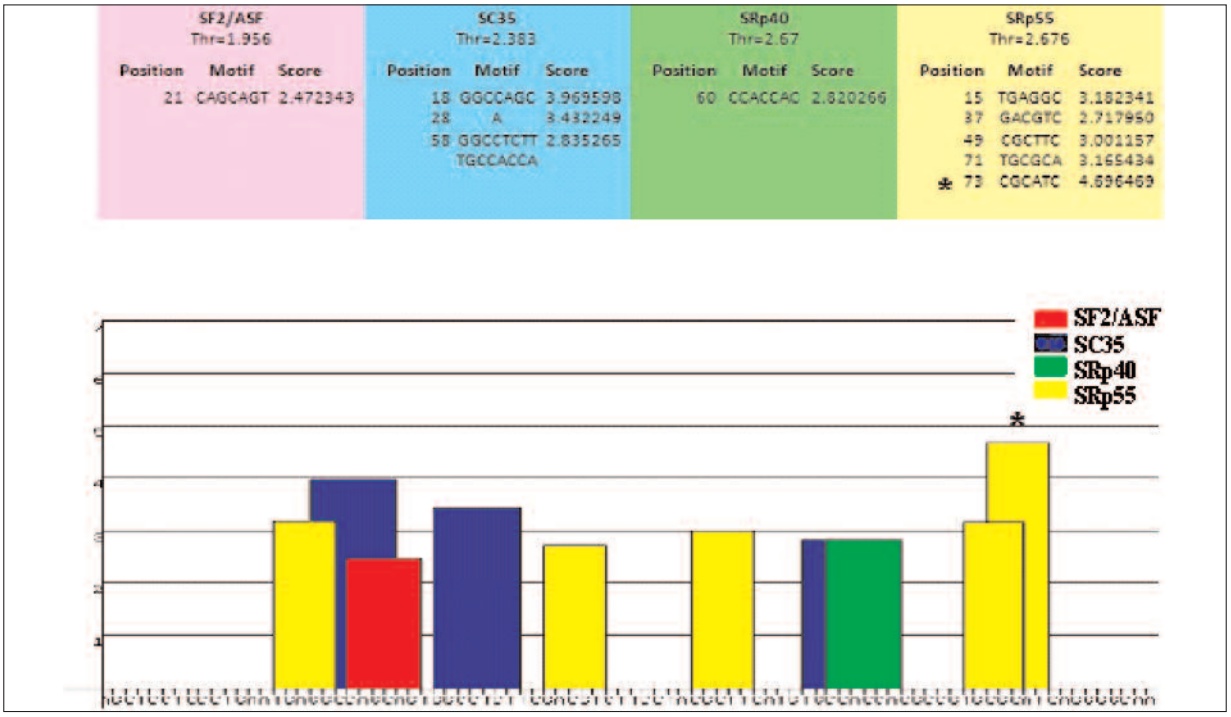


**Figure 10.** Graph output of hTERT exon 8 by ESEfinder2.0. (*Indicates SRp55 protein gets the highest score of 4.696469 in position 73, yellow bar).

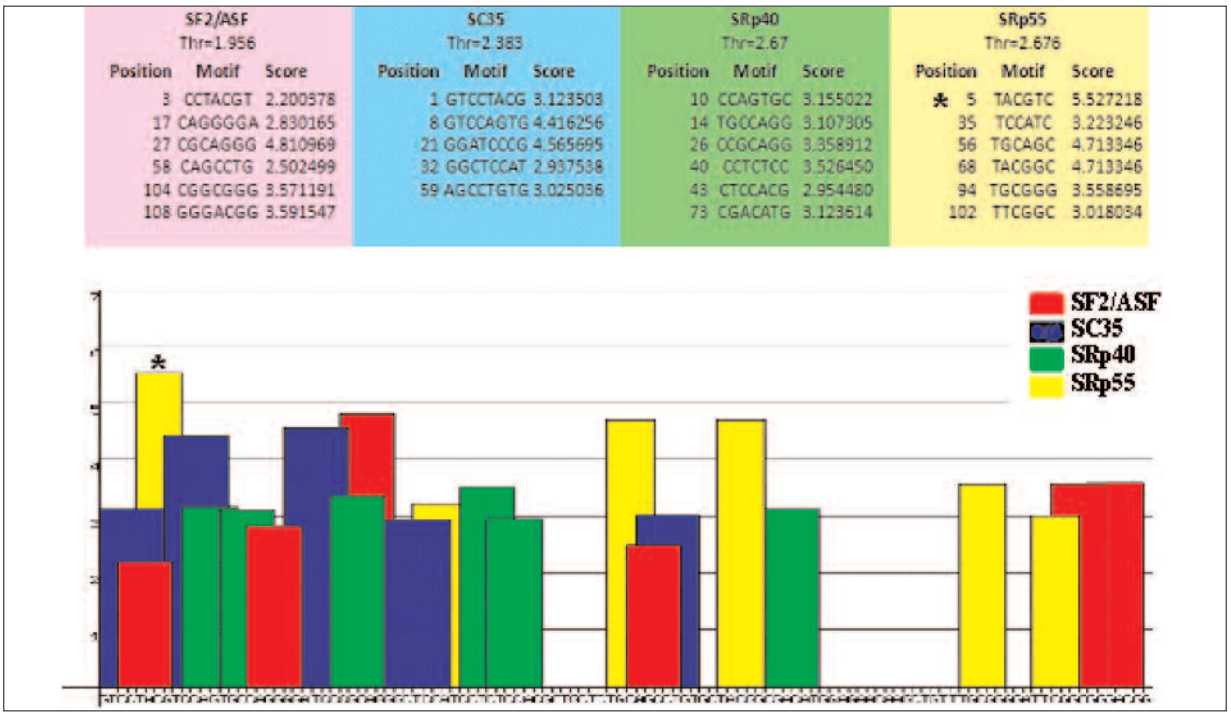


**Figure 11.** Graph output of hTERT exon 9 by ESEfinder2.0. (*Indicates SRp55 protein gets the highest score of 5.527218 in position 5, yellow bar).

thought to be involved mainly in protein-protein interactions. ESE-bound SR proteins can promote exon definition by directly recruiting components of the splicing machinery through their RS domain, and/or by antagonizing the action of nearby silencer elements. There are basically two models of SR protein action in exonic-splicing-enhancer-dependent splicing, one is an RS-domain-dependent mechanism and the other is an RS-domain-independent mechanism. These models are not mutually exclusive, and the splicing of some introns might involve a combination of these mechanisms[33,34].

The ESE motifs are identified using human SR proteins in ESEfinder program and their relevance to other species depends on the extent of conservation of each SR protein. The presence of a high-score motif in a certain sequence does not necessarily identify that sequence as an ESE in its native context. For instance, a nearby silencer element may prevent the SR protein from binding. The default threshold values are still somewhat arbitrary, although they are based on statistical analysis and empirical data. In the results of ESEfinder, the maximum score is not necessarily the most effective ESE. The score values of ESEs

**Table III.** Comparison of prediction of each hTERT exon with highest score by ESEfinder3.0 and ESEfinder2.0.

| Exon of hTERT | Program version | SR protein | Position | Motif | Highest score |
|---|---|---|---|---|---|
| Exon 5 | ESEfinder3.0 | SRp40 | 108 | CCACAGG | 5.96771 |
| | ESEfinder2.0 | SRp40 | 108 | CCACAGG | 5.96771 |
| Exon 6 | ESEfinder3.0 | SRp55 | 92 | TGCGTC | 6.13588 |
| | ESEfinder2.0 | SRp55 | 92 | TGCGTC | 6.13588 |
| Exon 7 | ESEfinder3.0 | SF2/ASF | 22 | CAGCCGT | 4.99607 |
| | ESEfinder2.0 | SF2/ASF | 22 | CAGCCGT | 4.99607 |
| Exon 8 | ESEfinder3.0 | SRp55 | 73 | CGCATC | 4.69647 |
| | ESEfinder2.0 | SRp55 | 73 | CGCATC | 4.69647 |
| Exon 9 | ESEfinder3.0 | SRp55 | 5 | TACGTC | 5.52722 |
| | ESEfinder2.0 | SRp55 | 5 | TACGTC | 5.52722 |

corresponding to different SR proteins cannot be compared to each other. The program currently searches the ESE motifs corresponding to four SR proteins, including SF2/ASF, SC35, SRp40 and SRp55. Yet there are several other SR proteins for which the ESE motifs have not yet been identified[35,36].

The prediction of ESEs has great value not only for genetists to better understand the effect of mutations on mRNA splicing, but also for clinical researchers to design new therapeutic approaches based on splicing interference. An example is the exon-skipping strategy used in Duchenne Muscular Dystrophy (DMD) or gene and exon silencing through regulating of mRNA splicing. It is very crucial to identify the most appropriate antisense sequences to be able to provide the highest possible skipping efficiency. Antisense oligonucleotides (AOs) can be used to correct the disrupted reading frame of Duchenne muscular dystophy (DMD) patients. Aartsma-Rus et al[37] reported that they synthesized 121 AOs, of which 79 are effective in inducing the skipping of 38 out of the 79 different exons. All AOs are located within DMD exons and act by steric hindrance of SR protein binding to ESE sites. Predicted by the ESEfinder program, retrospective *in silico* analysis of effective versus ineffective AOs indicated that the efficacy of AOs was correlated to the presence of putative ESE sites. The ESE predicting software program was a valuable tool for the optimization of exon-internal antisense target sequences[37-40]. Oligonucleotides complementary to exonic splicing enhancer elements (ESE) have been shown to induce exon skipping[41]. This approach has been shown to induce an isoform switch from an anti- to a pro-apoptotic form of Bcl-X for cancer therapy[42], isoform switching to a proapoptotic form of WT1 for leukemia[43], and from a transmembrane to a cytoplasmic form of FOLH1 for prostate cancer[44].

By using ESEFinder software, we predicted the ESEs of hTERT and found 5 motifs in exon 5 to exon 9. They were at position 108 located in hTERT exon 5, at position 92 located in exon 6, at position 22 located in exon 7, at position 73 located in exon 8 and at position 5 located in exon 9. In our future study, we will design and optimize exon-skipping antisense oligonucleotides that are complementary to the sequences of the ESEs. This could potentially increase β site hTERT ASV (or other non-functional hTERT ASVs) and decrease the full-length hTERT, ultimately inhibiting the telomerase activity and shortening the telomeres of cancer cells. Furthermore, we consider designing other antisense oligonucleotides that can increase the full-length hTERT and prolong telomeres in senescent cells for some neurological diseases.

## Conclusions

Five ESEs were predicted in exon 5 to exon 9 of hTERT. They were at position 108 located in hTERT exon 5, at the position 92 located in exon 6, at the position 22 located in exon 7, at position 73 located in exon 8 and at position 5 located in exon 9. Compared the ESEfinder 3.0 and ESEfinder 2.0 program, there were no significant differences between the two program versions in our case. The finding of these potential ESEs of hTERT might be helpful for the design of the antisense oligonucleotides, which could modulate hTERT alternative splicing and finally inhibit telomerase activity.

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### Conflict of Interest

The Authors declare that there are no conflicts of interest.

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