Construction of Minigenes of *LMNA* to Study Progeria

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Abstract:

Progeria is a premature aging syndrome that was first described by Dr. Jonathan Hutchinson in 1886 and Dr. Hastings Gilford in 1897. The condition was later named Hutchinson–Gilford progeria syndrome (HGPS). The term progeria is often applied specifically in reference to HGPS [1]. As an extremely rare genetic disorder, HGPS resembles aspects of aging at a very early age. This early onset, acute disease will cause wrinkled skin, severe cardiovascular problems, kidney failure, loss of eyesight, and premature death [2]. Those born with progeria only live to their mid-teens or early twenties. In this project, I generated minigenes of *LMNA* carrying progeria causing mutations. A better understanding of HGPS may reveal clues about the normal aging process.

Introduction:

HGPS is caused by mutations in the *LMNA* gene. The *LMNA* gene encodes for a structural protein which undergoes a series of processing steps before attaining its final form, called lamin A. Lamin A makes up the nuclear lamina to provide structural support to the nucleus. The protein is farnesylated in cytoplasm and is transported to the interior of the nucleus. The farnesyl group allows lamin A to attach temporarily to the nuclear rim and then is removed by a protease. If the farnesyl group cannot be removed, lamin A is permanently affixed to the nuclear rim. So, the nuclear lamina is unable to provide the nuclear envelope with adequate structural support. Chromatin organization during mitosis is interrupted, leading to compromised cell division [3].

Most HGPS cases are caused by one of two dominant, *de novo*, point mutations p. G608G (GGC > GGT) and p. G608S (GGC > AGC). The mutation p. G608G does not change an amino acid. The mutation p. G608S results in a substitution of serine for glycine.

A study in 2003 looked further into the normal sequence surrounding codon 608. By comparing this region to the consensus splice donor sequence, they proposed that both observed HGPS mutations result in activation of a cryptic splice site within exon 11 (Figure

1) [4]. Consequently, the terminal 150 bases of exon 11 are removed, resulting in the removal of 50 amino acids of the protein, producing a truncated protein (progerin or LaminAΔ50) [5].

The purpose of this study was to generate minigenes of *LMNA* that carry the progeria causing mutations. Using the mutation-containing minigenes, we can study the impact of these mutations on the splicing of exon 11.

Material and Methods:

DNA extracted from two cell lines (HL-60 and U2OS) was used to amplify the exon 10 to exon 12 region of the wild-type *LMNA* gene. Forward primer: 5'-CACCGAAGTGGCCATGCGCAAG. Reverse primer: 5'-GCCAGGGGTAGAAACAAC. PCR reaction was performed with *pfuUltra* II Fusion HS DNA polymerase. The conditions for thermocycler were as following: 95°C for 2m, 45 cycles of 95°C 20s, 58°C 20s and 72°C 45s and final extension hold for 3m. Purity and concentration of DNA product were measured with spectrophotometry. Products were then analyzed in a 1% agarose gel and sequenced by Sanger sequencing in Genewiz.

This amplified product was inserted into the pcDNA3.1/V5-His TOPO vector to generate the wild-type-containing construct, following the manufacturer protocol. Plasmids were extracted using QIAprep Spin Miniprep Kit. Purity and concentration of plasmids were measured with spectrophotometry. Insert of the generated plasmids was sequenced by Sanger sequencing in Genewiz.

HEK293 cells were transfected with wild-type-containing minigene construct. RNA was extracted using the Qiagen RNeasy plus mini kit, following the manufacturer protocol. Purity and concentration of RNA product were measured with spectrophotometry.

RT-PCR was performed using the Qiagen One-Step RT-PCR kit to examine plasmid derived transcript. Ten nanograms of RNA were amplified in a 20ul reaction with 0.5uM primers. Forward primer: 5'-CTGGCTAGTTAAGCTTGGTACC. Reverse primer: 5'-GATTACATGATGCTGCAG. The conditions for thermocycler were as following: 1st hold at 50°C for 30m, 2nd hold at 95°C for 15m, 40 cycles of 94°C 30s, 58°C 30s and 72°C 30s and final extension hold for 2m. Products were then analyzed in a 1% agarose gel and sequenced by Sanger sequencing in Genewiz.

To generate minigenes with either the p. G608G (GGC > GGT) mutation or the p. G608S (GGC > AGC) mutation, site-directed mutagenesis of the wild-type-containing construct was performed using QuickChangeXL Site-Directed Mutagenesis Kit, following the manufacturer protocol. For generating p. G608G (GGC > GGT) mutation, forward primer: 5'-CTCAGGAGCCCAGGTGGGTGGACCCATCTCCTCTG, reverse primer: 5'-CAGAGGAGATGGGTCCACCCACCTGGGCTCCTGAG. For generating p. G608S (GGC > AGC) mutation, forward primer: 5'-CTCAGGAGCCCAGGTGAGCGGACCCATCTCCTCTG, reverse primer: 5'-CAGAGGAGATGGGTCCGCTCACCTGGGCTCCTGAG.

Mutagenized plasmids were extracted using QIAprep Spin Miniprep Kit. Purity and concentration of plasmids were measured with spectrophotometry. Mutagenized plasmids were sequenced by Sanger sequencing in Genewiz.

All sequencing results of minigene constructs and transcript were verified using the NCBI's database.

Results:

DNA template was obtained from HL-60 and U20S cell lines. Primers were designed to amplify genomic region from the exon 10 to exon 12 of *LMNA* gene. The PCR products were analyzed in 1% agarose gel. The size showed in Figure 2 is 2.5kb as expected. Construct was sent out for sequencing. Sequencing results were verified with NCBI's database.

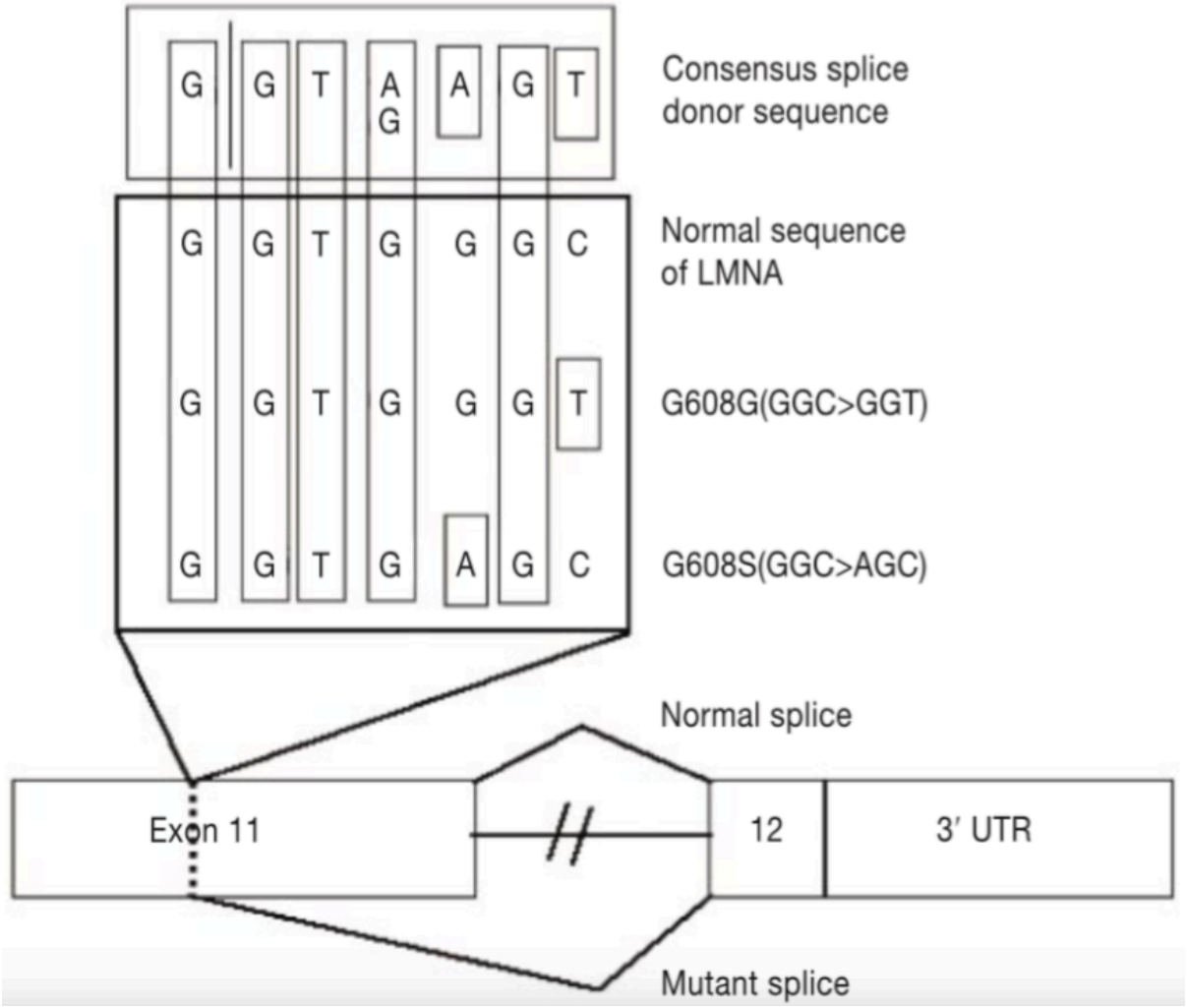

Figure 1. Hypothesis for activation of a cryptic splice donor site in exon 11.

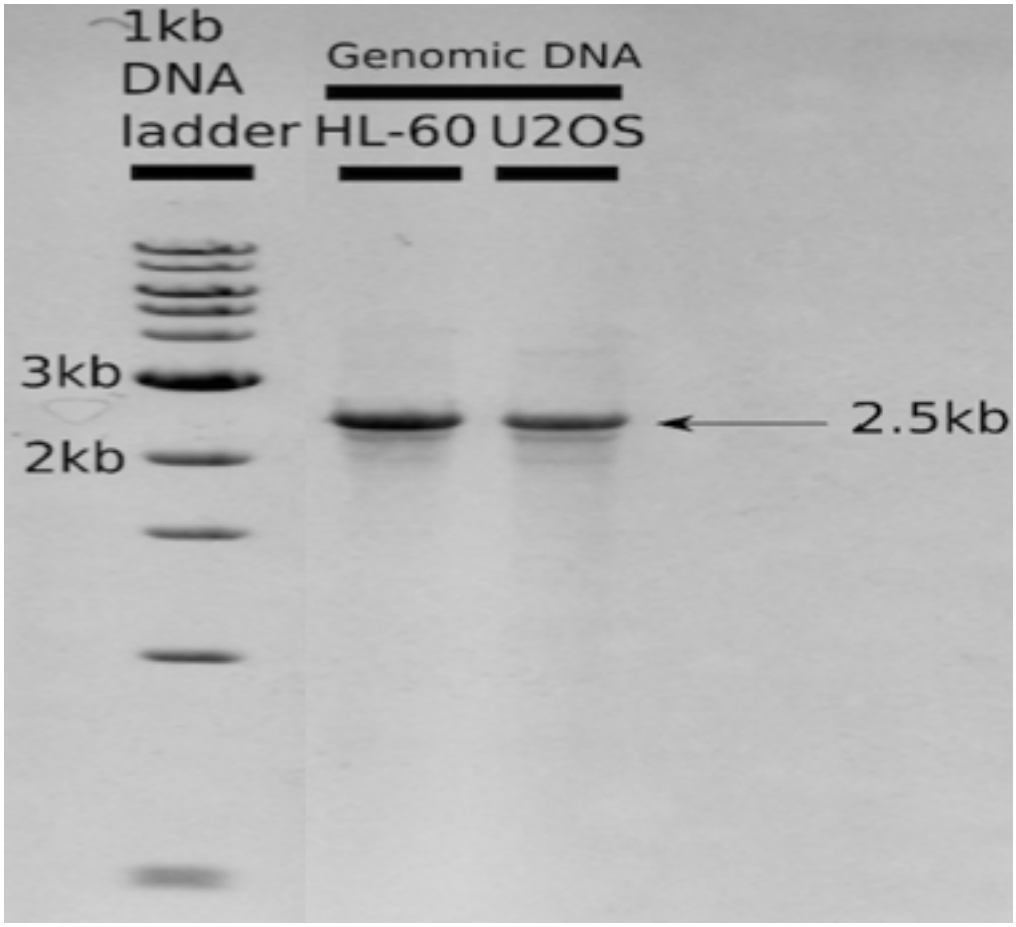

Figure 2. Successful amplification of the exon 10 to 12 region of *LMNA.* Amplicon is 2.5kb as expected.

Cells were transfected with wild-type-containing minigene. RNA was extracted. RT-PCR was performed to examine plasmid derived transcript. Primers were designed to amplify region from the vector to the beginning of exon 12. The RT-PCR product was analyzed in 1% agarose gel. The size showed in Figure 3 is 463bp as expected. RT-PCR product was purified and sent out for sequencing. As shown in Figure 4, sequencing result confirmed that wild-type-containing minigene generated the expected transcript.

Figure 3. Amplification of the plasmid derived transcript. Amplicon of transcript is 463bp as expected.

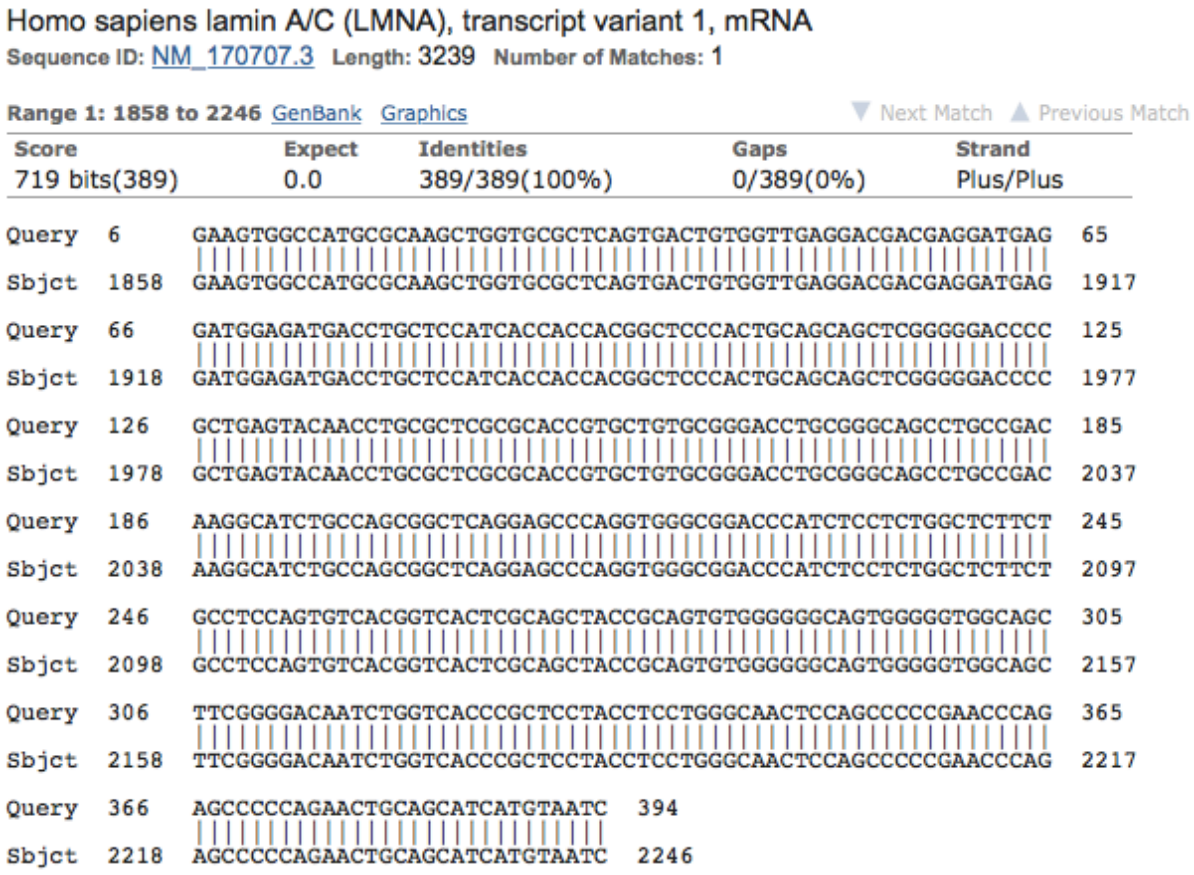

Figure 4. Transcript expression in cells containing the wild-type minigene construct is verified with NCBI's database.

Using site-directed mutagenesis, minigenes that contain the two progeria causing mutations were generated. Sequencing results were verified with NCBI's database, as shown in Figure 5.

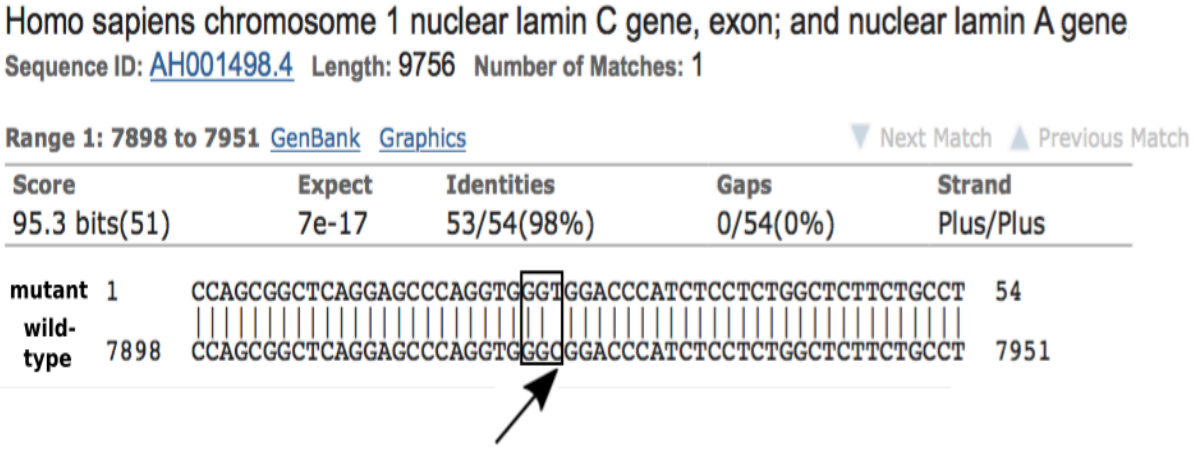

Figure 5a. Minigene construct that carries p. G608G (GGC > GGT) mutation.

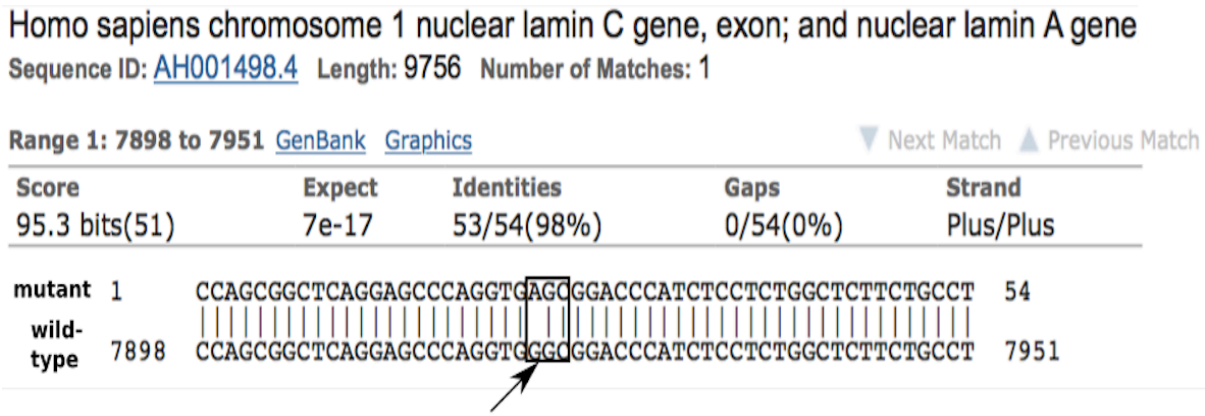

Figure 5b. Minigene construct that carries p. G608S (GGC > AGC) mutation.

Figure 5 (a, b). Using site-directed mutagenesis, minigenes that contain one of the two progeria causing mutations were generated. Sequencing results confirmed that these two minigene constructs carry desired mutations, respectively.

Discussion:

In this study, I successfully amplified the genomic region from exon 10 to exon 12 of *LMNA* gene. Wild-type-containing construct was generated. Two progeria causing mutations constructs were generated by site-directed mutagenesis of the wild-type-containing construct, respectively. Meanwhile, transcript expression analysis showed that wild-type-containing minigene generated the expected transcript.

Alternative splicing of pre-mRNA is a critical step for gene expression in eukaryotic cells. These three minigene constructs provide a valuable tool for researchers evaluating splicing patterns of progeria *in vivo.* Now we can study the impact of these mutations on the splicing of exon 11. Based on current knowledge, the regulatory elements are usually located within several hundred nucleotides on both sides of the regulated exon [6]. A rapid assay for loss-of-function and gain-of-function analyses can be used to identify potential *cis*-regulatory elements and *trans*-regulatory factors that affect *LMNA* gene expression, upon expression of minigene pre-mRNA by transient transfection.

Further work may include examining the *LMNA* transcript of cells transfected with the mutation containing construct. Small molecular compounds may be applied to cells transfected with these minigene constructs to change the *LMNA* expression level.

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