Investigation of the Effects of a Rare Mutation in LXH4 in a Family with Pituitary Dysfunction

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Abstract

Many proteins are required for the proper development and function of the pituitary gland. The aim of this research is to understand the genetic basis of pituitary dysfunction in a family with 2 affected children: one with Isolated Growth Hormone Deficiency (IGHD) and one with Combined Growth Hormone Deficiency (CPHD). Previous research identified a rare point mutation in the gene LHX4 in both affected siblings. The rest of the family members were analyzed for this variant. The father and the 2 unaffected siblings do not carry this variant, but the mom is a carrier. In order to determine the effects of this mutation on protein function, a PCR-based site-directed mutagenesis was performed to create the variant (c. G37A) in the type LHX4 cDNA. The c.G37A mutation replaces the amino acid valine with isoleucine at position 13 (V13I). Wild type LXH4 protein and the V13I variant will be produced using an in vitro translation system in order to compare the function of the 2 forms of the protein.

Introduction

Changes in the LHX4 gene, which comprises more than 45 kb of the q25 region of chromosome 1 and six coding exons, are frequently associated with diseases such as Combined Pituitary Hormone Deficiency (NCBI 2019). Researchers previously studied a family in West Palm Beach, Florida and determined that both affected children are heterozygous for a rare variant in the LHX4 gene (Dougherty et. al 2019). The mutation is found in Exon 1 (G37A), replacing the amino acid valine at position 13 with isoleucine (Dougherty et. al 2019). PCR amplication of Exon 1 in the remaining family members, followed by DNA sequencing, showed that neither of the unaffected siblings carry the mutation, supporting the hypothesis that this mutation is associated with pituitary dysfunction. In an effort to better understand a potential link between mutations in the LHX4 gene and pituitary disorders, PCR-based site-directed mutagenesis was performed to create the G37A variant of human LHX4 cDNA.

Methods

This research was conducted in the MacArthur Laboratory at Palm Beach Atlantic University in West Palm Beach, Florida. A plasmid containing the human LXH4cDNA was the generous gift of S. Rhodes. New England Biolabs Q5 Site-Directed Mutagenesis Kit was used to create the G37A variant in the human LXH4 cDNA. The Q5 Site-Directed Mutagenesis Kit is specially designed for performing insertions, deletions, and substitutions in DNA, and it operates in three phases: amplification, incubation with kinase, ligase, and DpnI digestion, followed by a transformation into competent cells (New England Biolabs 2018). Verification of mutagenesis efficiency was conducted by a control reaction and TACGene DNA sequencing. The transformation of New England Biolabs 5-alpha Competent *Escherichia coli* followed the recommendations of Addgene (2017). Modifications included using a Lysogeny broth (LB) agar plate containing 50 mg/mL of ampicillin, 1 µL of DNA, 20 µL of New England Biolabs 5-alpha Competent *E. coli*. Other modifications included icing for 30 minutes, heat shocking at 42°C for 30 seconds, and adding 950 µL of Super Optimal broth with Catabolite repression (SOC) Outgrowth Medium. Plates were created using 50 µL of transformed E. coli and incubated at 37°C for 18 hours. Two control plates (LB agar plates containing 50 mg/mL ampicillin) were also prepared. Untransformed E. coli was streaked on the first plate to act as a negative control. Previously transformed *E. coli* was streaked on the second plate to act as a positive control.

Methods (continued)

Two milliliters of nutrient broth were added to 20 µL of 10 mg/mL ampicillin in a test tube, and this process was repeated for two additional tubes. Tube 1 was then inoculated with one colony of the previously transformed *E. coli*. Tube 2 was inoculated with a colony (Colony A) of the newly transformed *E. coli*. Tube 3 contained untransformed *E. coli* to act as a negative control. A streak plate of transformed *E. coli* was created using Colony A as a positive control. One day prior to conducting the mini-prep, 100 µL of 10 mg/mL ampicillin was added to 10 mL of nutrient broth. One loopful of bacteria was added, and the tube was shaken overnight. The protocol for the mini-prep generally followed the guidelines of the Monarch Plasmid Miniprep Kit Protocol (New England Biolabs 2018). Modifications included using 1.5 mL of bacterial culture, centrifuging for five minutes at 16,000 x g (13,000 RPM), and using 30 µL of DNA Elution Buffer. The final concentration of DNA was determined using a spectrophotometer and the DNA Elution Buffer as a blank. The amount for each used was 2 µL. The protocol for site-directed mutagenesis followed the guidelines of the Q5 Site-Directed Mutagenesis Kit (New England Biolabs 2018). Modifications included using a unique forward primer (GGAAGGGGCTaTCAAGGGGCTC) and reverse primer (GCGGGGACAGTCGCACTC). PCR was performed with a denaturation at 98°C for 30 seconds, 25 cycles at 98°C for 10 seconds, 72°C for 30 seconds, 72°C for 2 minutes and 17 seconds, and a final extension at 72°C for 2 minutes. After transformation, a spread plate was made using 100 µL and incubated overnight at 37°C. PCR of the control reaction was performed with an initial denaturation at 98°C for 30 seconds, 25 cycles at 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 2 minutes and 17 seconds, and a final extension at 72°C for 2 minutes. A spread plate was created using 100 µL of the control mixture and incubated overnight at 37°C. In preparation, 10 µL of 100 mg/mL ampicillin was added to 10 mL of nutrient broth. One loopful of bacteria was added, and the tube was shaken overnight at 37°C. The protocol for the Monarch Mini-Prep Kit was then repeated approximately 24 hours later (New England Biolabs 2018). Modifications included using 1.5 mL of bacterial culture, centrifuging for five minutes at 16,000 x g (13,000 RPM), and using 30 µL of DNA Elution Buffer. The final concentration of DNA was determined using a spectrophotometer and the DNA Elution Buffer as a blank. The amount for each used was 2 µL. A glycerol-stock of bacteria was created using 500 µL of 50% glycerol and 500 µL of overnight culture and frozen at -80°C.

Results

Following site directed mutagenesis and transformation, 2 colonies grew on the LB-amp plates. Plasmid DNA from the 2 coloines was isolated, and the cDNA sequenced in the region where the G37A variant should occur. Both of these plasmids contained the desired nucleotide change, as shown in Figures 1 and 2.

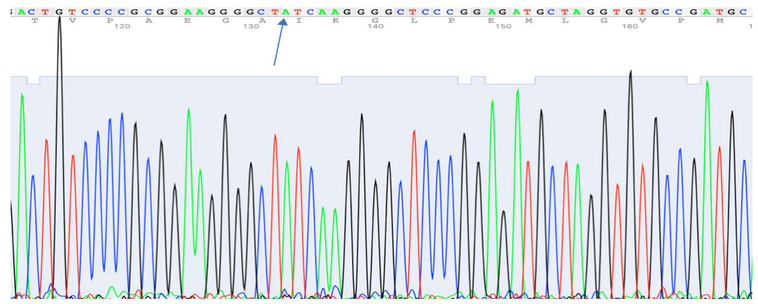

Figure 1. A Chromatogram of Mutant 1. This chromatogram shows the G to A mutation, which takes place at location 133 in this image.

Figure 2. A Chromatogram of Mutant 2. This chromatogram shows the G to A mutation, which takes place at location 134 in this image.

Discussion

The purpose of creating the LHX4 cDNA G37A variant is to explore the effects of this mutation on protein function. This variant has been previously reported in a CPHD patient in the ClinVar database where the variant is listed as “unknown clinical significance” (ClinVar) because the effects of the mutation have not been studied. With the variant cDNA available, it is possible to use *in vitro* methods to produce the variant LXH4 protein, as well as the wild type LXH4 protein. These proteins will be used in functional studies to determine if the mutant protein is able to bind DNA as well as the wild type protein. If the mutant protein is deficient in its DNA-binding capacity, this may provide the basis of how this mutant protein interferes with proper pituitary development. It would also be the first known case of a mutation in LHX4 causing IGHD.

References

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