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Title: RNA studies demonstrate that the *LDLR c.2389+4A>G* intronic variant causes familial hypercholesterolemia through an RNA splicing impact

Background: Familial hypercholesterolemia (FH) is an inherited disorder characterized by high plasma levels of low-density lipoprotein cholesterol (LDL-C). Mutations in the *low-density lipoprotein receptor* (*LDLR*) gene are the most common cause of FH. However, not all alterations in *LDLR* are pathogenic; some represent benign genetic variation, and the clinical significance of any given variant is not always clear. Variants of uncertain significance (VUS) require additional studies to elucidate their pathogenicity.

Objective: We sought to further characterize an intronic *LDLR* alteration originally classified as a VUS in a large family with clinical FH.

Methods: We performed multigene panel testing for four genes associated with FH (*APOB, LDLR, LDLRAP1,* and *PCSK9*) on the proband of a large family with a strong family history of high cholesterol and premature death; additional affected and unaffected family members were genotyped by Sanger sequencing for an identified intronic *LDLR* variant. The splicing impact of the *LDLR* variant was ascertained by RT-PCRseq performed on three affected individuals.

Results: Panel testing detected *LDLR c.2389+4A>G* in the proband. No other pathogenic variants, likely pathogenic variants, or VUSs were identified. Subsequent familial testing indicated that *LDLR c.2389+4A>G* cosegregates with disease. RNA studies demonstrated that this alteration causes skipping of exon 16, which is predicted to result in the in-frame deletion of 26 amino acids. This deletion removes part of the transmembrane domain and is expected to disrupt the ability of LDLR to insert into the membrane, resulting in loss of receptor function. Other pathogenic alterations impacting the same donor splice site have been shown to have a similar impact on splicing (*LDLR* c.2389G>T and *LDLR* c.2389+1G>T; Bourbon M et al. *J. Med. Genet.*, 2009 May;46:352-7; Holla ØL et al. *Mol. Genet. Metab.*, 2009 Apr;96:245-52).

Conclusion: The *LDLR* c.2389+4A>G intronic variant is a pathogenic mutation that results in a splice defect and cosegregates with disease. In this case, RNA and family studies resulted in a reclassification from a VUS to a pathogenic mutation and confirmed the molecular cause of the multigenerational FH. Clinical management recommendations exist for individuals with a confirmed FH diagnosis, and an accurate genetic diagnosis can improve patient outcomes through tailored treatments (Brown E et al. J. Clin. Lipidol.,2020 Jul-Aug;14:398-413). Our findings highlight the utility of familial testing and RNA studies in clarifying the classification of DNA variants.