A Splice Mutation in a Syrian Autosomal Recessive Hypercholesterolemia Family Causes a Two-Nucleotide Deletion of mRNA

To the Editor:

Autosomal recessive hypercholesterolemia (ARH) is a rare Mendelian dyslipidemia characterized by markedly elevated plasma low-density lipoprotein (LDL) cholesterol levels. ARH is caused by mutations in a protein that contains a phosphotyrosine binding domain (PTB) required for internalization of LDL in the liver.1,2 LDL receptor function is defective in liver and lymphocytes but not in fibroblasts from ARH patients. We reported a Syrian ARH family earlier in Circulation Research and identified a mutation in the splice-acceptor site of intron 1 that converts the guanine of AG into cytosine AC.3 Splice mutations may cause cryptic site activation, intron retention, creation of new splice sites, or exon skipping.4 However, we had no access to ARH gene-expressing tissue. We therefore constructed ARH gene fragments from an affected and a nonaffected individual and transfected the fragments into hepatocellular carcinoma cells (HEPG2).

DNA fragments containing the 3' part of intron 1, exon 2, the entire intron 2, exon 3, and the 5' part of intron 3 were PCR-amplified from nonaffected and affected probands. Both fragments were subcloned into TOPO-XL vector (Invitrogen). Fragments containing the 5' noncoding part of exon 1, exon 1, and the 5' part of intron 1 were also amplified and were subcloned upstream to the previously cloned fragment. The final partial ARH gene, with and without mutation, was cloned into pCMV3/Xi expression vector (Gene Therapy Systems, San Diego, Calif). We did not include the entire intron 1 because we were concerned that the entire intron could be retained upon ARH gene transcription when the splice-acceptor site of intron 1 is mutated. Amplifying the final transcript from the cDNA would be extremely difficult because intron 1 is about 6 kb. We included only the 5' and 3' parts of intron 1 in our constructs including the necessary sequences for splicing. The final fragments are shown in the Figure (top). The ARH gene cDNA was cloned into the same expression vector as a control.

We chose HEPG2 cells as a transfection target to resemble in vivo conditions. Transfection was optimized using Effectene (Qiagen) as a transfection reagent; 40% to 50% transfection rates were obtained. HEPG2 cells were transfected with the expression vectors containing constructed fragments from wild-type and mutant constructs, with both constructs, and with the ARH gene cDNA construct. Three days after transfection, the cells were harvested, total RNAs were extracted, and the transcripts from the transfected constructs were amplified by RT-PCR. We used primers that bind to sequences in exon 1 and to the polyadenylation signal in the vector.

A band of ~1100 bp came up from cDNA produced from the transfected ARH gene. This band confirms that the transfected ARH gene was properly transcribed. Using the same primers to amplify the transcripts produced from the transfected wild-type and mutant constructs resulted in two similar 600-bp bands. Direct sequencing revealed that these transcripts consisted of exon 1, exon 2, exon 3 with the 5' end of intron 3, and the polyadenylation sequence in the vector, see Figure (middle). The presence of the 5' end of intron 3 in the final transcript is due to the lack of splice-acceptor signal in intron 3, since the 3' end of intron 3 is not included in the construct. The fact that our wild-type construct was spliced correctly, exactly the same way the whole ARH gene appears in vivo, indicates that the sequences contained in our constructs were sufficient for normal splicing to take place and that no splice-activating sequences were missing in these constructs. The transcripts produced from HEPG2 cells transfected with the wild-type and both constructs, namely the wild-type and the mutant, produced the same signals. The only difference between the wild-type and mutant bands was the deletion of two nucleotides in the beginning of exon 2, namely AG (Figure, bottom).

The last 10 bases at the 3' part of intron 1 are GCTACCCCCAG. Exon 2 in the ARH gene again begins with AG. When the last G in intron 1 is mutated to C, the adjacent AG in the beginning of exon 2 is recognized by the spliceosome as a new splice-acceptor site and splicing of intron 1 will take place at this position. Thus, AG bases in the beginning of exon 2 will no longer belong to the coding sequence, leading to a frame-shift mutation. The mutation changes
the subsequent reading frame and leads to the appearance of the stop
codon TGA just two codons later. The resulting truncated protein
will consist only of 32 amino acids, leaving out the entire PTB
domain. The domain is crucial for LDLR internalization.

The mammalian 3’ splice site consensus can be divided into two
parts. One is a highly conserved YAG, where Y is a pyrimidine, at
positions −1 to −3 relative to the site of exon ligation. At position
−4, any nucleotide can be present. Another part is a polypyrimidine
tract (PPT), which starts from position −5 and extends for 10 or
more nucleotides back into the intron. The sequence context in the
vicinity of the mutated splice site brought the AG at the beginning of
exon 2 as an ideal splice-acceptor site (Figure 1, top). First, AG is
present in the beginning of exon 2 immediately adjacent to the
original splice site. Second, the splice-acceptor mutation in our
family converted the guanine into cytosine at the original 3’ splice
site. This cytosine occupies the position −3 in the newly emerged
splice site and is consistent with the consensus sequence of 3’ splice
site. This context helped to create a new 3’ splice site of intron 1 that
is perfectly in line with the 3’ consensus splice site.

The use of the new 3’ splice site at the beginning of exon 2 was
also predicted when we analyzed the available sequence from intron 1
with exon 1 and exon 2. We used the splice-finder software
(http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html). The
scores of the original splice-acceptor site and the new site that
emerged subsequent to the AC mutation are almost the same (83.7
versus 83) as calculated by the program. Had the mutation converted
the G of AG into T or A, the splice-finder program would also
recognize the adjacent AG in the beginning of exon 2 as a candidate
for splicing, but with lower scores, 74.7 and 71.6, respectively,
versus 83.7.

The consequences of the splice mutation are due to a deletion of
two bases from the beginning of exon 2 resulting in a frame-shift
mutation leading to a truncated protein. In this report, we verify how
the mutation we described earlier causes protein malfunction. The
approach we used serves as a paradigm to study splice profiles when
no patient tissues or cells are available.

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