Specific RNA Inhibition of Causal Alleles
A Potential Therapy for Familial Hypertrophic Cardiomyopathy

Robert Roberts

Allele-specific silencing of mutant Myh6 transcripts in mice suppresses hypertrophic cardiomyopathy
Jiang et al

For a recent article in Science, Jiang et al1 used RNA inhibition to suppress specifically the expression of a mutant allele known to cause human familial hypertrophic cardiomyopathy. The RNA inhibition treatment prevented the development of familial hypertrophic cardiomyopathy in the mouse. This is an exciting, innovative finding which could pave the way for specific treatment of this disease in humans. If shown to be safe and effective, RNA inhibition could be applicable for many inherited autosomal dominant diseases.

Golden Era of Single-Gene Discovery
For those of us in the field of Genetics, the past 20 years have been a golden era. The approach of DNA genotyping of pedigrees having familial disease followed by genetic linkage analysis to detect markers segregating with individuals affected with the disease have been extremely productive in the chromosomal mapping and discovery of genes responsible for disease. It is estimated that the gene for 2000 of the 6000 inherited single-gene disorders has already been discovered. Cardiology has benefited tremendously from the application of genetic linkage analysis. Familial hypertrophic cardiomyopathy (FHCM) was the first to have a chromosomal locus mapped,2,3 and in 1990, the gene was discovered.4 This was followed by familial dilated cardiomyopathy5 and familial arrhythmias including atrial fibrillation,6 Wolff–Parkinson–White syndrome,7 long QT syndrome,8 Brugada syndrome,9 and many others. Most of these diseases are inherited in a Mendelian pattern of autosomal dominance, and all occur in <1% of the population. Despite the low incidence of these gene defects, their discovery has contributed tremendously to overall disease management through genetic counseling and risk stratification. Importantly, these discoveries have also enhanced our understanding of the pathophysiology of several genetic diseases and syndromes. Transgenic mouse10 and rabbit models11 of the disease have been developed with transmission of human mutant genes to the offspring. These models exhibit clinical features of human FHCM consisting of cardiac hypertrophy, myocyte disarray, and extensive fibrosis. Administration of drugs such as simvastatin,12 angiotensin inhibitors,13 calcium inhibitors,14 and antioxidants15 in these animal models of FHCM has been associated with significant attenuation of the disease. However, for a variety of reasons, including lack of funding and lack of interest by the pharmaceutical industry, no appropriate randomized placebo controlled clinical trials have been performed with these drugs in humans. It was reasonable to expect that once genes were discovered and animal models developed, specific treatments would follow. Therefore, the lack of specific treatments is most disappointing for patients and families with these diseases and has tempered somewhat the search for further discoveries.

FHCM: A Major Cause of Death in the Young
FHCM is the most commonly inherited single-gene cardiovascular disorder, with a prevalence of 1 in 500. In a population of 7 billion, it is estimated that >14 million people worldwide carry a mutant gene for FHCM with >500000 individuals in the United States and 50000 in Canada. FHCM, the most common cause of sudden cardiac death below the age of 36 years, is also the most common cause of death among athletes. Unfortunately, these young individuals often die suddenly without prior symptoms.

Genetic Basis of FHCM
FHCM is primarily attributable to mutations in the proteins that make up the cardiac sarcomeres with the most common being the myosin heavy chain (MYH7), which encodes the β-myosin heavy chain protein. Almost all of the FHCM mutations are attributable to missense mutations, which substitute a single nucleotide in 1 of the 3 bases of a codon that code for a single amino acid. The substitution of a single amino acid in the protein is sufficient to alter sarcomere function. All individuals have 2 copies of each gene, 1 from each parent, referred to as alleles. Autosomal single-gene disorders are recessive, which means that to have any manifestation of the disease one must have both alleles defective, and thus the loss of function theoretically could be corrected by appropriate replacement. Recessive autosomal single-gene disorders only manifest the disease if both alleles are defective and, thus, the loss of function, and thus therapy is more likely to be developed for recessive over that of dominant disorders.
In autosomal dominant disorders, such as FHCM, treatment would be more difficult. The single abnormal allele is expressed over that of the normal (wild type) allele and is sufficient to induce manifestations of the disease. Treatment would require inhibition of the abnormal gene.

**Inhibition of the Mutant Allele Responsible for FHCM**

In their study, Jiang et al. have taken advantage of a potential therapeutic technique to specifically inhibit gene expression. A group of RNAs was discovered that does not code for protein and is referred to as noncoding RNAs. One class of these RNAs referred to as microRNAs or silencing RNAs consists of 20 to 26 nucleotides in length and inhibits or attenuates mRNA expression, either through inhibition of mRNA translation or enhanced mRNA degradation. These silencing RNAs, through complex mechanisms, seek out their target of complementary sequences, located in the 3 prime untranslated region of the mRNA and proceed either to cleave the mRNA or by other mechanisms inhibit the translation of the mRNA into protein.

In this study, FHCM was induced in the mouse using the p.R403Q mutation in the Myh6, a well-known common cause of human FHCM. Mice expressing the mutation developed FHCM with hypertrophy, myocyte disarray, and increased myocardial fibrosis similar to the pathological features observed in familial HCM in humans. This model is well described and used by several investigators to elucidate the pathogenesis of FHCM. Because FHCM is an autosomal dominant disorder, it requires only one of the pair of alleles to be defective to induce the disease. Knowing that one allele is adequate for normal cardiac function, the investigators attempted to prevent the expression of the abnormal allele specifically while leaving intact the expression of the normal allele. This was attempted by the RNA inhibition (RNAi) technique. Several short hairpin RNA constructs were generated with a sequence complementary to the p.R403Q mutation in an attempt to target the RNAi specifically to the mutant allele. RNAi is specific and can detect single nucleotide differences between alleles. The RNAi constructs were incorporated into adeno-associated virus serotype-9 vector with a cardiac troponin T promoter to direct it to the heart. The whole cassette was injected into the thoracic cavity of the mice at 1 day of age and was documented by enhanced green fluorescent marker, which was increased primarily in the heart. These mice were followed serially by echocardiography and electrocardiography and on euthanasia by pathology.

In vitro analysis showed the RNAi inhibited the expression of the mutant allele by 80%, whereas in vivo, there was only 28% inhibition of expression compared with the normal (wild type) allele. Animals with RNAi exhibited no myocyte disarray, and fibrosis was significantly reduced in comparison with FHCM controls. In controls with FHCM, the ECG showed QRS prolongation and other features of ventricular hypertrophy, but in the mice with the p.R403Q RNAi inhibition, such changes were not observed. Similarly, left ventricular hypertrophy markers of Nppa and Nppb were increased 2.5-fold in control mice with FHCM, compared with those treated with RNAi. The protective effect of RNAi, however, did dissipate with time, and by 11 months, the RNAi treated group was comparable with the controls. RNAi in animals with established FHCM for 3 weeks did not show any difference compared with FHCM controls, suggesting that the therapy was ineffective in reversing established disease.

**What Are the Prospects for Treating Human FHCM?**

What is the likelihood that RNAi would be successful in human FHCM? There are many barriers to overcome, as already noted by the investigators. The investigators selected adeno-associated virus serotype-9 vector, which has the advantage that it is more specific for the heart. It, however, has the disadvantage that most humans develop an immune reaction, which precludes a second injection. Another major concern is the off-target pleiotropic effects of the RNAi. The same mutant myosin is present in skeletal muscle, and any uptake in skeletal muscle could be benign or deleterious. An additional concern is getting enough of the vector cassette into the human heart. In mice, the abnormal mutant expression was reduced only by 28%, which may not be enough in human. The cassette must reach the nucleus for replication to provide a steady supply of the RNAi. High concentrations of the RNAi could, however, overload the Dicer-Complex as has been observed in studies with macular degeneration. Nevertheless, even if this therapy is effective only when given the first or second day of life, it might be applicable to humans. In today’s world in which many of the families have been genotyped and are aware that they have the disease, their children can be genotyped, and if the gene is present, early therapy could be initiated. The other observation not well documented, but often suspected from clinical data, is that the disease accelerates its manifestation with onset of puberty. So there is already some potential for hope, even if the disease can only be prevented in its early stages.

The allele specificity and potential complications from off-target inhibition will require considerable effort, both in determining the appropriate vector and the appropriate dose, along with extensive serial testing and monitoring. One of the complications of RNAi therapy recognized in treating macular degeneration was the activation of the toll receptors and the inflammatory pathway. This complication seems to be one that could and should be avoided in future applications.

Finally, it may be too soon to discard the efficacy of RNAi for established disease. The half-life of the myosin protein in humans is 3 to 5 days, and one would expect that using a vector that is safe with repeated injections of the RNAi, more and more replacement of the mutant protein would occur because of increased expression of the normal allele. This possibility is certainly still worth pursuing at least in mouse models. One may not be able to eliminate existing fibrosis, but could turn off further production of collagen and abnormal myosin. This is an exciting story and one worth following.

This is an innovative and ingenious approach to a familial disease. We are hopeful for many reasons that RNAi might have a role in preventing this disease in humans, and this is only the beginning. Jiang et al. have documented a dramatic effect when given early to mice that have the mutant gene.
This is still early for RNAi, and with time, the technology will certainly most likely improve.

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