A Father and Daughter's Journey Through the Genomics of Disease

Illumina exome sequencing may have uncovered the mutation responsible for a young girl's rare genetic disorder.

Introduction

Hugh Rienhoff, M.D. earned his medical degree from Johns Hopkins University and trained as a clinical geneticist under the "father of clinical genetics," Victor McKusick, M.D. For over a decade, Dr. Rienhoff worked in research and clinical medicine before moving on to become a biotechnology investor and entrepreneur. The birth of his daughter, Beatrice, took him back to his roots, when it became clear she wasn't developing normally. Physicians were at a loss to explain Bea's disparate symptoms, including hypertelorism (widely spaced eyes) and an inability to gain weight. Dr. Rienhoff refused to accept this lack of an answer. Using the training he received at Johns Hopkins, and the contacts he made there, he began his search for an explanation for Bea's condition. In the end, dogged determination and Illumina exome sequencing enabled him to identify the gene mutation that appears to be responsible for it all.

A Diagnostic Saga Begins

When Bea was born, one of the first things Dr. Rienhoff noticed were her long feet. He remembered that long limbs and extremities are hallmarks of Marfan syndrome, a genetic connective tissue disorder. "I dismissed the thought," Dr. Rienhoff said. "She appeared healthy, had normal Apgar scores, and I was thrilled to have a daughter. The pediatrician didn't mention her feet, but noted the port-wine birthmark on her face (nevus flammeus) and her stiff, contracted finger and toe joints (arthrogryposis)."

While the birthmark faded, the joint contractures remained along with other anomalies such as blue sclera, mild pectus excavatum, low strength and muscle tone, and diminished reflexes. When Bea was three months old, she was evaluated for failure to thrive.

In the following months, Bea was examined by a number of specialists, from orthopedists to geneticists. None could diagnose her condition. She seemed to have a mix of symptoms. Her long, twiggy stature and flat feet were typical of Marfan, while the joint contractures were similar to those seen in distal arthrogryposis, and the muscle weakness similar to Beals-Hecht syndrome. Yet, she didn't possess the complete list of hallmarks of any of these disorders. "I was at my wit's end," said Dr. Rienhoff. "The physicians and researchers who examined Bea were very thoughtful, but this was something they had never seen before."

While Bea's disorder is unique, she is not alone. Rare diseases and disorders are defined as those that affect fewer than 200,000 people[1], yet taken all together they impact millions of people worldwide. More than 80% of them are caused by genetic mutations.

Taking Charge

Dr. Rienhoff became Bea's champion, investigating every possible explanation for her condition. "To be fair, academics work for high profile papers and grants," Dr. Rienhoff said. "A single case is just not that interesting. Most want to conduct research on genetic disorders that impact larger numbers of patients."

He was concerned that while she didn't exhibit all the signs of any one disorder today, more symptoms might appear as she grew older. Because some of her symptoms resembled those of Marfan, he feared that she might develop the disease's life-threatening cardiovascular issues later in life. He decided to take her to Johns Hopkins.

"I realized that all of Bea's symptoms were clues," said Dr. Rienhoff. "I wanted a medical snapshot in time that could be used to identify

Dr. Hugh Rienhoff and his daughter Beatrice who is now nine years old.

Dr. Gary Schroth leads the RNA research group at Illumina. Exome sequencing with an Illumina next-generation sequencing system identified a mutation that may be the cause of Beatrice's rare disease

changes in her symptoms or alert us to the development of new ones. At Johns Hopkins in Baltimore, Bea had an old-fashioned, very thorough physical exam. The physician measured the distance between the length of each digit and the proportion of each digit to the hand, and determined the rotation of Bea's ear on her skull. It's a skill that's become increasingly rare, with only very clinically oriented people still performing this type of exam. I think there's great value in clinical phenotyping such as this. The data will ultimately help us chart Bea's progress."

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Dr. Rienhoff also took Bea to see David Valle, M.D., a pediatric clinical geneticist he met while training at Hopkins. Dr. Valle and his team quickly realized that Bea's features and symptoms closely matched those of a newly described disorder called Loeys-Dietz syndrome. Patients suffering from the syndrome share many Marfan features including aortic defects. They also possess a bifud uvula, something they observed in Bea, but had been missed on previous medical exams. Both syndromes are caused by mutations in genes that encode ligand receptors in the TGF-ß superfamily. This large protein family plays a role in cell regulation and growth, as well as in tissue and immune system development. While Marfan syndrome results from a mutation in the gene encoding connective protein fibrillin-1, Loeys-Dietz syndrome is caused by a mutation in the genes encoding TGF-ß receptors 1 or 2.

Dr. Valle recommended that Bea have an echocardiogram and drew blood to sequence her *TGF-ß1* and *2* receptor genes. By good fortune or by some premonition, Dr. Rienhoff had already scheduled such a heart study that week in San Francisco. He watched apprehensively as the echocardiogram visualized a completely normal heart and aorta. Several months later, the results of the gene sequencing showed that Bea didn't have any mutations in the TGF-ß receptor. The normal echo and TGF-ß receptor genes effectively ruled out Loeys-Dietz syndrome.

Focusing on Bea's Genome

It was now 2007, Bea was three years old, and they were back at square one. This time with a new focus—Bea's genetic makeup. "I had this brilliant hypothesis that it must be a mutation in one of the myostatin receptors," Dr. Rienhoff said. "Myostatin (GDF8) is a ligand in the TGF-ß super family and controls muscle differentiation and growth. A mutation there would explain Bea's inability to develop normal muscle tissue while her cardiovascular system was unaffected. There were only three genes to sequence, which was relatively manageable for someone like me, and I had all the resources I needed to sequence them. I identified a mutation in one of the GDF8 receptors (ACVR1B), but found nothing in the critical regions of that gene where I had expected to see a change. The other two GDF8 receptor genes were completely wild-type."

It was around that time that Dr. Rienhoff learned about recent advances in next-generation sequencing technologies from Nobel Laureate, Andrew Fire, Ph.D. "We were friends from when Andy lived in Baltimore and we reconnected when he came out to California to become a professor at Stanford," Dr. Rienhoff said. "He mentioned that he was sequencing 100 genes at a time and asked if I had ever thought of sequencing 100 of Bea's genes. I realized that I wouldn't know which 100 to pick, but that I might not have to. I could instead sequence her whole genome. About six months later, I bumped into Jay Flatley at a meeting."

Jay is the President and Chief Executive Officer of Illumina, and knew Dr. Rienhoff from his days at Molecular Dynamics. Upon hearing Dr. Rienhoff's story, Jay suggested that he contact Gary Schroth, Ph.D. who leads the RNA research group at Illumina. Dr. Schroth was working on transcriptome sequencing and agreed to sequence the transcriptomes of Bea, Dr. Rienhoff, and his wife Lisa Hane. "Surprisingly, Hugh was more passionate about the transcriptome than most people," Dr. Schroth said. "He looked at transcriptome sequencing as an inexpensive and efficient way to view what was occurring in the exome, while at the same time learning how mutations were impacting the expression of proteins. He was convinced that it was the way to identify what was happening with Bea."

By this time, Dr. Rienhoff had talked with enough experts that he was confident that the genetic answer could be found somewhere in mutations encoding one of the members of the TGF-ß superfamily. Using blood samples, transcriptome sequencing was performed with an Illumina next-generation sequencer. "Only about 55% of the exome is represented in the RNA found in white blood cells, but we hoped it would provide a clue" Dr. Rienhoff said. "We looked at all the reads that might be related to TGF-ß signaling."

The only mutation unique to Bea and not the rest of the family was found in *CPNE1*; Bea was homozygous for a deletion. "I had assumed that the mutation would be heterozygous dominant since neither my wife nor I had the condition," Dr. Rienhoff said. "Marfan, Beals-Hecht, and Loeys-Dietz syndromes are also the result of heterozygous dominant alleles. The *CPNE1* change was however the best candidate we found, so I investigated it."

Dr. Rienhoff reached out to Alan Beggs, Ph.D. at Harvard Medical School and with the help of his team, interrogated an additional 400 chromosomes for the *CPNE1* mutation. They tested for Hardy-Weinberg disequilibrium and found there was no disequilibrium, suggesting that the allele was not deleterious. "In addition, the frequency of *CPNE1* mutation homozygotes in the population is 1%," Dr. Rienhoff said. "At that frequency, there would be a lot more Beatrices in the world, which there are not. As more of the 1000 Genomes sequence has come online, it's become clear that the *CPNE1* gene is now considered a pseudogene. It's full of missense mutations and deletions."

Exome Sequencing Provides the Answer

Dr. Rienhoff found himself back at square one again, but not for long. "It was more than a year since we had initiated sequencing Bea's transcriptome," Dr. Schroth said. "In the interim, we had begun performing exome sequencing on Illumina systems. We suggested we give that a try and Hugh agreed. So the samples were processed

through the service lab that was in Hayward at the time and one of our bioinformaticists, Dr. Irina Khrebtukova, helped Hugh with the interpretation."

Dr. Rienhoff soon received a call from Dr. Khrebtukova saying that they had found a mutation in the TGF-ß3 ligand. "While I suspected some aspect of TGF-ß signaling was involved, I had no clue that the mutation would be in one of the ligands," Dr. Rienhoff said. "I really thought it was going to be somewhere else downstream, a receptor that we hadn't thought about or one of the intracellular signaling molecules. It was really a surprise to everybody."

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To determine how the *TGF-ß3^{G1226A}* allele alters TGF-ß signaling, RNA from wild-type human *TGF-ß3* allele and the *TGF-ß3^{G1226A}* allele were injected into fertilized Xenopus (frog) eggs at various ratios and concentrations. Using this sensitive assay system, the mutant *TGF-ß3^{G1226A}* allele when co-expressed with wild-type *TGF-ß3*, was shown to reduce TGF-ß signaling activity to approximately 40%, suggesting that the phenotype is a consequence of a hypomorphic allele.

While the *TGF-ß3^{G1226A}* gene mutation downregulates TGF-ß signaling, there is no compensatory increase in TGF-ß1 or TGF-ß2 secretion or in any mechanism downstream of the receptor. Dr. Rienhoff wondered how Bea could have none of the cardiovascular defects of Marfan, but have so many other Marfan-like features. "I think there are two possibilities," Dr. Rienhoff stated. "One is that for the tissues that are affected, such as the palate and the vascular system, the *TGF-ß3^{G1226A}* mutation is truly a hypomorph and is downregulating TGF-ß signaling. For other tissues, such as bone and muscle, the mutation might upregulate signaling. The flip side is that Marfan syndrome may not be due completely to hypersignaling issues. The fibrillin mutation may only be hypermorphic in the aortic root tissue where it negatively impacts vascular smooth muscle development, while it is hypomorphic in bone and muscle tissue. That's a possibility that's never really been ruled out."

TGF-ß3 is the most abundant TGF-ß isoform in developing skeletal muscle. A deficiency during early myogenesis could result in hypomyoplasia throughout development that Marfan syndrome clinically mimics, but is etiologically and histologically distinct from the myopathy caused by excess TGF-ß signaling. There is a considerable inflammatory component in the muscle of Marfan patients that may account for most if not all of the muscle pathology. TGF-ß is a potent regulator of cell-mediated immunity including autoimmunity. However, no inflammation was observed in a biopsy of Bea's muscle. Dr. Rienhoff concluded that normal muscle mass and palatal development appears to require a minimum of TGF-ß3 signaling.

"When Irina told me the exome sequencing analysis was finished and that it was a *TGF-ß3* mutation, I really couldn't believe it," Dr. Schroth said. "I just remember the first conversation that I had with Hugh. He'd given me a lecture on TGF-ß and its pathways. That's what made the results of the exome sequencing so amazing. They completely validated and seemed to fit Hugh's original model of the disorder."

"We didn't find evidence of the mutation in the transcriptome sequencing because the TGF-ß3 protein isn't expressed in cells found in blood, it's probably expressed in muscle cells," Dr. Schroth added. "For functional genomics, transcriptome sequencing is best performed with cells or tissues that are impacted by the disease. We had at one time considered performing transcriptome sequencing on muscle biopsies. Fortunately, we never had to go down that path."

Finding Another Bea

For over a decade, Dr. Rienhoff has searched for other people with Bea's condition. He created a website[2] and recently published a scientific paper[3], but has yet to find anyone that shares Bea's mutation and profile. "The paper covers the constellation of findings to alert the clinical community to look for patients who may not have vascular disease, but may have this specific kind of muscle and palate problems," Dr. Rienhoff said. "I know they're out there. This type of mutation is not uncommon; it is a cysteine to tyrosine. Patient's that possess *TGF-ß3* mutations may not have come to medical attention because they actually have a benign course. Indeed, my hope is to find a bunch of octogenarians who have *TGF-ß3* gene mutations."

For Bea, exome sequencing provided the answer. "Being able to work with Gary, Irina, and the rest of the team at Illumina made all the difference in the world," Dr. Rienhoff added. Despite the more than 100,000 exomes that have been sequenced, there is currently no way to check how often this gene is mutated. "Many large centers around the world like The Broad Institute, the University of Washington, Baylor College of Medicine, and Washington University, along with very large projects like The Cancer Genome Atlas (TCGA) project have sequenced tens of thousands of exomes over the past couple of years," Dr. Schroth said. "I'm not sure if anybody has created a central exome database of all that data, but it would be helpful for cases like this if they did."

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In the meantime, there are now mice with Bea's allele knocked in. "They're just pups now, so I don't know much about them," Dr. Rienhoff said. "Over the next six months, we're going to spend a lot of time looking at these pups to determine the functional effects of this gene mutation."

References

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2. http://www.my daughtersdna.org
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