Usher Syndrome Coalition | Small Molecule and Gene Therapy Approaches to Mitigate Hearing Loss in Usher Syndrome III, Kumar Alagramam, PhD

Hi, my name is Kumar Alagramam. I'm an associate professor and Director of Research at the University Hospital Ear, Nose, and Throat institute. University Hospital, Cleveland Medical Center Case Western Reserve University School of Medicine Cleveland, Ohio.

I'm going to talk about small molecule and gene therapy approaches to mitigate hearing loss and Usher syndrome type III. Usher type III is characterized by post-lingual progressive hearing loss, a variable presence of balance disorder, and variable onset and severity of retinitis pigmentosa, which leads to loss of vision.

Mutation in the clarin-1 gene is linked to Usher type III. Clarin-1 gene calls for clarin-1 protein, which is a membrane protein as depicted in the lower right corner of the slide. Please note, the yellow molecule attached to the extracellular loop of clarin-1. This sugar molecule is attached to amino acid asparagine, single letter called "n." I will talk a bit more about it in the next slide. The two common mutations in clarin-1 are, clarin-1 Y176X, also referred to as a Fin-major mutation. The other common mutation is N48K. The amino acid asparagine, single letter called "n" at position 48, is replaced by lysine, single letter called "k." Therefore N48K. This mutation is common among Usher patients of Ashkenazi Jewish descent.

To have an idea of what these two mutations means, I depicted them in form of the key, shown in the bottom of the slide. In one case, the N48K, the protein is made, but it's unstable. The other case, the Y176X mutation introduces a premature stop, resulting in premature translational stop, or a truncated protein. This is similar to a broken key or a null effect.

Some symbols that you might encounter in the slides are CLRN1 representing clarin-1 protein, HL, hearing loss, delta symbol for mutation or change, and squiggle to represent similar to.

Why hearing loss happens with clarin-1 mutation. So I'll talk about that using the mouse models. I'll then move on to the small molecule we developed to mitigate hearing loss associated with the N48K mutation. The second part of my talk will focus on gene therapy to preserve hearing in the clarin-1 mutant background.

When I refer to mouse models, I mean mice carrying mutation in clarin-1 and showing hearing loss. These mice, or these mouse models, do not develop eye disorder. We do not know why.

The focus of my talk will be about hearing loss and mitigation of hearing loss associated with clarin-1 mutation. More than 10 years of research compressed in a few slides that you're going to see, therefore the details are kept to a minimum. If you have any questions, please write to me at kna3@case.edu.

This slide shows human anatomy compared to the mice. Particularly, the focus is on the human Organ of Corti, which is in the cochlea. The main cells, or the sensory cells, in the Organ of Corti, the rows of inner hair cells and outer hair cells, this structure is identical to the structure in the mouse. Therefore the mouse provides a good model to study hearing and deafness associated with hair cell defects.

Why do we call them hair cells? The sensory cells in the ear have hair-like projections on the apical surface. Therefore, the nickname hair cells. The hair cells can merge sound vibration, or vibration from the head movement, into electrical signals, which are then conveyed to the brain via the auditory nerve or the vestibular nerve. Any defect to the hair cell genetic or envrionmental will result in hearing loss.

So why hearing loss with clarin-1 mutation? What do the mouse models tell us? To the right of the slide, the top panel shows a scanning electronmicrograph of hair cells from a normal mouse. You see that the hair bundles are organized as an inverted v shape and they are arranged in a neat fashion. And the bundles organized, the hair bundles are organized.

When clarin-1 is mutated, the hair bundle organization is disrupted as seen in the bottom right panel. The hair bundle is the mechanosensory structure of the hair cell. Therefore mutation in the clarin-1 results in hearing loss as shown in the panel to the left. The graph on the panel to the left shows hearing of the normal mice at the bottom. So the green line these mice can respond at about 35 decibel sound intensity. What is 35 decibels equal to? Approximately sound intensity of a whisper.

In contrast the null mice, clarin-1 null mice at the top, and red line does not respond even to 100 decibel sound intensity. 100 decibel sound intensity would be the sound you hear at a wood shop. The N48K mice do show some degree of hearing, the earlier stages. This is indicated in the blue line in the middle of the plot. However they quickly lose all of their hearing. The bottom line of the slide is loss of clarin-1, or mutation of clarin-1 affects hair cells, which in turn results in loss of hearing.

In this slide, I'm showing in vitro studies to understand why clarin-1 N48K is dysfunctional. The top panel shows cells that express normal clarin-1 protein fused to a reporter GFP protein.

The proteins do not have any presence of their own, so fusing it with a reporter is a standard laboratory technique. The top panel shows the normal clarin-1 protein locates or goes to the cell membrane. The bottom panel shows a similar experimental condition where the clarin-1 N48K protein fused to GFP is expressed and observed.

First, clarin-1 N48K protein can be seen in the cell only if we add chemical agents to block protein degradation. Second, we notice that the clarin-1 N48K protein is stuck inside the cell. That is, it does not reach the membrane.

So these are two critical pieces of information. Combined with the in vitro data that you saw in the previous slide in the mice, we hypothesized that increasing the stability of clarin-1 N48K would help the mutant protein reach the proper site in the cell and rescue clarin-1 mediated function in the affected cells.

Now proteasome inhibitors can increase clarin-1 N48K stability in cell culture. But we cannot use these as drugs for disorders like Usher III, because it is toxic. Therefore we need to find small molecules of some other drug that can specifically protect N48K version of clarin-1 and assist the protein to reach the membrane.

In this slide, I show the first page of our recently published paper in *Nature Chemical Biology.* The title of the paper is, "A Small Molecule Mitigates Hearing Loss in a Mouse Model of Usher syndrome type III. This was published April 25, 2016.

I have put a box around all of the authors' names to emphasize the point that this is a team effort. If you need more details about the small molecule therapy for Usher type III, please look up this paper.

This slide shows essentially a schematic of our effort to screen a small molecule library consisting of about 50,000 small molecules. Here, cells expressing the clarin-1 N48K protein fused to a reporter gene screen against each of those 50,000 small molecules. After a couple of years of hard work, we arrived at one compound, which is labeled O03. This compound stabilized clarin-1 N48K and it was found not to be a proteasome inhibitor. So it was stabilizing clarin-1 N48K and a portion of the protein was seen in the membrane in the presence of this small molecule.

So this molecule O03 is a lead compound. The structure of this is shown in this slide to the left. And at a concentration of two micromolar the EC50 or 2 micromolar for this lead compound. However we would like to bring the dose of this lower. That is, we would like to lower that EC50 while we keep the potency the same.

Medicinal chemists were involved for more than two years working with this compound to come up with the most ideal, or more optimal, small molecule labeled and shown here to the right, which is BF844. So this compound stabilized the N48K and assisted some of the protein to reach the surface of the cell. This is in cell culture. And it could do this at a much lower concentration than the lead compound, as indicated in EC50 below.

So we have now thus far at this point in our work, we have obtained a small molecule that is able to stabilize the N48K mutant protein. The next big question is whether this small molecule can preserve hearing in the mouse model with the N48K mutation.

This slide shows simply the schematic for an auditory brain stem response, one of the tests used to check the hearing sensitivity. The top right, start with the sound provided to the ear. The ear then provides a signal to the auditory nerve, which is then passed on to the brain. And from the brain stem you have an electrical response

which is recorded by subdermal probes that are placed in the subject's head. And the hardware connected to this recorder converts that information to wave form as shown in the bottom left panel under normal mouse.

So this is the wave form you get when you have a normal hearing subject. The left, the numbers shown here 46, 51, 56, shows the increasing intensity of sound. As the sound intensity increases, you see the amplitude of the wave form also change.

So in contrast to the normal mice, if you look at the lower middle panel, the Usher III mouse, at about 100 days essentially shows no response whatsoever even at the highest intensity. Basically flat line. That means the animal is profound and displays deafness.

The right lower panel indicates what we expect would happen if a particular treatment is successful. We will essentially restore those waveforms. This slide shows the actual test of the small molecule in the clarin-1 N48K mice. The orange diamond at the bottom represents response of normal mice a/b or tests. These mice can respond at about 35 decibel sound pressure level. What does that mean? That sound intensity is equal to a whisper.

In contrast, the blue square shown at the very top represents the results from the N48K mice untreated. Even at 100 decibels sound, a sound that similar to if you're standing in a wood shop, that's how much sound you would get, even at that sound, these mice do not respond.

When you treat the N48K mice with the small molecule, the results are dramatic. This purple triangle shown in the middle, three purple triangles in the middle, indicate that the hearing in the N48K mice treated with a small molecule has significantly improved. How significantly? Our calculations show that it is 10,000 fold improvement.

To summarize the first part, we have developed the first targeted therapy for an Usher syndrome, particularly for the hearing loss. BF844 preserves hearing in clarin-1 N48K mouse. Since the clarin-1 N48K causes both hearing and vision loss, BF844 administered systemically could in principle prevent both sensory deficits. That is the vision loss and hearing loss in patients with Usher syndrome type III associated with the N48K mutation.

This slide summarizes all of our effort in the small molecule project. At the 12 o'clock position we start with the N48K subject and going down to the 2 o'clock, 3 o'clock, 5 o'clock, 7, and 9 o'clock position, where we end up showing efficacy of the small molecules using the mouse model in vivo.

So that's how far we have. And at this time the Usher III initiative has taken the small molecule BF844 and they are proceeding with additional preclinical studies that are necessary for regulatory approval. And hopefully in the future we will be able to take this to clinical trials.

I'm going to shift gears and go to my second part of the talk. This is a more exciting development, more recent. It's gene therapy tested in Usher III mice mouse model. This paper was published 18th October 2017 in the journal called *Scientific Reports* from the publishers of the journal *Nature.* The title of the paper is "Modeling and Preventing Progressive Hearing Loss in Usher syndrome type III." Again, all of the authors I put a box around to indicate the contributions of many and this is a team effort.

So what is gene therapy? The normal copy of a gene is replaced, transplanted if you will, into the mutant cells of the target organ to correct the genetic disorder. Why gene therapy for Usher type III? Especially after I talked about the small molecule. Now small molecule, BF844 in this case, won't work for all mutations in Ushers. For example, the Y176X essentially no protein is made. 844 is only useful if there is a mutant protein present and with a particular type of mutation. Whereas gene therapy will enable the synthesis of clarin-1 protein, irrespective of the nature of the mutation.

Then you can ask the other question, why did we bother with the BF844 clarin-1 N48K if gene therapy looks like it might work? In case of the clarin-1 N48K functional protein is made. But it needs help to keep up, to deliver clarin-1 mediated function. BF844 assists the mutant protein to do so. But if you don't have any protein, BF844 won't help.

The second thing is, in case of the clarin-1 N48K, BF488 can reach both the eyes and both the ears and treat all sensory organs at the same time. Whereas in gene therapy you have to treat one eye and one ear at a time. So the advantages of the small molecules, it depends on the nature of the mutation.

So in this slide, I'm showing the mouse model that we developed. In the knockout background of clarin-1 with a progressive hearing loss. The black line represents the response from the normal mouse so these can respond at about 25, 30 dB whereas the clarin-1 knockout shown in the red at the very top fails to respond at the highest intensity of sound presented. The blue line represents the new progressive hearing loss model in the clarin-1 knockout background. So early on, at 20-30 days, it shows relatively normal hearing. More to the left of this trap marked by P22 more time the hearing gets worse as indicated by the blue line going at nearly a 45 degree angle.

So the new model shows delayed onset progressive hearing loss in the clarin-1 null background whereas the knockout mouse alone, the older model showed early onset profound hearing loss. So we used a new model to test our gene therapy because this progressive hearing loss is similar to what is seen with the Usher III patients. This slide shows the clarin-1 gene therapy construct that is used with the AAV viral gene therapy vector. So this cDNA clarin-1 cDNA is in the middle of the construct.

And this viral gene therapy vector was injected into the ear of mice that are 2-3 days old. Then after that injection,

we wait for about a month and look at the hearing over a period of time. So this is similar to what I showed before in terms of the hearing test that was done. One point I want to emphasize is that this is a very similar test done in humans as well. So we are using comparable tests to determine the efficacy of either the small molecule or the gene therapy.

So this shows the actual results of the gene therapy that we did. The black line in this graph represents the hearing of normal mice which is not treated with anything. And these mice can hear at about 35 dB. And the hearing is sustained, the x-axis shows the days from day 27 to day 150 when the study was stopped.

The blue line in this graph represents the progressive hearing loss model with Usher type III. Earlier on, the hearing is around 40 dB and slowly hearing sensitivity decreases and you need to provide more and more sound. And by about 90 days, even at the highest intensity of sound provided in this test, these Usher III untreated mice failed to hear. So these are the ones with the knockout background.

The red line is what you need to focus on. This is the progressive hearing loss model. This is the Usher III in the knockout background. This is the new mouse model. When the AAV virus is injected in the ear of these mice they seem to A, sustain their hearing over a period of time. The AAV viral gene therapy has prevented the progression of hearing loss compared to the untreated mice. And their hearing sensitivity is stable over a long period of time as shown here until 150 days when we stop the study.

So what this data shows is that gene therapy with clarin-1 gene is very effective. And when the viral vectors are delivered before the onset of hearing loss, you can see hearing preservation is robust.

Conclusion of part two, we have developed a progressive hearing loss mouse model for Usher III in the knockout background. We have developed a gene therapy approach that is very effective in curtailing progressive hearing loss in the Usher mutant mouse and the effect remains stable. Gene therapy vectors were introduced very early, i.e., Before the onset of hearing loss in this model. And that seemed to be very effective.

The next step in gene therapy for Usher III will gene therapy work in the new Usher III mouse model? If the viral vector was introduced in adult mice. If so, how well? And we also would like to test gene therapy in Usher III mouse model using new generation viral vectors such as Anc80. And hopefully, if everything goes well, apply for regulatory approval for a trial.

So the papers that I described these two yellow hyperlinks can take you to the two papers. They are free and if you're interested in more information you can go there and also you can send me an email if you have questions. I would like to thank Usher III intiative for funding this project and some of the support also came indirectly from NIDCD. And also I'd like to thank the University Hospital Cleveland Medical Center for financial support as well.

And with that, I'd like to thank everybody. Thank you very much.