Unveiling HDAC6: A Promising Therapeutic Target for SPG4 - Insights from Human and Animal Models

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Dr. Qiang began by thanking The Spastic Paraplegia Foundation for their invitation to participate in this Annual Conference and have “really heated” discussions with scientists about the important diseases of HSP and PLS. He also thanked Norma Pruitt for creating a platform where the scientists could relate with patients and learn from them.

Dr, Qiang’s talk today will have three parts. The first part will have to do with what they are doing in terms of therapeutics. They are trying one compound that they have found inhibits some enzymes that are found aberrantly enhanced in the disease.

The second part of his talk will be about High Throughput Analysis (HTA). With HTA they can look at hundreds of proteins and thousands of genes in a very short time span. It saves a lot of time, is very efficient to allow them to find new pathways that may be novel to develop new compounds and develop vectors to treat these pathways.

The third topic will be about how we can learn from different research arenas and apply their therapeutic approaches to HSP and PLS. He pointed out the two logos on his screen which were The Drexel University and his spinal cord research center at his lab at Drexel University. They have a group of more than 15 scientists that are working on studying spinal cord related diseases especially spinal cord injury. A lot can be learned about HSP and PLS from studies on spinal cord injury.

First of all, what are the drug screenings that they are working on? When you start with drug screening or testing, you want to find models to do it with. The contemporary strategy is to target

things and they actually have three important components. Animals are important. Previous speakers have spoken about rodent animals but bigger mammals, like monkeys and cows are very useful.

A breakthrough happened in early 2012 when Dr. Yamanaka earned the Nobel prize for induced pluropotent stem cells. That technology can turn skin cells into stem cells, and then any desired kind of body cell needed. This helps scientists to model the disease. This is fascinating because you can take the patient’s skin cells and then modify them to create disease models to better understand the mechanisms that are going wrong. Studies on Parkinson's disease, ALS and Alzheimer's are moving remarkably fast because of this technology. We have bio banks of human post Mortem tissues that are invaluable in this type of scientific study.

With these three models, scientists are able to do mechanistic based research. They build a hypothesis and it test on one of these three models. By hypothesis, they can really target certain pathways. They can use a mix of proteins with high throughput analysis to be very efficient. This gains them tons of information and with this information discover new pathways. This lets them do more analysis and find their target. After they find their target, they can work with chemical analysts to find a compound to attack this target whether it is inhibitory or activating. After they develop the compound, using these same models, they need to develop biomarkers to measure its effectiveness.

Another method which is not hypothesis driven, is do use the thousands of compounds approved by the FDA to test them on the different models with the high throughput analysis to hope-fully find compounds that are effective. After that, clinical trials take place, and if they work out, it can lead to a big improvement. If the clinical trial fails then you go back to your mechanistic studies to determine more effective compounds.

In his lab, they mostly do hypothesis driven studies. After talking with other scientists in the field to develop hypothesis to work with they can progress. The other method of doing drug screening and use AI to eliminate the non-relevant ones but it takes a lot of time and resources to perform those kinds of studies.

In his lab, they are using animal models and the principal mouse models are those they inherited from Dr. Baas. This mouse model has both a loss of function and gain of function modality such that they can really use it to test different hypothesis. They use a catwalk to measure the mouse's walking ability and they use over 50 output measures to identify exactly what is improving or worsening in the mouse gait. They can really detect very subtle changes just by looking intensively into the data longitudinally.

Two parameters that they have found are the most important is "stand duration" and "step width". After they look at the behavior and know the functional deficit of the animal, they can also study the mouse anatomically. They can measure both the axon diameter, and the axon numbers.

There are several differences between rodent models and human models with the actual anatomy and so it is very important to have actual human cells to work with. To do this, they take human skin cells from SPG4 HSP patients, reprogram them into human induced pluripotent stem cells (hiPSCs) and turn them into an Embryoid body and turn them through Dorsomorphin, SB431542 EGF, bFGF neural induction into either of eight different types of human cells. 1. CHIR, insulin, B27 like those in the human Cerebrum (no region identity). 2. BDNF, NT3, IWP-2 like those in the human Forebrain. 3. Transferrin, progesterone, GDNF like those in the human brainstem. 4. WNT3a, PMS, SHH like those found in the human Midbrain. 5. Retinoic acid, GDF-11 like those found in the human spinal cord. 6. FGF19, SDF1 like those found in the human Cerebellum. 7. BMP-7 like those found in the

human Hippocampus. 8. BMP-4 like those found in the human Choroid Plexus.

They then can do disease modeling and drug screening on these different cells. They have been maintaining and growing many of these cell types in their labs for over 1.3 years. As far as Outcome Measures for these human organdies, they look for molecular markers for early axonal degeneration (which is really an anatomical analysis) and functional readout for axonal degeneration (electrophysiology). They are actually studying the electrical arrays to understand the activities of these neurons.

The molecular markers for early axonal degeneration include SMI32, NeuN and Cleaved Caspase-3. For the functional readout for axonal degeneration (electrophysiology) they use the MEA system to measure voltage levels over time. He showed a diagram comparing the electrical activities of normal versus degenerated cells and there is a very noticeable lack of electrical connectivity. Their models showed a significant reduction of stable microtubules in the SPG4 models. Stable microtubules are very important for the stability of the axons.

They found HDAC6 elevated in the cells of the m-KO-Het mouse (that is a SPAST mouse with loss-of-function and greatly elevated in the dHet mouse which is a cross between the hSPAST-C448Y, gain of function toxicity and the Spast mouse (having loss of function and gain of function toxicity). So, this is likely to be a gain of function target.

Regarding the two different isoforms of the SPAST gene, M1 and M87, it is only the longer one, M1, that shows these high levels of HDAC6. HDAC6 is actually Histone Deacetylase 6 and it deacetylates microtubules. Are there any drugs to target HDAC6? Fortunately many drug companies, working on cancer research, have developed several compounds that target and affect HDAC6. One of them is called tubastatin A (Tub A).

One of the reasons that tubastaten A is attractive for us is that it can pass through the blood brain barrier. The project of testing Tubastatin A and 5% DMSO + 95 % corn oil was done by two very talented graduate students in his lab, Neha Mohan and Skandha Ramakrishnan. They are testing these drugs on symptomatic animals, or animals that are old enough to have the HSP's symptoms.

It took a long time to get enough animals for this study. Three weeks after the drugs were applied to the animals, they were given a behavior test and then they were sacrificed. He showed a film, comparing walking ability of the mouse, models, and the mouse displayed at the top of the screen was pre-treatment, and the mouse at the bottom of the screen was the same mouse after treatment, and there was a noted improvement in its ability to walk.

Their actual measurements showed a remarkable improvement in the two principle measures of “stand duration” and “step width”. These are early, prepublication results. So, this is really exciting! This information is still very new and they haven’t finished examining the anatomy of the six mice. He showed some preliminary data on the anatomy measurements of one mouse with a table entitled: “Tub A Rescues Axonal Defects in SPG4 Mice. It showed that Tub A greatly improved the degenerating axons in the SPG4 mouse. These two students are continuing to examine the mice, and they hope to find an improvement in all mice on an anatomical level, as well as the behavior level.

They also tested TubA on human forebrain organoids of SPG4 humans and Dr. Qiang put up a chart entitled “TubA Alleviates Neurodegerative Defects in Human Forebrain Organoids of SPG4. The Treatment Regimen dose was 100uM TubA in DMSO. The treatment Duration was daily for 3 days and the organic age was 6 months. This showed that TubA can decrease the degen-

eration but there was not a full rescue. Additionally, they did not detect any noticeable adverse affects in the SPG4 mice.

Conclusion:
TubA did not induce any noticeable adverse effects in SPG4 mice or human brain organoids, when administered systemically.

TubA treatment partially rescued the gate deficiency and axonal pathology in SPG4 mice.

TubA treatment rescued the early axonal degeneration in the human forebrain organoids harboring the spastin mutation.

The two avenues they are pursuing with their High throughput studies are **proteomics** and **single cell RNA sequencing** (see definitions below). These two experimental approaches are very meaningful, to look at the protein level and RNA level.

Wikipedia:

Proteomics is the large-scale study of proteins.[1][2] Proteins are vital parts of living organisms, with many functions such as the formation of structural fibers of muscle tissue, enzymatic digestion of food, or synthesis and replication of DNA. In addition, other kinds of proteins include antibodies that protect an organism from infection, and hormones that send important signals throughout the body.

The proteome is the entire set of proteins produced or modified by an organism or system. Proteomics enables the identification of ever-increasing numbers of proteins. This varies with time and distinct requirements, or stresses, that a cell or organism undergoes.[3]

Proteomics is an interdisciplinary domain that has benefited greatly from the genetic information of various genome projects, including the Human Genome Project.[4] It covers the exploration

of proteomes from the overall level of protein composition, structure, and activity, and is an important component of functional genomics.

Proteomics generally denotes the large-scale experimental analysis of proteins and proteomes, but often refers specifically to protein purification and mass spectrometry. Indeed, mass spectrometry is the most powerful method for analysis of proteomes, both in large samples composed of millions of cells[5] and in single cells.

Single cell RNA Sequencing: RNA sequencing (RNA-seq) is a genomic approach for the detection and quantitative analysis of messenger RNA molecules in a biological sample and is useful for studying cellular responses. RNA-seq has fueled much discovery and innovation in medicine over recent years. For practical reasons, the technique is usually conducted on samples comprising thousands to millions of cells. However, this has hindered direct assessment of the fundamental unit of biology—the cell. Since the first single-cell RNA-sequencing (scRNA-seq) study was published in 2009, many more have been conducted, mostly by specialist laboratories with unique skills in wet-lab single-cell genomics, bioinformatics, and computation. However, with the increasing commercial availability of scRNA-seq platforms, and the rapid ongoing maturation of bioinformatics approaches, a point has been reached where any biomedical researcher or clinician can use scRNA-seq to make exciting discoveries. In this review, we present a practical guide to help researchers design their first scRNA-seq studies, including introductory information on experimental hardware, protocol choice, quality control, data analysis and biological interpretation.

Dr. Qiang showed a diagram of the Spastin protein and showed how it binds to other proteins. All proteins have to interact with other proteins to carry its function. They are learning what proteins bind to M1, M87 and to both of them. They are

comparing the functions of the normal spastin with the functi ons of the mutated spastin to better understand what is going on. They are thinking that M1 is actually “the bad guy”. Mutated M87 has problems but not near as bad as mutated M1. We want to see what proteins bind to the normal M1 compared to what proteins bind to the mutated M1. He said that he did not have the time to explain all of their data but his diagram showed that 54 proteins bound with M1 and 47 proteins bound with M87 and 23 of those proteins combined with both. What he found really interesting is that there are only 14 common proteins between the mutated M1 and mutated M87 protein but that did not bind to the normal M1. Several proteins they pulled out from these proteomic studies were actually HSP genes. These proteomic studies are helping them to understand how the different forms of HSP might be related to each other. He said these studies are revealing a lot of very important information and he is very excited about this project.

The second high throughput analysis they are doing in their lab is called single cell RNA sequencing. They are doing this with both their mouse models and their forebrain organoids. They look at thousands of genes in just one cell. They are working with a lab at A&M University in Texas to look at the gene expression changes. He showed a chart of some results they just received a month ago demonstrating single cell RNA sequencing of a SPAST protein. It showed 6 different categories: 1. Forebrain glutamatergic neurons. 2. Cortical Neurons. 3. Cortical astrocytes, 4. Glutamatergic neural progenitors. 5. Basal intermediate neural precursors. 6. GABAergic projenitors. 7. Oligodendrocyte progenitors. 8. Neural Progenitor cells. They are doing a lot of analysis of this right now and what is fascinating here is that they are preliminarily finding out that one population in the cells in the mutant version of the SPAST that is not in the normal SPAST protein. He can’t reveal what that is right now because they are still doing a lot of analysis on that. Comparing

this disease to other types of disease, this is not surprising because in other diseases as the disease is developing, there are different populations in the cells. The field is moving really rapidly to look at neurodegenerative disorders. Both HSP and PLS might have developmental deficits but we are just now getting the information to be able to understand the changes at the very early stages. He promises to share more about their single cell RNA analysis in the future.

The last thing he wanted to talk about what they are doing in their lab is a collaboration they are doing with The Cure-SPG4 Foundation. Their objective is to reprogram skin cells derived from SPG for patients into functional cortical neurons, which enables personalize, drug screening and in-depth analysis of the underlying mechanisms. It is an ongoing effort: collecting skin fibroblasts from SPG for patients who donated their skin fibroblasts to NIH/Coriell Institute. They can use this approach, both for drug screening and for personalized medicine.

Last but not least….We need to learn from other research arenas, especially spinal cord injury (SCI). As we know, SPG4 HSP is a Neurodegenerative disease. As HSP progresses, the axons die back, and it is very difficult to get them to grow back.

The task of trying to determine how to change neurodegeneration into Neuroregeneration is very important in the field of spinal cord injury. They have learned a lot about activity based therapies like exercise which can help spinal neurons to regrow somewhat.

They are also learning how to apply gene therapies to augment axonal regeneration in adults. With this gene therapy, they are targeting some of the genes that inhibit or promote regrowth.

The third one is cell therapy. Cell therapy is a rapidly growing research field and a lot of companies are interested in promoting this research. We know, in spinal cord disease, the upper motor neurons descend their axons through the spinal tract. They not only innervate into the lower motor neurons but there is a vast population of a huge variety of inter-neurons in the spinal cord. These interneurons actually are modulating the descending tracts with the lower motor neurons.

It is important to know that these interneurons are playing a big role in the functions of your motor movement. They are starting to look at interneurons with the SPG4 HSP mice. Just weeks ago, they looked to see if there is any interneuron loss. The interneurons can be inhibitory or excitatory. They carry different functions. Interneurons have been ignored by scientists, studying HSP, and he thinks they are very important. If they discover that there is interneuron loss in the spinal cord, they can replace the interneurons, and form the relay to connect the upper motor neuron with the lower motor neuron in a better way. That has been done in the spinal cord injury field.

People are also talking about Epidural and muscle stimulation. This is heavily involved in spinal cord injury therapies. There are actually clinical trials using spinal cord stimulation on HSP patients in China. This is a semi-unexplored field right now. These are just semi-invasive.

Dr. Qiang closed by thanking the associates in his lab. He also thanked his collaborators which include: Dr. Peter Baas, Dr. Emanuela Piermanini and Dr. Michael Lane at Drexel University; Dr. Nicholas Canaan from MSU; Dr. Celeste Karch from Washington U; Dr. Lana Zholudeva from Gladstone; Dr. Frank Bennett, Dr. Berit Powers and Dr. Jacki O'Rourke from Ionis; Dr. Mei Liu from Nantong U; Dr. Miguel Esteves and Dr. Heather Gray-Edwards

from U. Mass; Dr. James Cai from Texas A&M and Dr. Bill Seeley from UCSF. Those marked in red are his HSP collaborators.

Dr. Qiang received one question from Dr. Hande Ozdinler. She asked about the fact that his display only showed one dose of the drug that was so effective and wondered whether they had considered a higher dosage? The answer was that they did not have enough mice to test different dosages but they plan to continue testing higher dosages with their human stem cell models.