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Mutations and Biological Behavior of NF2-Associated Schwannomas and Meningiomas and Potential Therapies

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Steve Reason: I'm going to introduce Dr. Long-Sheng Chang. He comes to us from The Research Institute at Nationwide Children's Hospital and The Ohio State University College of Medicine. Dr. Chang has collaborated with Dr. Brad Welling over the past 18 years. And I don't know if any of you remember two years ago, NF Midwest presented Dr. Welling with a research grant for AR42. And so I'm sure we're going to hear a little bit about that, an update on that, as well as other things, exciting things happening in Dr. Chang's lab.

I can say to you from what I've learned, I've gotten to know Dr. Chang over the last few days, this man is a very dedicated, committed man who's in our corner. I think everyone in this room can easily say he's a friend of the NF2 community.

He's also a member of the quote/unquote "Dream Team" with Synodos. So I hope we learn a little bit about that as well. But I consider it an honor and privilege to introduce to you Dr. Chang. Dr. Chang, thank you so much for coming. Welcome.

[Applause.]

>> DR. CHANG: Thanks, Steve, for the nice and kind introduction. It is my great pleasure to share some of our research with you, particularly regarding the current status of the NF2 treatment options the research community has come up with.

First, I would like to say to you that I attended the NF2 Ohio Gathering, which was held about two weeks ago. This is one of the shirts (with the printed message "Never give up") I bought at this event. I would like to share with you that we have identified some potential NF2 treatments. With your dedication and support, I believe that we'll, all together, be able to come up with something to cure this debilitating disease in the future.

I'm going to cover quite a few different topics. First, I will go over the recent findings on the preclinical and preliminary clinical

study of AR42. I think some of you may have heard of it before. We have been studying this experimental drug (AR42) from preclinical to clinical evaluation, and we have some data suggesting that, particularly in meningiomas, it was quite effective, while in schwannomas, it also slowed tumor growth.

I will also talk about genomic mutation analysis. A comforting message from that study is that NF2 tumors have very few mutations. From there, we have suggested some additional therapies, particularly for NF2 tumors that have mutations in other gene(s) in addition to the *NF2* gene.

Then, I'm going to go over the morphoproteomic analysis of NF2-associated vestibular schwannomas that we have done. It is like genomic analysis, but instead of analyzing DNA, we examine proteins in the tumors. So, we are looking at protein expression in the cell, using the so-called proteomics analysis. We study various key proteins in the cell, particularly the proteins that regulate tumor growth. From the analysis of a patient's tumor growing over time, we have also come up with some other potential therapies. For example, we have studied an NF2 patient who had a tumor debulked two years ago. Then, two years later, she had tumor regrowth. So, we compared these tumor specimens for any changes in the expression of various signaling proteins. We also studied a patient who had a tumor removed following AR42 treatment. So, we also looked at what has changed in the tumor after the treatment.

Then, I'm going to go over another new study. As you know, the current treatment for NF2 tumors is inadequate. In light of the lack of a primary vestibular schwannoma cell line, we have generated some pluripotent stem cell lines from an NF2 patient's vestibular schwannoma. We plan in the future to develop more lines of these induced pluripotent stem (iPS) cells from different patients' tumors and to use them to search for a therapy that is common to all tumors or specific to each tumor in light of the personalized therapy.

Also, I will talk about some natural antineoplastic compounds we have been working on. Last year, I received an email question from the NF2 community, asking about whether natural compounds are good to use. So, I will tell you about the progress that we have made on natural compound testing. Natural compounds are not much different from synthetic compounds because they have defined chemical structures and mechanisms of biological action, and I will tell you how we approach natural compounds as potential therapies for NF2 tumors.

And then finally, as Steve mentioned, I am part of the Synodos

project team, and I would like to report to you the current progress. In addition, I will go over some additional potential therapies that we are studying.

As described by the previous speaker, current treatment for NF2-associated tumors relies on observation, surgery, or radiation; however, these treatments are inadequate to address the preservation of hearing and balance and other cranial nerve and facial nerve functions. Ideally, you would like to have some medical therapies or drugs; however, none of the therapies that are being tested right now are specifically designed for NF2. So, we would like to develop some novel medical therapies specific for NF2.

Some people consider radiation treatment risky. However, there are new technologies available, such as high-intensity focused ultrasounds, which can heat up a tumor to 85 degrees to kill those tumor cells; neuroblades, which use a specific laser that can focus on a specific tumor location to operate on the tumor; and nanoknife, which utilizes high voltage, low energy DC current as a way to penetrate the cell membrane and punch holes in it to lead to tumor cell death. Additionally, there is an interest in using bacterial injection into the tumor, i.e., directly injecting certain bacteria which are usually normal flora and do not cause any problems unless you are immunodeficient. These bacteria only grow in anaerobic (without oxygen) conditions. Normally, the environment in tissues is under aerobic (with oxygen) conditions, which are not favorable for the growth of these bacteria. However, tumors tend to be more hypoxic and allow bacterial growth. When these bacteria grow, they produce toxins and kill tumor cells. Hopefully, some of these new technologies will also be incorporated into NF2 trials in the future, including in combination with a targeted therapy – such as in combination with AR42.

First, I will present our AR42 study.

The way we approach drug trials is to make sure that we have various kinds of cell culture and animal models so that we will be able to thoroughly assess drug efficacy before putting humans at risk in clinical trials. We have generated a variety of human or mouse cell lines. We engineered mouse schwannoma cells lacking *NF2*. As we have been able to acquire human vestibular schwannoma tissues, we have used them to prepare primary schwannoma cell cultures for further drug testing. With these different types of cell cultures available, we test therapeutic compounds in mouse cells with or without *NF2* and then in human schwannoma cells (lacking *NF2*) and normal Schwann cells. If the compounds of interest show efficacy in

these culture models, we further evaluate them in animal models.

For animal testing, you want to have animal models to efficiently develop tumors. Previously, *NF2* knockout models in Schwann cells have been generated; however, these models develop tumors only in a fraction of mice. We have been able to engineer mice with *NF2* gene deletion at specific time points of development. In our model, when we delete the *NF2* gene in neuroprogenitor cells, about 50% of the mice develop schwannomas.

Also, we have developed meningioma mouse models. Here, this is an immunoblot (Western blot) for NF2 protein expression in several human cell lines. This one is called Ben-Men-1 benign meningioma. This one is called KT21, which was derived from a malignant meningioma. We found that these two meningioma cell lines do not express the NF2 protein. Then, we engineered these *NF2*-deficient meningioma cells to express a firefly luciferase enzyme. When you give these luciferase-expressing meningioma cells a chemical called luciferin, these cells will glow and emit light. This allows us to detect light-emitting tumor cells when implanted in mice and to quantify tumor size as tumors grow over time.

The Ben-Men-1 meningioma model behaves very similar to a benign meningioma tumor found in a human patient; it is slow-growing. As you can see, when we injected Ben-Men-1 cells in the skull base here, the tumor just grew slowly along the skull base, never invading the brain; similar to what you see in benign human meningiomas. Here, you can see how the Ben-Men-1 tumor grows over time. This is on a month scale; so you can see that the Ben-Men-1 tumor grew slowly; every month it grew a little bit larger.

Once we have all different cell culture and animal models, we begin to evaluate potential therapeutic compounds. One way is to screen a compound library, similar to what you heard from Dr. Christina Fernandez-Valle last year. Basically, you can screen chemical compound libraries and find the lead anti-tumor compounds. Alternatively, you can first identify pathway-specific compounds. So, we have been looking for drugs that can inhibit signaling pathways that are frequently activated in NF2 tumors. Particularly, we and others have previously shown that the AKT pathway is frequently activated in NF2-associated schwannomas and meningiomas.

Here is a slide showing vestibular schwannoma tumor sections that overexpress an activated kinase enzyme called p-AKT, an activated AKT or phosphorylated AKT (or phospho-AKT). We used an antibody to stain tumor sections for this protein. You can see that the p-AKT protein is strongly expressed in the tumors compared to adjacent normal

tissues, indicating that the AKT pathway is frequently activated in the tumors. Studies have shown that this AKT signaling pathway is important for cell growth and survival. If you inhibit this pathway, tumor cells may stop growing. So, we have tried to identify drugs that inhibit this signaling pathway.

At Ohio State, there were two drugs developed to inhibit the AKT pathway at the time when we established our NF2 models. One is called AR12, which inhibits an upstream kinase, the PDK-1 enzyme, that phosphorylates AKT to activate the AKT enzyme. The AR12 compound was originally developed as a derivative of Celebrex, which, as you know, is a commonly-used anti-inflammatory drug. However, the problem you have heard of with Celebrex is that it can increase the risk of having heart attacks. The AR12 compound was developed to have a higher affinity to PDK-1, but it doesn't have that anti-inflammatory side-effect.

The other compound is called AR42, which is an inhibitor of histone deacetylases (HDACs), enzymes that are frequently over-expressed in leukemia, lymphomas, and other types of cancers. AR42 can also inhibit the interaction between HDACs and protein phosphatase 1 (PP1). For this reason, AR42 can down-regulate the AKT pathway by disrupting interactions between PP1 and HDAC6, consequently allowing free PP1 to dephosphorylate, and thus deactivate, AKT. So, in cells, AKT is activated by phosphorylation by an upstream kinase. However, when PP1 is present, it can inactivate AKT by dephosphorylation. Therefore, we examined whether blocking AKT activation by AR12 inhibition of PDK1 or inactivating AKT via PP1 by AR42 would have any effects on the growth of NF2 tumor cells.

Both AR42 and AR12 are orally available. We thought that oral bioavailability would be important for treatment design as NF2 patients might require long-term treatment. If you have to perform drug injections, patients would have to come to the doctor's office frequently, and it may not be as convenient. If patients can take the drug orally, we may be able to maintain patients on the drug for a longer period of time.

>> Question from audience: So you can't put them together and make a 54?

>> DR. CHANG: Hopefully, but we have not tried it yet.

So, we basically blend the drug into animal diet and feed it to mice to see whether tumors in mice will stop growing.

Here, just to show you an example. We first tested these drugs (AR42 and AR12) in our cell culture models and found they effectively inhibited cell growth. Then, we ran them through our animal models.

Here, just to show you the results in the *NF2*-deficient mouse schwannoma allograft model. As you can see, the tumors in the control group (without drug treatment) grew very quickly. However, the tumors in the treatment groups remained small. MRI (magnetic resonance imaging) confirmed these findings.

For the human vestibular schwannoma xenograft model, we implanted a piece of human tumor directly from a patient into a mouse and watched for tumor growth, plus/minus drug treatment over time. Without drug treatment, some of these human tumor tissues remained stable in mice perhaps because of the slow-growing nature of human vestibular schwannomas. Some of them exhibited slow growth while most others did not grow at all. Some of the tumors shrank slightly. In contrast, with treatment, almost all of the tumors substantially reduced in size.

These images show the genetically-engineered *NF2*-knockout mouse model. As I described before, these mice develop schwannoma tumors, and when we treated them with the drug, we observed that the internal region of the tumor became whitish with fewer cell nuclei. Pathologists told us that these are so-called necrotic tissues. It is typically observed for drug-induced necrosis or unregulated cell death. So, the drug kills the tumor cells, and the whole region exhibited massive necrosis.

This slide shows our meningioma model. We injected tumor cells in the skull base here, and the tumor just grew along the skull base, but never invaded the brain, characteristic of a benign tumor. We tested the compound in this model the same way as I described for the schwannoma model. As you can see here, when you fed mice normal diet, the tumor grew bigger along the skull base. When we treated mice with AR12, meningioma tumor growth was reduced by about 50%. But, when you treated mice with AR42, it caused tumor regression, and the tumors were pretty much gone after treatment. This tumor shrinkage occurred over time, month by month. In contrast, the control tumors continued to grow larger every month. In conclusion, we found that AR12 treatment reduced tumor growth by about 50% after six months, while AR42 effectively caused tumor regression.

More interestingly, if we treated tumor-bearing mice with AR42-formulated diet for about six months (which substantially shrank the tumor to a very small size), then removed the drug (by giving mice normal diet), and followed these mice for another six months, we found that the tumor did not grow back and remained very small. These results have important implications as this would allow patients to take a break from treatment if needed.

As Steve mentioned before, Dr. Welling, with whom I have collaborated over the last 20 years, has conducted a phase 1 AR42 clinical trial. Also, several other investigators at OSU have been testing AR42 in other advanced or recurrent solid tumors and lymphomas. Dr. Welling's trial focuses on vestibular schwannomas and meningiomas.

So far, Dr. Welling has treated five patients. Two of the patients have had extensive follow-up. One of them has been treated for about one year. Both of these two patients have multiple tumors. The first patient has two schwannoma tumors that were followed by monthly MRI. As you heard from Eva's talk before, the volumetric MRI is the gold standard to monitor schwannoma tumor growth. So we used volumetric MRIs to measure the volume of each tumor. Prior to treatment, the size of the first tumor (a neck schwannoma) was about 12 cm^3. Following 10 months of treatment, the tumor remained similar in size. As the changes in the size of this tumor are within the margin of error for MRI, we considered that the tumor remained stable with little growth. However, it should be noted that prior to the treatment, this tumor was actively growing. I just don't have the data to show you right now. The second tumor in this patient was a vestibular Schwannoma. It was a small tumor. Interestingly, following treatment, this tumor pretty much shrank by about 65%.

The second patient had quite a few more tumors. We monitored five of them. Two of them were meningiomas, and the other three included two vestibular schwannomas (right and left) and one ependymoma. As summarized in this Table, these two meningiomas were monitored prior to treatment for two months from September to November; one of them grew 40%, and the other one grew about 5% within two months. However, following treatment for about 10 months, the first meningioma shrank by 36% in tumor volume.

>> It's okay to ask?

>> DR. CHANG: Yes.

>> Can you please help me understand a single treatment protocol of the AR42 is doing double duty on two separate tumor types within the same patient?

>> DR. CHANG: It is a single treatment, but the drug is delivered three times a week. Because of its oral bioavailability, AR42 can go through the blood and circulate through the body. This drug can penetrate the brain and reach any tissue.

>> So it targets multiple targets?

>> DR. CHANG: Yes.

>> So vestibular Schwannomas are growing, still, because it's not

affecting it?

>> DR. CHANG: Yes, but it slowed down schwannoma growth. You're ahead of me.

[Laughter]

The other meningioma, before treatment, increased 5% in size; but, after treatment it shrank by 25%. If you ask Eva, she will tell you that these changes in tumor volume are beyond the margin of error for MRI measurement because radiologists usually consider a significant change in tumor volume to be greater or less than 20%. So, the changes in meningioma tumor sizes following AR42 treatment are already beyond the margin of error. Therefore, we concluded that the changes in meningioma tumor sizes were a real treatment effect of AR42.

For the vestibular schwannomas (VS) in this patient, the right VS tumor grew before treatment by about 24% in two months. Following treatment, this tumor pretty much remained stable and only increased by 1%. Although the tumor seemed to grow a little bit more, its growth rate substantially slowed down compared to pretreatment. The other tumor, the left VS, grew 32% in the two months prior to treatment. Following treatment for 10 months, we observed that the tumor only grew 17%. So, for the vestibular schwannomas in this patient, AR42 did not shrink these tumors; however, while these VS exhibited some growth following treatment, their growth rates were substantially reduced.

>> I see Participant 1 and Participant 3. What happened to Participant 2?

>> DR. CHANG: As I mentioned before, Dr. Welling has recruited five patients for this AR42 trial. Some patients withdrew from the trial. For Patient No. 2, he only received two doses. Because he lives very far away and felt the commitment was too much, he did not continue.

>> I'm sorry. I was missing…

>> DR. CHANG: That's what I mentioned before; the treatment design is very important for patient recruitment. One of the patients experienced some adverse side effects and had to discontinue treatment. We thought that it may be because the patient was treated with a high dose of AR42. Dr. Welling gave some patients different doses of AR42 because he wanted to determine the maximal tolerable dose. This patient happened to receive the highest dose and showed adverse side effects; therefore, Dr. Welling had to stop treatment for this patient.

>> And the percent change following treatment, so that I understand those, Patient 3, for example, has been followed since July of last

year. So 1 year plus?

>> DR. CHANG: If you follow this Table here, from September 2012 to July 2013, it was about 10 months. So, this patient #3 was treated for about 10 months. However, because this patient's right VS was getting too big, he underwent surgery to remove his tumor. That's what I am going to tell you next. We have compared the signaling changes in this tumor after AR42 treatment.

So, this is a summary of the Phase 1 AR42 trial. Although there are some toxicity issues with AR42 treatment, particularly at a high dose, the side effects were manageable. Because Dr. Welling happened to be recruited away from Ohio State, this Phase 1 trial for AR42 was stopped. However, some of the patients are being followed up. Also, Dr. Welling has started another AR42 trial which has been recently funded by a DoD clinical trial award. This new AR42 trial is a Phase 0 study. Instead of going from Phase 1 to Phase 2, Dr. Welling and colleagues thought that, if AR42 demonstrates target inhibition in this Phase 0 trial, they will be able to quickly move into a Phase 2 study as AR42 has been evaluated in a Phase I dose escalation study in some hematological malignancies and other solid tumors at OSU and has shown a tolerable toxicity profile.

The design for this Phase 0 trial is to treat patients with AR42 for a short duration (three weeks) followed by tumor removal. Then, we will perform pharmacokinetic analysis to determine whether a sufficient concentration of AR42 reaches the target tissues (i.e., tumors). Also, we will examine whether AR42 inhibits specific signaling targets in treated tumor tissues. In addition, Dr. Welling's team will assess any audiological changes prior to surgical removal of tumor. This Phase 0 trial is currently recruiting patients at several of Dr. Welling's collaborating sites. I am a co-investigator in this trial.

>> Was there a study done on when that trial stopped if the tumor grew again after the drug was stopped?

>> DR. CHANG: I am not aware of such a study in humans. The new Phase 0 trial is basically designed to have a short duration of treatment and then, regardless of whether the tumors grow or not, they will be surgically removed for pharmacokinetic and pharmacodynamic studies.

>> The other study. When you stopped...

>> DR. CHANG: The other Phase 1 study was originally designed, as Eva mentioned before, to study safety and toxicity. The efficacy was not the primary objective there; so, mainly it was to investigate the safety issue. That is why that study was to determine whether

patients can sustain the treatment; however, Dr. Welling has continued to follow some of the patients.

I'm going to shift gears to the second area of my talk regarding genomic mutations. We have examined what changes occur in NF2-associated VS at the DNA level because the *NF2* gene may not be the only gene that is mutated. Also, we do not know when these tumors are going to grow. It is possible that tumors may acquire additional genetic changes to increase growth. So, if there are additional genomic changes, we would like to know.

As I mentioned before, two of the NF2 patients who were on the Phase 1 AR42 trial had their tumors removed. So, we sent their tumors to a company called Foundation Medicine, which has designed a new kind of genomic sequencing test called the FoundationOne test. Instead of sequencing the entire human genome, this FoundationOne test examines only cancer-related genes. The problem with sequencing the entire human genome is that once you sequence the entire 3 billion base-pairs of the human genome, it is a big task to find out which changes are real and which ones are really affecting proteins. So, rather than sequencing the entire human genome, Foundation Medicine chose to sequence about 400 genes that are frequently mutated in human cancers. The majority of these genes is involved in tumor growth and is more likely to be changed. Most importantly, this test only takes about two weeks. They can identify the genomic changes quickly and based on the genetic changes identified, Foundation Medicine will suggest some potential targeted therapies.

We submitted these two tumors for the FoundationOne test. It is comforting to tell you that these NF2-associated vestibular schwannomas had very few mutations. As predicted, both tumors have *NF2* mutations, and the results confirmed the previous mutational findings because one of these two patients was previously tested in Alabama and the other in Arizona; in other words, both of these patients had *NF2* mutation analysis done before.

For Patient No. 1, the only known mutation identified from the FoundationOne report was in the *NF2* gene. The second patient had two known mutations, one was in *NF2* and the other was a duplication in exons 2-3 of the *MYC* oncogene, which encodes a transcription factor important for cell growth. This duplication (in *Myc*) has been found in human cancers previously.

So, if you find your tumors have mutations in other genes in addition to *NF2*, this information may suggest additional potential targets for therapies. Drugs that inhibit the pathways affected by

these mutations may be tested so that you are not limited to drugs that only affect the known *NF2*-related pathways.

Although these changes (in *NF2* and *Myc*) are considered as real mutations in these tumors because they have been previously studied, there are several other changes that were identified as variants of unknown significance (VUS), changes in the DNA sequence whose significance has not yet been defined. It is possible that these VUS may be genetic polymorphisms or natural variations that have no adverse effects. Some of the changes may be silent or may only change one amino acid. If the resulting amino acid change is very similar, it may not affect protein structure and function. For example, you could have isoleucine change to valine or leucine. These are all non-charged amino acids, and their structures are very similar. So, such changes may not affect protein function. In that regard, we don't think that those kinds of VUS are true mutations.

Intriguingly, we found that, coincidentally, the tumors from both patients had mutations in the *NUP98* gene that resulted in single amino acid changes. Also, the mutated residues in the NUP98 sequence in these two tumors were located in close proximity in the protein. So, what I'm trying to say is that NUP98 could be another important gene frequently mutated in NF2 tumors.

Here is a copy of the standard report that we receive from Foundation Medicine. Just to give you an example, the report tells us the mutations identified and the information about the mutated genes and their regulated pathways. It also tells us the FDA-approved drugs or drugs in clinical trials that target the pathways affected by the identified genomic mutations. For these two cases, Foundation Medicine has provided us these clinical trials to consider. For example, here are the drugs in clinical trials that target the *NF2* pathway. We heard some of them from Dr. Machado's talk before. And you may be familiar with some of them. For patients with *MYC* mutations, these are the available clinical trials. The NF2 patient with a duplication in exons 2-3 of the *Myc* gene may also consider these trials as the drugs in these trials directly target the Myc pathway.

This slide summarizes what we know about NUP98. It was originally discovered as a nucleoporin protein, a key component of the nuclear pore complex. Nuclear pores allow transport of materials in and out between the cytoplasm and the nucleus. Recent studies suggest that the NUP98 protein is not only working as a nucleoporin protein but also playing important roles in gene expression, mitotic checkpoints, and pathogenesis. *NUP98* mutations have been found in leukemia and

several types of solid tumors. If we can prove that the *NUP98* mutations that we have identified have to do with schwannoma tumorigenesis, the NUP98 protein could become another therapeutic target to consider. So we should start thinking about potential treatments that target this protein.

Another question you might have is whether these *NUP98* mutations can be found in the germline of these NF2 patients; i.e., whether these patients inherited the *NUP98* mutations. Alternatively, the *NUP98* mutations may be tumor-specific. Also, it will be interesting to see whether these *NUP98* mutations confer a growth advantage to tumor cells. We are presently working on these questions and hopefully will understand whether there is any role for NUP98 in NF2 tumors.

Next, I will talk about morphoproteomic analysis of NF2-associated vestibular schwannomas. Morphoproteomic analysis uses standard immunocytochemical staining techniques. It is just like when a pathologist prepares a tumor section, stains it with some dye, and looks at tumor morphology in order to tell you what kind of tumor you have. Immunocytochemistry means that you use an antibody to stain a tumor section for a particular protein of interest. So, in addition to using chemical dyes, you use antibodies to stain for particular signaling proteins. In our case, we stained for more than 30 different key signaling molecules that may be affected by NF2 or drug treatments.

We first looked at the signaling changes in the tumors before and after treatment. Then, we searched the PubMed database for the functions of the altered signaling pathways in schwannomas and other tumor types and for the available drugs or clinical trials that target these pathways. Here we are looking for the drugs that are frequently used and have little or no common side effects; i.e., some commonly-used anti-diabetic drugs that may also have anti-tumor activities.

For example, this patient No. 1 had a previously debulked tumor, which was previously removed. Then, the debulked tumor regrew. About 2-1/2 years later, she participated in the AR42 clinical trial. However, she only received one cycle of treatment due to side effects. Subsequently, she underwent surgery to remove the tumor. We compared the signaling changes in the previously debulked tumor with those in the tumor that regrew. These tumors were confirmed as vestibular schwannomas with typical spindle cell histopathology.

In this slide here, we examined the activation of the insulin-like growth factor 1 receptor (IGF-1R). As you may know, this IGF-1R

receptor signaling pathway is also frequently activated in vestibular schwannomas. IGF-1R is an RTK (receptor tyrosine kinase) like the EGFR family of RTKs that you have heard of frequently. However, it is a different RTK as it is activated by the insulin-like growth factor 1 (IGF-1). Both the debulked tumor and the tumor that regrew showed strong expression of activated IGF-1R.

For patient No. 2, we obtained a right vestibular schwannoma and a geniculate ganglion mass after AR42 treatment. We did not have tumor specimens from before AR42 treatment for this patient. The right VS tumor was monitored during the AR42 trial. When this tumor was removed, a geniculate ganglion tumor was also found and removed. This geniculate ganglion mass was not monitored during the clinical trial. Histopathology confirmed that this tumor was a mixed meningioma with schwannoma. We compared the signaling differences in these tumors. As you can see, activated IGF-1R was strongly expressed in these tumors, suggesting that AR42 doesn't seem to affect this pathway.

The RTK pathway that is most frequently activated among various tumor types is the EGFR (epidermal growth factor receptor) family, which includes EGFR, ERBB2 (HER2), ERBB3 (HER3), and ERBB4. In patient No. 1's debulked tumor, the EGFR signal was relatively low; however, following tumor growth, its intensity substantially increased. These results suggest that during tumor regrowth, EGFR is frequently activated. Consistently, the downstream signaling molecules of this RTK were activated. For example, this tumor exhibited strong activation of phospho-ERK (p-ERK), a downstream signaling molecule of the EGFR pathway.

As I mentioned to you before, AR42 can also down-regulate AKT, another downstream signaling molecule of the EGFR pathway, but it doesn't directly affect EGFR. Interestingly, in patient No. 2's tumors following AR42 treatment, the activated p-AKT signal was rather mild, compared to patient No. 1's tumor that regrew. However, this patient No. 1's previously debulked tumor had much lower levels of p-AKT than the tumor that regrew. These results suggest that AR42 treatment substantially reduced expression of activated AKT (p-AKT).

In this morphoproteomic analysis, we examined about 30 to 40 signaling molecules. You can study as many proteins as you like depending on the signaling pathways in which you are interested. Here is another example. We also observed that vestibular schwannomas tend to express several proteins associated with inflammation, such as NFκB, COX2, and STAT3. These proteins are induced by cytokines and were strongly expressed in both patients'

tumors.

Based on the signaling molecules/pathways that are activated in these VS tumors, we searched PubMed for drugs that can inhibit these pathways and are commonly prescribed for other indications. We identified metformin, statin, Celebrex®, and melatonin. These are drugs commonly used for many years. In the literature, metformin has been shown to suppress IGF-1R, AKT, and mTOR. In addition, it also decreases ERK signaling and reduces COX2. All of these signaling molecules were strongly expressed in both patients' tumors. So, metformin may be considered for treatment in these patients.

The literature also suggests that metformin can suppress tumor growth in some tumor types. However, it was not known whether metformin will suppress vestibular schwannoma growth. So, we decided to test this possibility. We found that while metformin, by itself, did not inhibit schwannoma cell growth, it gave rise to a synergistic growth inhibitory response when combined with AR42. So, in the future, it may be possible to combine some of these commonly prescribed drugs with targeted agents to treat NF2 tumors.

I would like to switch gears to talk about schwannoma-derived induced pluripotent stem (iPS) cells. As I mentioned before, currently, a human vestibular schwannoma cell line that maintains benign growth behavior is not available. Most of the vestibular schwannoma cell lines that you have heard of have been generated using viral oncogenes. However, transformation by viral oncogenes changes the tumor cells' growth behavior and alters their responses to drugs. For example, HEI-193 is a vestibular schwannoma cell line that was established by the HPV (human papilloma virus) E6 and E7 oncogenes. Once these schwannoma cells are transformed by these viral oncogenes, they change the characteristics of Schwann cells. In addition, if you give these cells some drugs; for example, RAD001, which normally can inhibit schwannoma cell growth, they are no longer inhibited by this mTOR inhibitor. Therefore, transformation by the HPV oncogenes changes the properties of schwannoma cells and renders HEI-193 not suitable for drug testing. Ideally, any drugs should be tested in a human cell system before they are evaluated in clinical trials. So, establishment of a reproducible model for NF2-associated VS that could be used to study disease pathogenesis and to faithfully predict drug response would significantly accelerate NF2 translational research.

Another commonly used method to establish a human cell line is to use telomerase, an enzyme that repairs the ends of chromosomes (called telomeres) during DNA replication. If the chromosome ends

are not repaired, telomeres will become shorter after each cell division. Once telomere shortening reaches its critical limit, cells will stop dividing and undergo senescence. In differentiated somatic cells, telomerase expression is turned off. This is the reason why primary human cells cannot grow indefinitely in culture. However, if you re-express telomerase in cells, they may resume growth and become immortal. This approach of cell immortalization has been used to establish a variety of human cell lines. The Ben-Men-1 benign meningioma cell line that I mentioned earlier was established using this approach. However, so far we have not succeeded in using telomerase alone to immortalize human vestibular schwannoma cells.

Alternatively, we have used a new technology called induced pluripotent stem (iPS) cells to develop a human vestibular schwannoma cell model. By definition, stem cells are capable of indefinite growth and can be differentiated into many cell lineages. iPS cells allow access to cell lineages that are difficult to culture, such as Schwann cells. Differentiated iPS cells can be used to model diseases with patient-specific mutations and can constitute a reproducible source of cells for high-throughput drug screening. In addition, it is possible to use genetic techniques, such as the CRISPR/Cas9 genome editing technology, to correct *NF2* mutations in patient-derived iPS cells to generate an isogenic pair of cell lines, one containing the patient's *NF2* mutation and the other with a normal *NF2* gene. This would allow side-by-side comparison of drug sensitivity due to *NF2* loss.

We have been able to convert an NF2 patient's vestibular schwannoma cells into iPS cells using several reprogramming factors. This slide shows you a colony of iPS cells growing over time. These schwannoma-derived iPS cells expressed several stem cell markers, such as TRA-1-60, SSEA3, OCT4, and NANOG. Also, several of these iPS clones exhibited a normal human chromosome profile. As I mentioned before, iPS cells can be differentiated into all different cell types and have been used to model human diseases; for example, ALS (amyotrophic lateral sclerosis or Lou Gehrig's disease) and Parkinson's disease. Researchers have been trying to use differentiated iPS cells for stem cell therapy. But, in our case, we are using patient-derived iPS cells as a renewable source of Schwann/schwannoma cells. If successful, we will use this model to test drugs in the future.

In addition, we plan to establish a panel of patient schwannoma-derived iPS cell lines carrying different *NF2* mutations and to use them to test drugs to see if they work in one or all tumors. These iPS cells derived from NF2 patients' vestibular schwannomas would

signify an advance in personalized healthcare in which the effects of unique patient mutations can be studied and therapeutic testing can be customized. Development of a panel of VS-derived iPS cells would facilitate identification of effective therapies by accounting for variations in treatment response among individual patients.

Next, I would like to talk about natural compound testing. I am going to tell you about the progress that we have made, particularly on a natural compound called silvestrol.

Over the past few decades, several hundred natural compounds have been identified to possess antitumor activities. Some natural compounds also possess antioxidant and anti-inflammatory properties. Natural compounds are a particularly attractive solution for cancer treatment as patients often take them as dietary supplements and for health enhancement purposes. Some of you probably have been using natural supplements or antioxidants to improve your health or may be thinking about using them to prevent tumor growth.

Silvestrol was first isolated from an *Aglaia* tree found in the tropical rainforest in an inner island of Indonesia. It shows a nanomolar cytotoxic potency similar to other natural anti-cancer drugs, such as camptothecin and paclitaxel, for a panel of cancer cell lines and possesses potent anti-tumor effects in multiple cancer models. Our collaborator Dr. A. Douglas Kinghorn at The Ohio State University College of Pharmacy is one of the international leaders on the isolation and structure elucidation of this natural compound and other related rocaglates. He has led a natural compound program project over the last 10 years. We have been fortunate to collaborate with him.

We began by screening a library of natural compounds that Dr. Kinghorn has isolated from either tropical trees, marine organisms, and fungus over the last few decades. Here is a table showing the first 23 compounds that we tested in a panel of human and mouse Schwann and schwannoma cells and human meningioma cells.

On the bottom part of this Table, you can see natural compounds that you have heard of and which are commonly used; for example, caffeic acid phenethyl ester (CAPE), which can be found in bee hives; curcumin, which is an oriental spice; resveratrol, which is present in grape juice and red wine; and sulforaphane, which is obtained from cruciferous vegetables, such as broccoli. However, these natural compounds, when they were tested in vestibular schwannoma and meningioma cells, were not very potent. It would require quite substantial amounts of these compounds to inhibit the growth of these tumor cells.

Here, these columns show you the so-called 50% inhibitory concentration, or IC50. An IC50 value of 10 micromolar is generally considered as a cutoff for the drug potency in this kind of testing. If a compound has an IC50 greater than 10 micromolar, the NCI Therapeutic Testing Program usually will not consider the compound active. As the IC50 values of these four natural compounds (CAPE, curcumin, resveratrol, and sulforaphane) in schwannoma cells are about 20 micromolar or greater, in order to achieve these concentrations in the blood you would need to consume a large amount of these compounds to achieve efficacy.

However, when you look at the rest of the 19 natural compounds, some of them, particularly those highlighted in yellow, including silvestrol and related compounds, have IC50 values of just a few nanomolar. Nano- vs micro-molar is 1,000-fold difference. So, in the case of silvestrol, you need 1000-fold less of compound to kill 50% of schwannoma and meningioma tumor cells, compared to the four commonly-used natural compounds (CAPE, curcumin, resveratrol, and sulforaphane) that I mentioned before. Silvestrol is one of the most potent anti-neoplastic compounds that we have ever tested in NF2 tumor cells.

In addition to vestibular schwannomas, we also tested silvestrol in malignant peripheral nerve sheath tumors, aggressive soft-tissue sarcomas that can occur spontaneously in the general population or in patients with NF1. We have found that silvestrol also efficiently inhibited the growth of both *NF1*-deficient and *NF1*-expressing MPNST cells, except at a slightly higher IC50 value but still at the nanomolar range.

The following slide shows you images of schwannoma, meningioma, and MPNST cells treated with silvestrol or DMSO (dimethyl sulfoxide), which is used as a solvent to dissolve compounds. Without silvestrol, these cells grew as a monolayer in culture dishes. In contrast, in the presence of silvestrol, they stopped growing. At a higher concentration, most of the cells died and detached from the dishes. You can see the effect in all three cell types (vestibular schwannoma, meningioma, and MPNST cells).

Subsequently, we tested silvestrol in animal models. To generate an orthotopic, quantifiable MPNST model, we injected luciferase-expressing, *NF1*-deficient MPNST cells into sciatic nerves of immunodeficient mice so that the MPNST tumors would grow in a location/environment similar to that seen in humans. Using bioluminescence imaging (BLI) as described earlier for the Ben-Men-1 meningioma model, we quantitated the amount of light emitted from the

tumors over time. BL images show that initially all tumors were relatively small prior to treatment. Without treatment for four weeks, tumors became very large. In contrast, the tumors in mice treated with silvestrol were substantially smaller.

This graph shows quantitation of light intensity emitted by the tumor cells. Previous studies have shown that BL intensity correlates with tumor size. It should be noted that the graph is shown on a log scale. As you can see, the BL signals from the silvestrol-treated group are clustered in the lower part of this graph, while those from the untreated group were substantially higher. The differences in BL intensities between the untreated and treated groups were at least 300-fold.

To confirm the BLI results, we performed MRI on tumor-bearing mice prior to and after treatment with silvestrol for four weeks. MR images show that prior to treatment, the tumors were relatively small. In control mice that only received the vehicle (HPβCD or 2-hydroxypropyl-beta-cyclodextrin), tumors readily grew to a large mass after four weeks. HPβCD is commonly used in preclinical trials as a solvent for drug formulation. Below the MR images, you can see the tumor volumes calculated by volumetric MRI measurement. Consistent with the BLI results, tumors in mice treated with silvestrol for four weeks remained very small. These results indicate that silvestrol exhibited potent anti-tumor effects in MPNSTs.

We also tested the anti-tumor efficacy of silvestrol in an orthotopic, *Nf2*-deficient schwannoma model. As expected, without treatment, the control tumors grew larger over time. However, treatment with silvestrol noticeably reduced tumor size. Quantitation of BL signals showed that silvestrol inhibited schwannoma growth by about 60~99% in treated mice. Collectively, these results indicate that silvestrol effectively inhibited the growth of *Nf2*-deficient schwannomas.

Histological analysis showed that silvestrol-treated schwannomas contained less cell density, compared to vehicle-treated controls. Also, silvestrol-treated schwannoma cells exhibited chromatin condensation, reminiscent of pyknosis, a condition often seen in dying cells. In addition, silvestrol increased apoptosis in treated tumors as determined by TUNEL staining, a method to detect programmed cell death. It has been hypothesized that silvestrol inhibits protein translation of selective messenger RNAs (mRNAs) important for growth. Silvestrol can inhibit the expression of specific signaling molecules, such as AKT, which is important for cell survival. Western blot analysis showed that silvestrol inhibited the level of

the AKT protein in VS, meningioma, and MPNST cells. In addition, we also observed that silvestrol inhibited the expression of Aurora A, a mitotic checkpoint kinase important for cell division.

In 2007, silvestrol was accorded DDG IIA status by the National Cancer Institute (NCI) Drug Development Group, which is responsible for pre-clinical and clinical decision-making and is now in the NCI's Experimental Therapeutics Program (NExT) for preclinical development. Hopefully, this program will enhance the translation of silvestrol to the clinic.

Here is the chemical structure of silvestrol, which is somewhat complex. To test it in clinical trials in the future, we will need a large quantity. Dr. Kinghorn has acquired a program project grant from the NCI, which has a contract with the Indonesian government for 500 kilograms of *Aglaia* trees, leaves, and bark to purify silvestrol. However, the amount of silvestrol they can obtain from this effort is only enough for a small clinical trial.

To obtain silvestrol in larger quantities, Dr. Kinghorn and collaborators have been examining the evolution of the *Aglaia* species to identify related tropical trees that may have higher concentrations of silvestrol. Ideally, chemical synthesis would be the sustainable route to generate large amounts of silvestrol for clinical trials. However, the current approaches toward total synthesis of silvestrol involve quite a few complicated steps, making its synthesis challenging.

Alternatively, we have explored silvestrol-related rocaglates with simpler structures (smaller molecular weights) but with comparable or stronger potency. Here are some of the silvestrol-related compounds. The major difference from silvestrol is that these related compounds do not have the dioxanyloxy ring, which makes the synthesis of silvestrol more complicated. As these silvestrol-related compounds are smaller in molecular weight, it is anticipated that they will be easier to synthesize. Therefore, we tested 10 silvestrol-related rocaglates for their growth inhibitory activities in schwannoma and meningioma cells and found that several of them still possessed potencies similar to or better than silvestrol. We are continuing to work on this area. Hopefully, we'll be able to synthesize these silvestrol-related compounds for further studies.

Finally, I would like to tell you about the Synodos project and some other potential drugs that we are investigating.

As you know, last year, the Synodos project for NF2 was initiated. The goal is "to better understand merlin's tumor suppressor behavior via molecular manipulation and pathway interrogation with and without

the *NF2* mutation through a systems biology approach using tissues derived from disease-related tumor types in order to select biologically-relevant agents to test in disease-relevant animal models, followed by translation to disease-specific problems in the clinic" (for detailed information, please see http://www.ctf.org/Research/Synodos.html). In other words, the concept is to take the findings from the bench to the clinic and then return back to the lab to further refine the treatment.

So, this is the team, which is composed of 12 investigators from eight institutions led by Drs. Jim Gusella and Scott Plotkin from Harvard University/Massachusetts General Hospital, Dr. Wade Clapp from Indiana University, and Dr. Jaishiri Blakeley from Johns Hopkins University. Each investigator brings different expertise to the study from interrogating gene function and identifying new targets and potential therapeutics to testing novel drugs in preclinical and clinical trials.

In the first phase of the Synodos project, we have evaluated a panel of new drugs that either were approved by the FDA for other indications or are currently in clinical trials. We have selected these compounds based on literature searches for drugs that inhibit *NF2* signaling pathways and then tested them in schwannoma and meningioma cells. Some of them block a specific pathway, while others inhibit multiple pathways. Preliminary results indicate that several of these compounds were very active in inhibiting the growth of schwannoma and meningioma cells. We are currently planning to test these compounds in animal models. If any of them show potent antitumor effects, they will be considered in future clinical trials. In addition, investigators have been looking into changes at the genomic, proteomic, and kinome levels in cells with or without *NF2* mutations and in tumors. So, this is the status of the Synodos project right now.

Finally, this slide gives you a summary of the NF2 pathways. Merlin, the *NF2* gene product, can inhibit various RTK (receptor tyrosine kinase) pathways, including the EGFR family members that I mentioned before. Activation or over-expression of these RTKS, which is frequently seen in NF2 tumors, triggers downstream growth signals through the mitogen activated protein (MAP) kinase pathway and the PI3K/AKT/mTOR pathway. Several RTK inhibitors have been previously tested but only showed modest efficacy in inhibiting NF2 tumor growth. Avastin (bevacizumab) acts as an angiogenesis inhibitor by inhibiting vascular endothelial growth factor A (VEGF-A). As a result, it blocks activation of VEGF receptor, which is also an RTK.

Dr. Plotkin at Harvard/MGH has shown that Avastin reduces schwannoma tumor size and improves hearing in about 50% of patients. Other RTK inhibitors, such as lapatinib and axitinib, are currently being evaluated in NF2 patients. Lapatinib blocks both the EGFR and ErbB2 receptors, and axitinib inhibits multiple RTKs.

Previously, we showed that lapatinib and erlotinib, an EGFR inhibitor, exhibited only moderate potency in vestibular schwannoma cells. One possible explanation for this observation is that other EGFR family member(s) is(are) activated in these tumor cells. Inhibition of EGFR and/or ErbB2 may not be sufficient to completely block schwannoma cell proliferation. So, we performed an activated RTK array analysis, which examines the activation of various RTKs in tumor cells. Interestingly, we found that activated or phosphorylated ErbB3 levels were frequently elevated in vestibular schwannoma tumors compared to paired vestibular nerve. In contrast, cultured schwannoma cells selectively activated EGFR expression. In addition, strong ErbB3 expression was confirmed by immunohistochemical staining of vestibular schwannoma tumor sections. These results suggest that ErbB3 may play an important role in schwannoma growth *in vivo* and may be a potential therapeutic target. We are currently exploring the possibility of inhibiting ErbB3 alone and in combination with other RTKs as potential treatments for NF2-associated vestibular schwannomas and meningiomas.

In summary, I have shown you that AR42 may be a potential treatment for NF2-associated tumors, particularly meningiomas. We have found that NF2-associated vestibular schwannomas tend to have very few mutations. In addition to *NF2* mutations, mutations in other genes, such as *NUP98*, have been identified and need to be further investigated for their potential roles in NF2 tumorigenesis. Morphoproteomic analysis and cell culture studies suggest that some commonly-used drugs may be considered in combination with targeted drugs for treating NF2 tumors. In addition, we have established iPS cell lines from an NF2 patient's vestibular schwannoma. These iPS cell lines may be used as a potential human schwannoma cell model for drug testing. Also, we are very excited about the anti-tumor potency of silvestrol and its related compounds. We are continuing to work toward developing these compounds for human use. Hopefully, the Synodos team and our efforts will identify some priority compounds for NF2 clinical trials in the near future.

I just want to leave you with these thoughts. This is one slide that I borrowed from Dr. Welling, showing one of his young NF2 patients. Here is another T-shirt with the printed message "F'N NF"

that I just bought from the NF Midwest front desk. We hope that one day we will be able to say these words loudly. Last night, Diana took us out to dinner. She told us how she came up with this message. She said this is her favorite that she has come up with so far. I hope that with your dedication and support, one day we can eliminate this debilitating disease.

[Applause.]

>> DR. CHANG: Thank you very much.

>> When did you get the DOD funding? What year?

>> DR. CHANG: DOD funding? We had DOD funding several years ago. The recent DOD funding awarded to Dr. Welling in August 2013 is for the AR42 clinical trial. It was supposed to start last year. But, negotiation with the DOD took some time; so it has only just started last month.

>> What about the Silvestrol project you're talking about? Do you know about the toxicity of it? Is it a lot better than the regular chemo drugs that are out there?

>> DR. CHANG: When we did silvestrol treatment in animals, treated animals did not exhibit any significant side effects; i.e, basically very similar to those treated with AR42 or AR12. It is well-tolerated.

The MTD (maximal tolerable dose) for silvestrol was first determined in mice. It is being evaluated in a dog model at OSU right now. Hopefully, these studies will guide the design of a human clinical trial.

>> I have a strange question. First of all, thank you for what you're doing for NF2. But I have a strange question. I serve on the Board of the National NF Network. And we just funded a project with the University of Wisconsin in Madison. And they're going to genetically engineer an NF1 swine model - miniature pig, basically. And I've spoken to them about doing the same thing for NF2. And for $150,000 they can genetically engineer an NF2 pig if it works. And my question to you is: If that was something I could find the funding for and pursue, would that be helpful to NF2 as opposed to using the mouse? Is there a limitation with the mouse that a swine would help? I can't get an answer from everyone.

>> DR. CHANG: Diana asked the same question yesterday. My answer to that question is basically there are pros and cons. Physiologically and evolutionarily, pigs are closer to humans than mice. However, when you use a larger animal model for testing drugs, the cost will be higher, it will require larger facilities, and everything resource-wise will be more costly.

If you have such a model, the FDA is going to ask you to evaluate your drugs in this model because pigs are closer to humans. This means that if you have an NF2 swine model, it is likely that any new drugs would need to be tested in this model prior to human clinical trials. If you need to test drugs in pigs, the cost and the resources will be higher.

>> My understanding is that you will have to do the research there (in Madison) because they have the barn, the veterinarians, and the surgical tables and everything else. Then, you wouldn't be able to take the pigs to Ohio State.

>> Unless they are willing to transfer the swine model to other institutions. And the institution is willing to come up with the resources to do those kinds of trials. Imagine when researchers need to use a monkey model for certain human diseases, each monkey costs $50,000 or more. You will also need a large space to care for these animals, plus you have to provide other enrichments, such as playing music for them.

>> Huge undertaking. I'll visit that facility in March. We'll see the NF1 pig.

>> There are a couple of other facilities. Emory's got one. And another... Both of them have pigs going full thrust.

>> For NF2?

>> But when it's all said and done, if there was an NF2 pig and there was this model, would it help move this kind of stuff along quicker and more efficiently, or not really?

>> DR. CHANG: I would also say that there are pros and cons. You will need to have another level of drug testing. So, it will take a little bit longer. But, it might be safer when it goes to human trials.

>> [Inaudible] you go to like synthesize an organ, human organ. Then specifically like a kidney. Then that's the closest you can get from the sense of human sized kidney.

>> My sister has a swine valve in her heart now.

>> A rib fest.

[Laughter]

>> Thank you very much.

[Applause.]

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