Interactomics Basics and Applications
Prof. Sanjeeva Srivastava
Dr. Joshua LaBaer
Department of Biosciences and Bioengineering
Arizona State University, USA
Indian Institute of Technology, Bombay

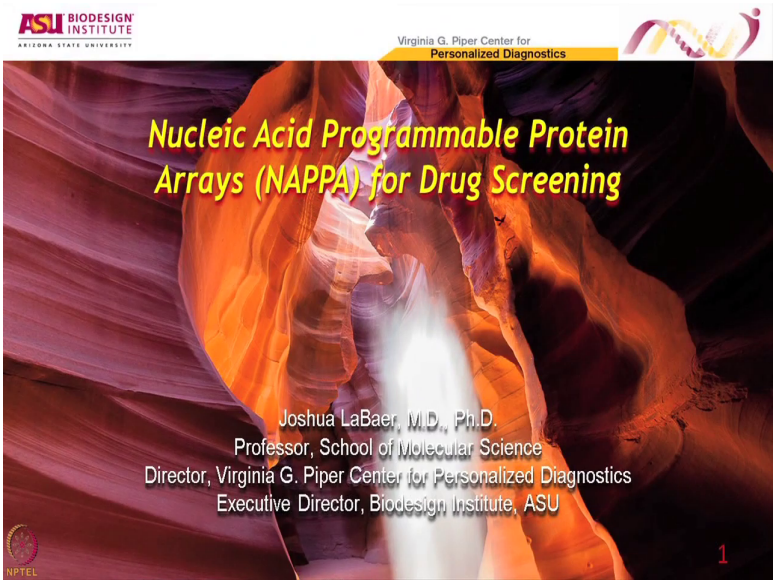
Lecture – 15
Using Functional Proteomics to Identify Biomarkers and Therapeutic Targets-II

Dr. Joshua LaBaer has taught you various good concepts about planning for experiments for biomarker discovery; how and very novel protein microarray platform like Nucleic Acid Programmable Protein Array or NAPPA can be utilized for many applications. Today he is giving his last lecture where he is going to talk to you about a case study in which how NAPPA technology could be utilized for the functional studies.

There are various type of modifications. Post translational modification happens which makes protein functional which gives them different properties and which are very crucial to study. However studying PTMs are not very straightforward not very easy, there are variety of modification happens as you are aware like phosphorylation, glycosylation, acetylation and there are some newer forms like addition of AMP, AMP isolation etcetera.

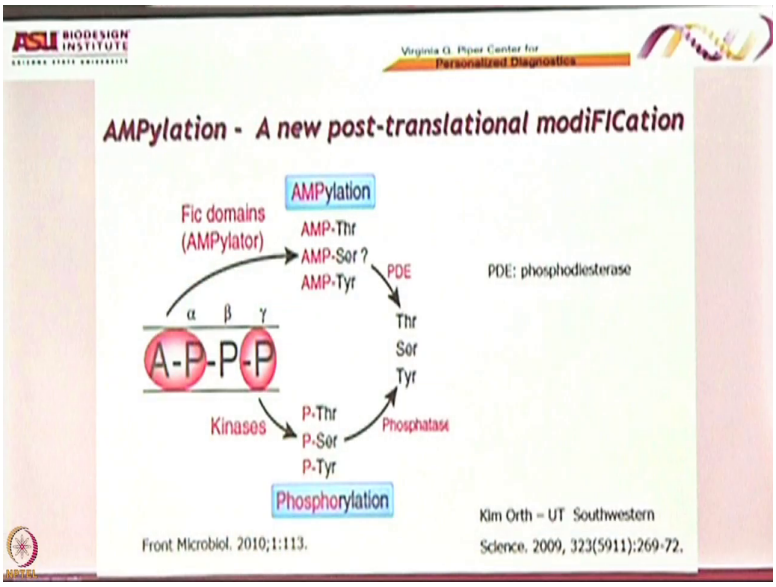
All of those are very crucial for understanding a given cellular context. Suppose (Refer time: 01:27) we will also summarize all the various studies which have been covered during his section, especially for the NAPPA technology as well as the biomarker discovery program and other clinical applications. So, let us listen Dr. Joshua LaBaer's lecture.

(Refer Slide Time: 01:53)



Since, it is the last lecture of the series let us focus a little bit more on sort of functional studies that we have been doing with the NAPPA with the fairly heavy emphasis on our most recent story. But, I just thought it would be useful to wreck you know one of the things that I keep saying is that the proteins on the array are active and I think probably one of the best bits of evidence for that is when we test function of these proteins, protein-protein interaction, enzyme substrate activity that sort of thing we usually get it and so that is kind of how we look at it.

(Refer Slide Time: 02:41)



So, the first one of the first stories that we looked at was AMPylation. So, you are all familiar with this part of the pathway, where you have ATP and the gamma phosphate and ATP is added to threonine series or tyrosines on proteins in a process that we call phosphorylation and that is usually catalyzed by an enzyme called a kinase right.

So, you are all, you have seen that a million times by now. So, it turns out that in some circumstances a slightly different, but very related reaction occurs in which the AMP, the adenosine plus the first phosphate get added to a protein. So, you get AMP threonine AMP serine maybe and AMP tyrosine and that is called AMPylation.

So, you are taking the opposite half of the molecule and you are adding it to proteins and it and it turns out that this process is remarkably well conserved, if you look through evolution. So, if you look at many many bacteria and even in eukaryotic cells there are classes of

enzymes called AMPylators that will do this reaction. We do not fully understand what the biology this interaction is, but one of the places where we see at the most often is when bacteria infect an individual and then the bacteria use this to modify host proteins.

So, it is possible that pathogens have used this as a way to regulate expression in cells. So, the challenge with AMPylation is that we do not really know what the targets are. It you know for years people have been trying to study one of the targets that are being modified by these enzymes and when we began this work there was probably one bonafide target that we really knew about.

There were 2 or 3 others that had been proposed, but not verified and the methodologies that people had used they had tried you know doing pull-down experiments they had tried doing mass spectrometry experiments. They had done various chemical linkage experiments, but it was very hard to figure out what the targets were. So, we had a very ambitious postdoc in the lab Sabo (Refer time: 04:59) and he wanted to see if he could use the protein array as a way of discovering what were the targets of these enzymes.

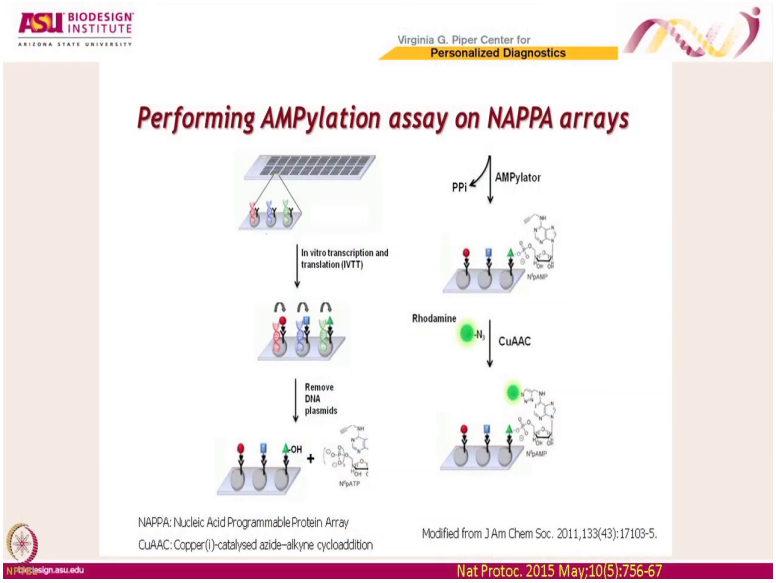
So, the approach that he took to do this was the idea would be you print an array you express the proteins and you treat it with an AMPylator that will add the AMP group to the proteins, then you come back later and try to determine which proteins have the AMP group on it. So, the initial approach that people thought would work would be you do this method, you have the AMP group and you come in with an antibody that recognizes the AMP here.

But, it turns out that the antibody did not work well, it was really not very selective and it did not pick up what we wanted and so he came up with a different strategy that was very creative. So, the strategy he used is based on click chemistry. Basically you take an alkyne group and an azide group. These are 2 chemical groups and they are reactive species, but they are very selectively reactive species.

And, in the presence of copper they will form a chemical reaction that creates a covalent linkage and it requires copper for activity, but it is very selective. So, if you run a if you have the azide group on one protein in a cell lysate and the alkyne group on the other protein even

amongst millions of other proteins only those 2 will linked and nothing else will. So, it is very selective.

(Refer Slide Time: 06:37)



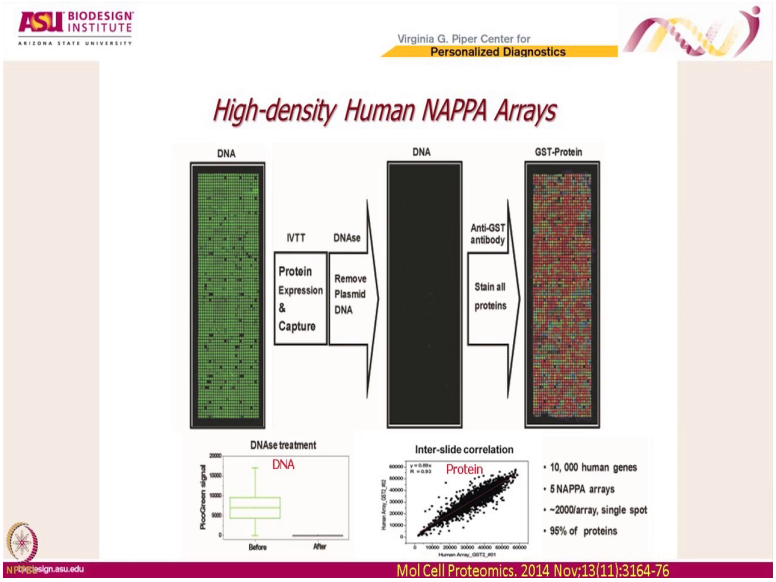
So, what he did was a he worked with a group Howard Wang in New York who had made a modified version of ATP in which he put this alkyne group right here that is the alkyne group on an ATP that was linked to the sugar. So, it was part of the AMP molecule ok. And so, the idea then would be you take a protein array, you translate the proteins you remove it turns out that one of the things that that Sabo discovered was that to get this to work, he had to remove all the DNA from the array.

So, you all realize that we print plasmids on the array to make the proteins. Once the proteins are made you do not really need the DNA anymore and for a variety of reasons at times if you need to you can digest away the DNA with DNAs and you still have your proteins left on the

array. So, that is what he did. And then he added this alkyne modified ATP along with an AMPylator that released a pyrophosphate and it added this modified AMP to whatever target proteins were there and now it is displaying this open alkyne group.

He then came in with an azide linked to rhodamine which is a fluorescent marker added that to the array and added copper and that added the this it added it caused the covalent linkage and displayed the fluorescent tag. So, essentially he was marking the modified proteins with his azide and then he came in with rhodamine with alkyne and he came with rhodamine linked azide to find the proteins; so, then only proteins that are targets of the enzyme will light up.

(Refer Slide Time: 08:28)



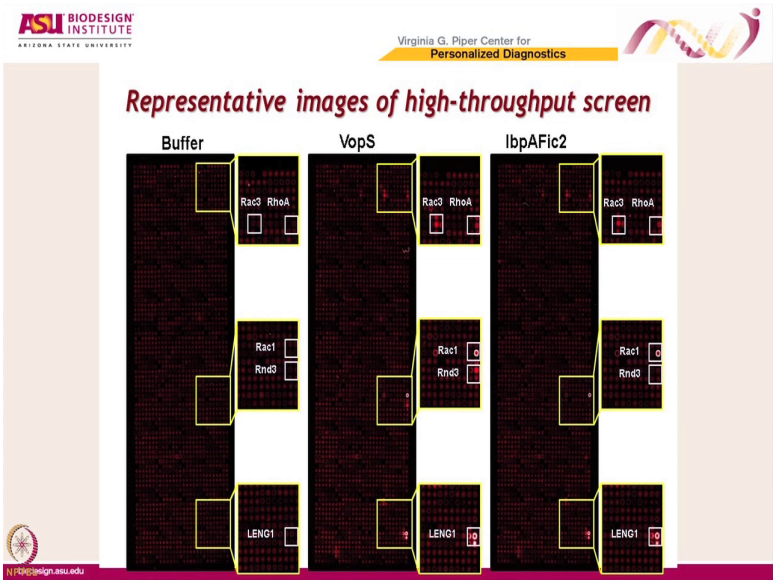
First thing he did was to make protein arrays. Here he shows you that he has the DNA remember we stained with a pico green to look for DNA that confirms to be a good printing.

Then he expressed the protein and captured it and then he digested all the DNA using DNAs. And so, if he stains for DNA again it is completely gone and then this is just showing you.

Here is the DNA level before and DNA level after treatment. Then he tested with anti GST antibody and showed that he still had all the proteins. So, this is kind of useful to know in some circumstances when you are going to be working with a protein array like NAPPA if you do not want the DNA around let us say you are doing a transcription factor study or something like that you can digest the DNA away and you are still left with the proteins and it is still a perfectly good protein array alright.

And then this just shows that when he did two different array studies he got very reproducible results ok. So, now he is got now he is got this whole protein array displaying protein no nucleic acid, no DNA on the chip and he wants to then treat that array with an AMPylator plus this alkyne modified ATP ok. So, here is the array.

(Refer Slide Time: 09:50)

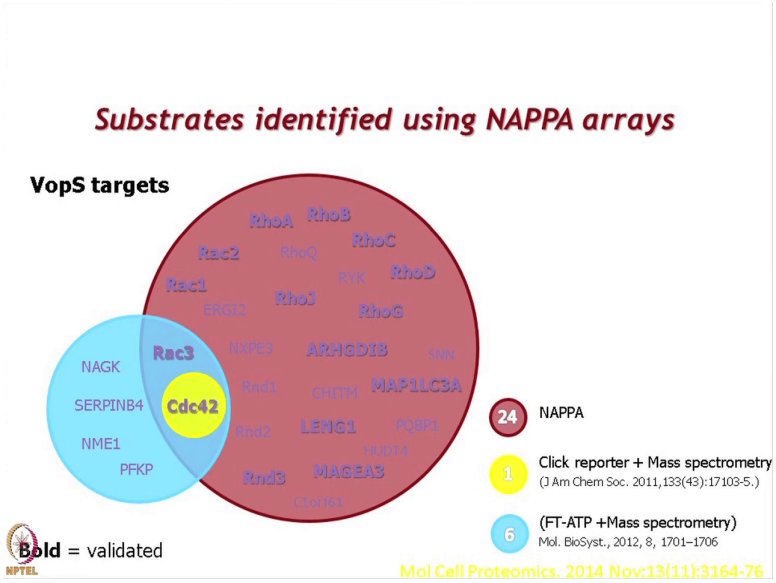


If he treats it with modified alkyne ATP and buffer alone; so, you do not see anything. That is good right you know if there is no alkyne if there is no AMPylator there you do not want to see a signal if you did then that would mean that you had contamination. VopS is a well known AMPylator and then ibpAFic 2 is also a well known AMPylator.

When he treats with those guys can you see that, all of a sudden a few not very many, but a few spots like these guys right here start lighting up. That is kind of the result that you are hoping for right when you are a researcher in the lab when you are a graduate student in the lab and you see only a few spots light that is what really gets you excited, because if everybody lit up then you know if there was a lot of background and it probably did not mean anything.

But if only a few selective ones light up that is a sign that you really found something. So, this is what those spots look like and of course, these are the identities of those spots. You can see that they have very clean signals right and of course, you do not see those signals over here on the control right.

(Refer Slide Time: 11:02)



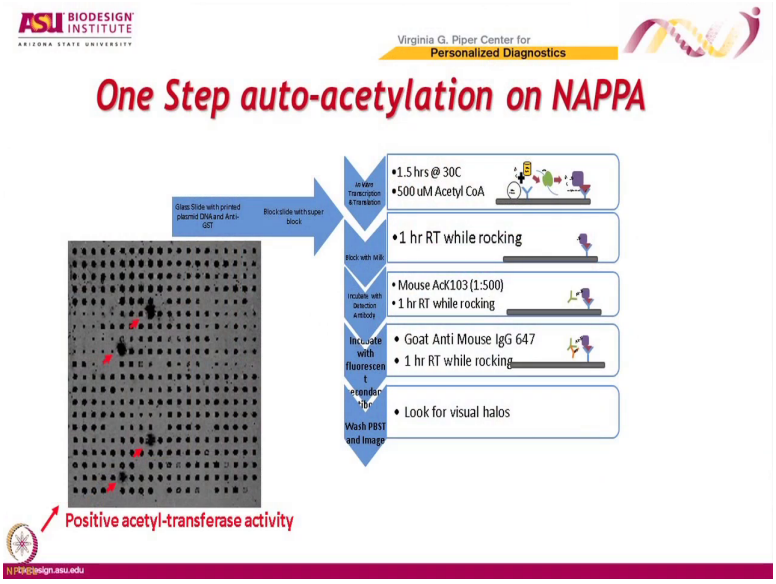
So, by the time he was done with these experiments and I am not going to walk if you all the studies he did of these experiments. This is what was known before he started that was the only known target and all of these targets in here were things that he uncovered by screening the arrays.

So, he found a couple dozen more new targets for this. When he actually looked at the targets and I do not think I have the slide to show you for that work, but he actually found that there was a sequence motif that was common to all the targets or at least most of the targets. And

so, he was able to identify what it was that the AMPylators were looking for when they modified proteins.

And a lot of the targets of these proteins turned out to be GTPs proteins. So, that is one example of how you can use the array to study for enzyme substrate type interactions.

(Refer Slide Time: 11:55)



Another assay and I am only going to show one slide from this because, it is still an early study is work that G Cho in the lab has been doing and again I do not know hopefully you can see the dark spot there the dark spot there the dark spot there. He basically was looking for proteins on the array that auto was satellite and so in this case he was using an acetyl group that was labeled and then you know he was using a acetyl group that could be detected by an antibody.

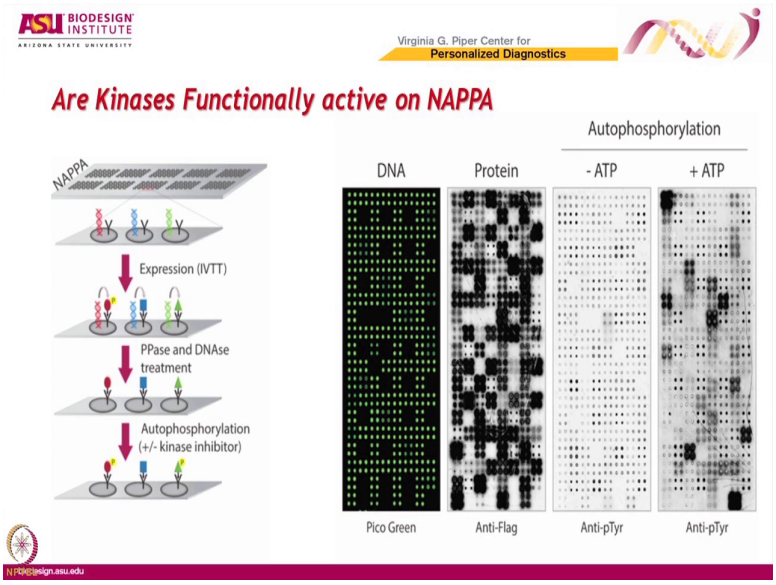
And, then he treated the array with acetylcholine right there, incubated the array and just allowed the proteins acetylate themselves, washed away all the reactants and then stained with the antibody and identified proteins that auto acetylate. So, this is again a way to look for enzymatic activity on the array ok. So, now I am going to a switch gears and talk about here the third application and I am going to spend a little bit more time on this one, because I want to kind of walk you through what I think you want to do you do these sorts of studies because one of the mistakes I see all the time as a journal editor.

And I can not tell you how often I see this [laughter] pretty much every day I am rejecting at least 2 papers for this. This people do once one screen with a proteomics technology maybe some aspects screen maybe it is a protein array screen, they get the results and then they write it up or they get the results they do some informatics and then they write it up. And, they do not follow up on any hypothesis they do not do any subsequent biology. They just simply say here was my screen here is what I got enjoy it [laughter].

And you know for me that is not really what scientists should be doing. They should be I using this tool to identify a hypothesis and then doing some kind of work to test that hypothesis. You do not have to follow up every lead, but you should follow up at least one or two. So, that by the end of your story you have shown something new you did not know before because that is really the goal of science.

And so, we you know we usually send those papers back to the office and say this is good start to preliminary go back and solve the problem and come back to us when you have a bigger story ok.

(Refer Slide Time: 14:09)



So, this is a NAPPA array and what we are going to do here is we are going to express proteins on the array. So, first here you see the array stained with pico green which by now you all know means it is the amount of DNA and the fact that it is pretty even in it is staining means we did a pretty good job of printing.

And then here what we have done is expressed the proteins and then stain them with an anti flag antibody. And the reason it is a flag antibody is in this particular circumstance these proteins which are all kinases happen to have the flag tag and not the GST tag.

And, it is just a good point to remind you that we are not wedded to any one tag we have we have done NAPPA with mik tag, with flag tag, with GST tag with halo tag it is a technology that can be used a variety of different ways. In this case we happen to have all the human

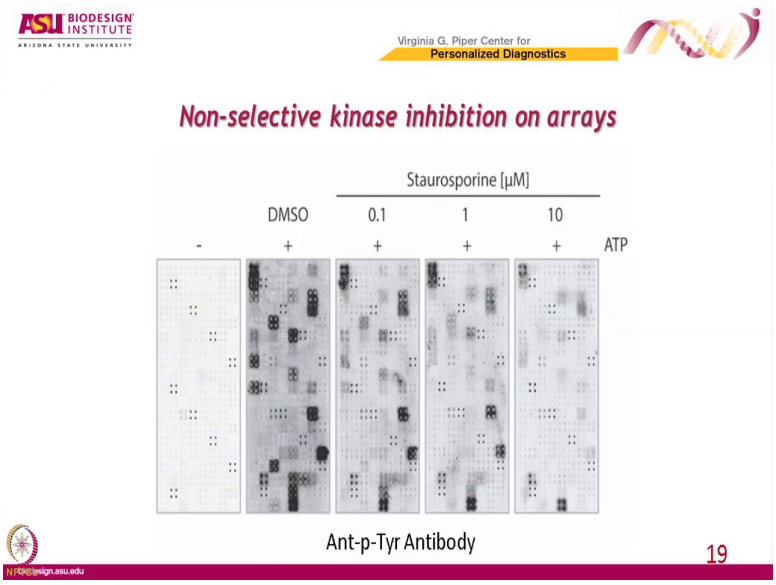
kinases in the flag tag. So, we use the flag tag and this gives you a sense that the proteins are well expressed where they should be right.

So, now the question we wanted to ask was are these proteins phosphorylated. So, we took this array and if we do not treat it with, if we treat it with buffer and just, but no ATP and you stain it with an anti phosphotyrosine antibody none of the proteins light up. So that means, that after if you strip the kinase if you stripped the proteins with phosphatase to remove the phosphates and stain with anti phosphotyrosine antibody, you would not see any that the proteins would not have phosphates that no surprise.

The question was where they where these proteins active and so if we added back ATP to the array and just incubate the array with ATP. Now some of the proteins are lighting up. All of these proteins are auto phosphorylating right because just by adding ATP to the protein on the array they are they are phosphorine themselves on tyrosine ok. So, that is really good evidence that all of these proteins are enzymatically active on the surface of the trip ok.

And it was evidence to us that we had the possibility at least of exploring now the function of these proteins in the array setting. So, one question that comes to mind is can you inhibit this activity using drugs.

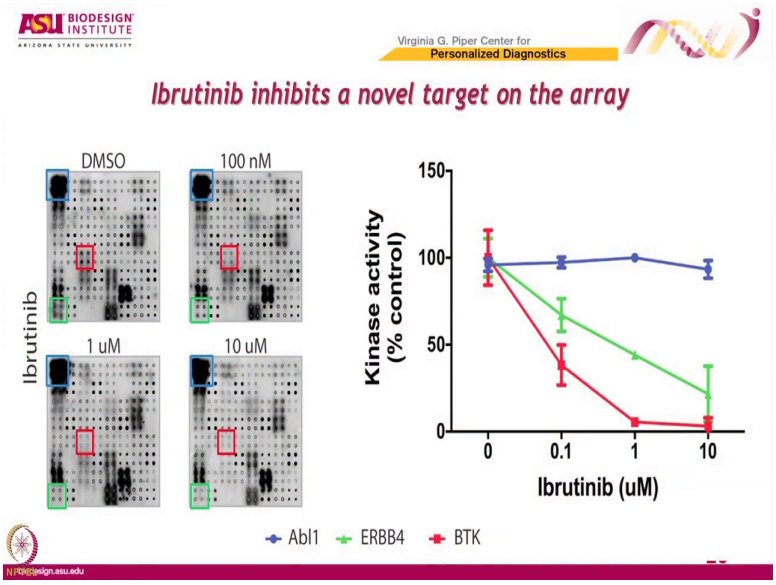
(Refer Slide Time: 16:23)



So, the first experiment that that Fernanda did this was my, this was a postdoc in the lab at the time where she took a broad spectrum kinase inhibitor called staurosporine and staurosporine inhibits most kinases. And she increased the staurosporine on the array and so here is no ATP, here is full ATP and then here is increasing amounts of staurosporine and as you can see the kinase activity is headed is decreasing due to the drug. It is not completely wiped out, but it is significantly inhibited.

So that means, that the enzymes are behaving as we expect them too. A more interesting question is can you selectively inhibit kinases. So, can you use a kinase inhibitor that knocks out one kinase, but not another kinase and will it also behave on the array right and that is this experiment here.

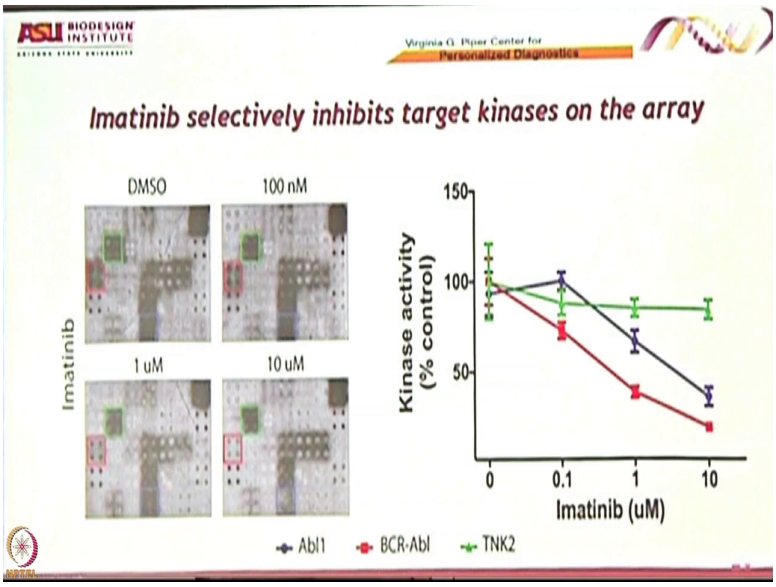
(Refer Slide Time: 17:12)



So, many of you are familiar with this drug imatinib. Imatinib is the same thing as gleevec. Gleevec is the was the first selective drug inhibitor chemical inhibitor ever used to treat a targeted pathway in cancer. So, I mentioned the other day that herceptin was the first targeted pathway that was an antibody. This was the first compound. This is brian druckers work he in you know essentially invented this molecule that selectively knocks out the BCR Abl protein.

The people with a type of CML get this translocated enzyme that links the BCR gene to the Abl kinase and it activates a kinase and it becomes an oncogene that turns on they creates cancer. And using imatinib you can put people into remission. In fact, there are long term survivors now with that disease who have been treated with imatinib and who have never gotten their cancer back. So, it is a pretty promising compound alright.

(Refer Slide Time: 18:14)

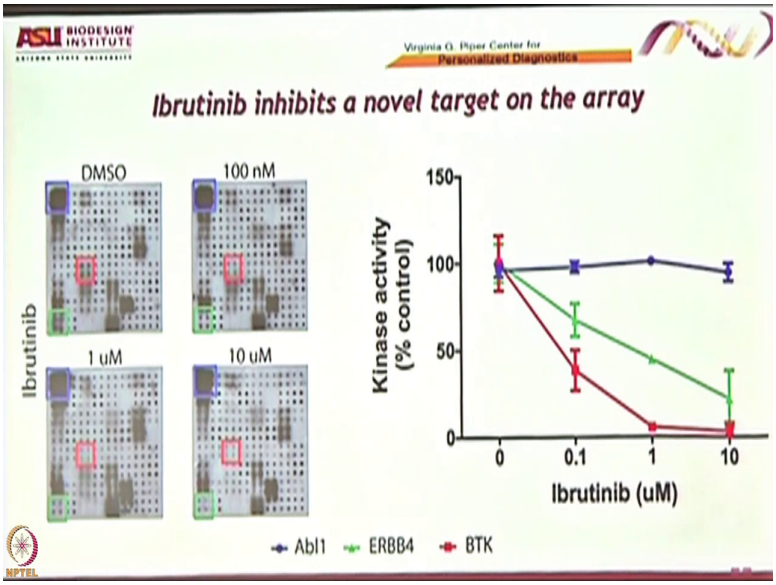


So, what I want you to look at first is this protein in the green circle a green square and you can see that this is the TNK 2 kinase and notice that no matter how much drug we add it is still active. So, the drug is not inhibiting TNK 2, but if you look at BCR Abl here it is here, now it goes down a little bit, now it is much down and now it is completely down. So, the drug is selectively knocking down BCR Abl, but it is not knocking down TNK 2.

Similarly, if you look at Abl which is right down here you can see that the Abl is also decreased ok. So, on the array these proteins are behaving exactly like you want them too. So, we did a number of studies like this to convince ourselves that the array platform was behaving as we expected and then we then decided now can we discover something new with that. So, we started treating the array with other kinase inhibitors and what we look for in

particular was were these kinase inhibitors ever hitting a kinase that we did not expect them to hit.

(Refer Slide Time: 19:14)



And one of the first ones that Fernanda found was this one. So, ibrutinib is a drug that is used to treat an enzyme called BTK or ibrutinib, tyrosine, kinase. BTK is an important enzyme kinase in the b cell pathway and it plays a role in a lot of b cell cancers. So, mantle cell lymphoma for example, relies on BTK and a ibrutinib turned out to be a very useful drug in treating those patients, it inhibits the BTK, it essentially stops the growth of that tumor and it is well tolerated by patients not a lot of side effects.

So, we asked you know does it ibrutinib inhibit anything else. So, the first thing I am going to point out to you is that and that is Abl 1. Abl 1 was the example in the last slide and you saw that Abl 1 was inhibited by imatinib, but Abl 1 is not inhibited by a ibrutinib. You can see

that the signal is the same in all 4 spots ok. So, then we asked well is it working for BTK which is the one of supposed to work for and that is in red and sure enough there is BTK it is going down it is going down even more it is going down even more.

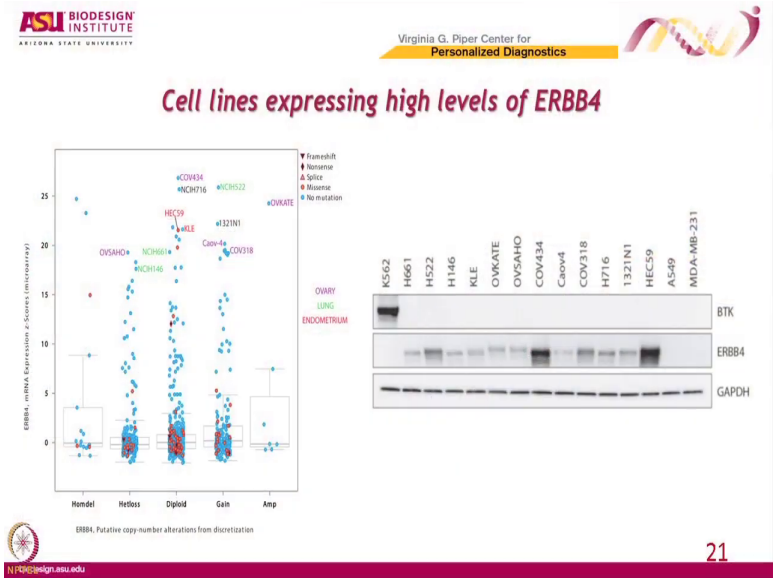
So, in this case even though it is not affecting this kinase it is affecting that kinase. And then what Fernanda noticed by carefully reviewing these slides was this guy down here. Strong signal over here, weaker signal here, weaker signal there and weaker still over here (Refer time: 20:41) that protein turned out to be ERBB 4. That was pretty exciting because we think about it the ERBB family right. So, EGFR receptor ERBB 2 (Refer time: 20:52) those are 2 of the most prominently known oncogenes in all of cancer studies right.

There are very successful drugs against both EGFR and ERBB 2 and now we found a drug against ERBB 4. There was not a lot of data on ERBB 4 and so that is why we decided to kind of pursue this story a little bit. So, the first question we wanted to ask was could this drug inhibit cell growth. So, the first thing we had to look for was cells that had ERBB 4 in it ok. Now the next thing we thought about was what about artifact what potential confounders could screw us up.

So, what else do we know about BTK is a drug what does it normally inhibit? The ibrutinib. So, it the main target the reason it was invented was to target what kinase? BTK right. So, if I put it if I use it in a cell and that has BTK in it then the reviewers are going to look at me and say how do you know it is due to the ERBB 4 it is probably due to the BTK. So, our first thought was we need it selling that has a lot of ERBB 4 and no BTK right ok.

So, it takes a little time to you have this when you do your experiments you have to think about them a little bit and make sure you are doing them in a logical way.

(Refer Slide Time: 22:15)



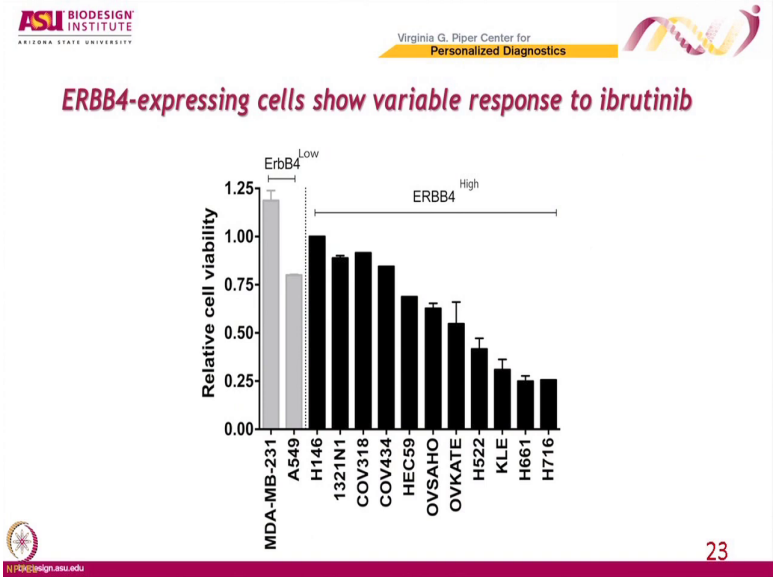
So, that is what we did we searched to all the CCLE is a website that has thousands of cell lines and their gene expression labeled and we scanned all those data specifically searching for proteins that had high levels of ERBB 4 and low levels of BTK and then we ordered a bunch of them. And, then the first thing we did was confirm by western blot that these cell lines were as advertised.

So, here we show here is our positive control that has BTK. All of these cell lines have no BTK. So, that we taken care of that that is not going to be a confounder and then all of them have varying levels of ERBB 4. So, the protein is definitely present and then this is just a loading control ok.

So, now, we know that we have some cell lines that have both and the first question we want to ask is will BTK will ibrutinib inhibit these cell lines because, our hypothesis now based on

our protein array study is that ibrutinib inhibits ERBB 4. And, we think that that might in some way inhibit some cell lines that rely on ERBB 4 for their cell growth. Keeping in mind that no ones ever really shown that before.

(Refer Slide Time: 23:33)

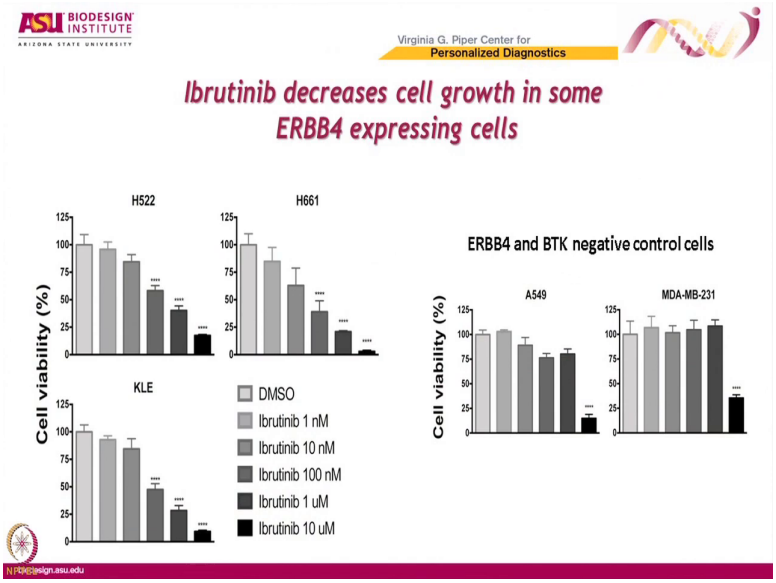


So, we treated all these cell lines with the ibrutinib and what we saw was a range of activity. So, some of them this is relative when I say relative cell viability what that means, is cell growth plus minus drug. So, if it is a 100 percent it means that with drug it is a same as without drug.

If it is down around 25 percent; that means, that with drug gets inhibited by 75 percent. And so, you can see that some of these cell lines out here were significantly inhibited by a imatinib a ibrutinib and these guys not so much. So, these guys look like they are resistant to drug

these guys look like they are sensitive to drug. You still with me ok. So, then we did a dose response curve which is the logical thing to do next and that is what you get.

(Refer Slide Time: 24:24)

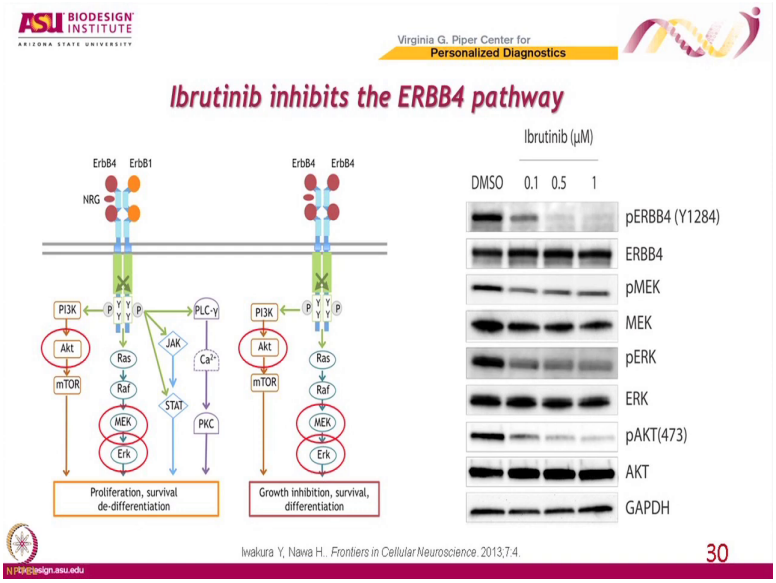


So, adding increasing amounts of drug you see increasing inhibition of cell growth. So, that looks like a ibrutinib is inhibiting a cell line that has higher ERBB 4 and no BTK. So, you can not argue that this is due to BTK ok. And then and then we did a couple of key controls. We looked at cell lines that were either ERBB 4 negative or BTK negative and sure enough neither of these cells were responsive to drug ok.

So, let us so let us think a little bit about the ERBB 4 pathway because one of the first questions that comes to mind is maybe you have added a drug that you think you have shown inhibits ERBB 4 on your protein array. How do you know that it is really inhibiting it in these cells in a way that affects the cell pathway the biochemical pathway by ERBB 4 because you

have to show that right. You can not just say well it inhibits in vitro, but I do not know what happens in vivo.

(Refer Slide Time: 25:26)



So, so let us look at the pathway. There are 2 pathways for ERBB 4 it is a growth survival pathway over here and there is a proliferation pathway over here. There they are kind of similar ERBB 4 also has an alternate pathway depending on the splice form that you use, but you can see there is signals directly is ERBB 4 like the other EGFR receptors is a dimer there are this is an irregular binding here. There other ligands that bind to this protein it sends signals through the Ras pathway the Ras ERK pathway it also sends signals through the PI 3 kinase AKT pathway via mTOR.

So, these kind of look very similar. So, if you were going to think about key parts of these pathways to test you might look at these guys. AKT it is a well known oncogene that that

drive cell division and then MEK and ERK which are also oncogenes and play a role in signaling through the transcription factors like the june foss and so on. The all 3 of these proteins AKT MEK and ERK get phosphorylated when they are active and we have good antibodies for those phosphoryl forms.

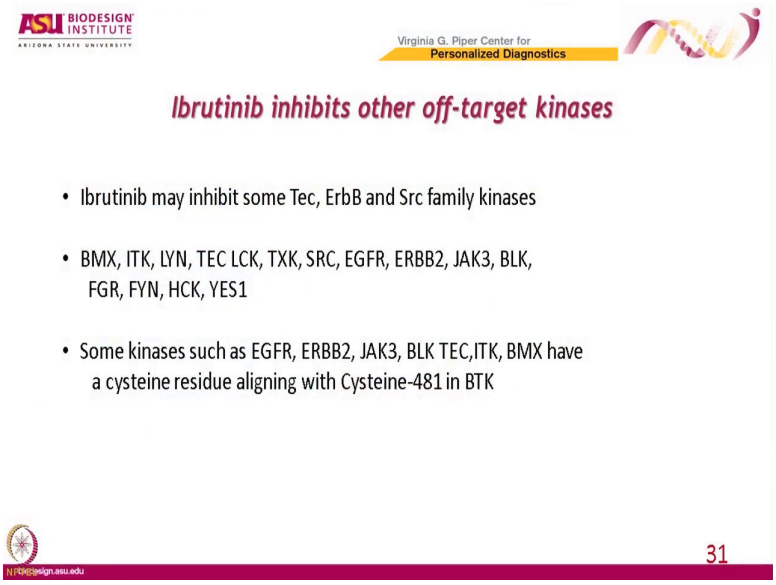
So, a good simple test would be when we inhibit the cells with a ibrutinib do we see a reduction in the phosphor form of these proteins which are downstream of ERBB 4 right. And I would not be telling you this if we did not do that experiment and that is the result. So, here you see that first of all ERBB 4 itself is less phosphorylated with drug. So, that tells you right there that it is already that that it itself is being inhibited by drug and then here you see phosphor MEK is going down not dramatically phosphor ERK is definitely going down and phosphor AKT is certainly going down.

So, and keep in mind that the level of AKT is the same. So, the protein is still there it is just not as phosphorylated. Same is 2 of ERK same is 2 of MEK. Although this is a little bit bigger than that I think, but you get the idea right. So, when we treat with them at when we treat you with ibrutinib it inhibits the AKT the downstream pathway of ERBB 4 ok. So, now what is the next objection we are going to get from the reviewers.

So, we have shown that it inhibits the kinase. We have shown that it turns off the biochemical signaling pathway right. We have shown that it is reliant on ERBB 4 expression in cells and it does not matter the BTK is not there what else do we have to worry about what about other members of the ERBB 2 family or ERBB family right. So, those proteins are all very similar there is a very good chance that a ibrutinib could also inhibit ERBB 2 could inhibit it could inhibit EGFR.

And so, one of the objections the reviewer might make is well how do you know that it is specifically through the ERBB 4 pathway and not through these other members of the of the ERBB family because after all they are really well known we know there are cancer proteins right. So, that was what we have occurred to us you know.

(Refer Slide Time: 28:38)



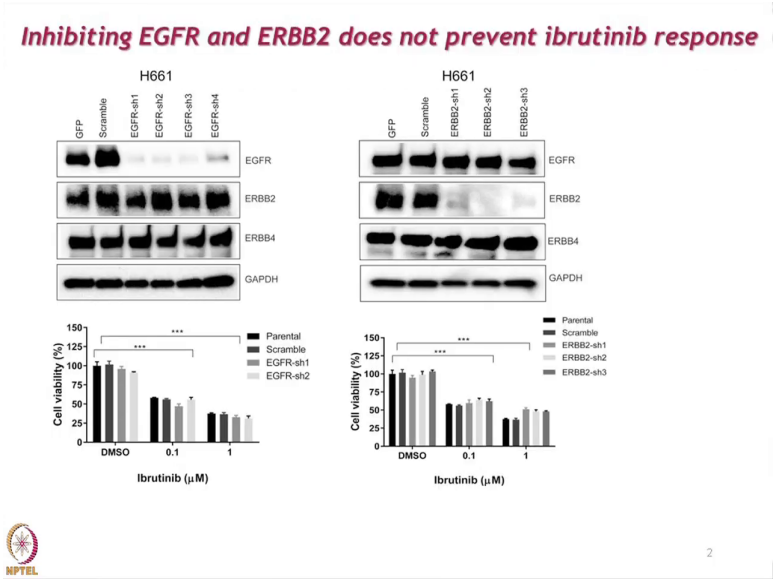
It might it might inhibit ERBB SRC family members some of these proteins in the literature had been listed by someone somewhere has being inhibited by ERBB by imatinib of ibrutinib. So, I keep confusing those although the data were not very strong.

So, and then some of these had this cysteine residue which is in the binding pocket where the drug seems to bind. And so, it is possible that because they have that cysteine like BTK like ERBB 4 they too might be inhibited. So, the question we want to ask was if we do you know could EGFR or ERBB 2 be contributing to this ibrutinib response could it be that this is all due to these guys and not ERBB 4 right. So, how would you rule that out?

Student: (Refer time: 29:25).

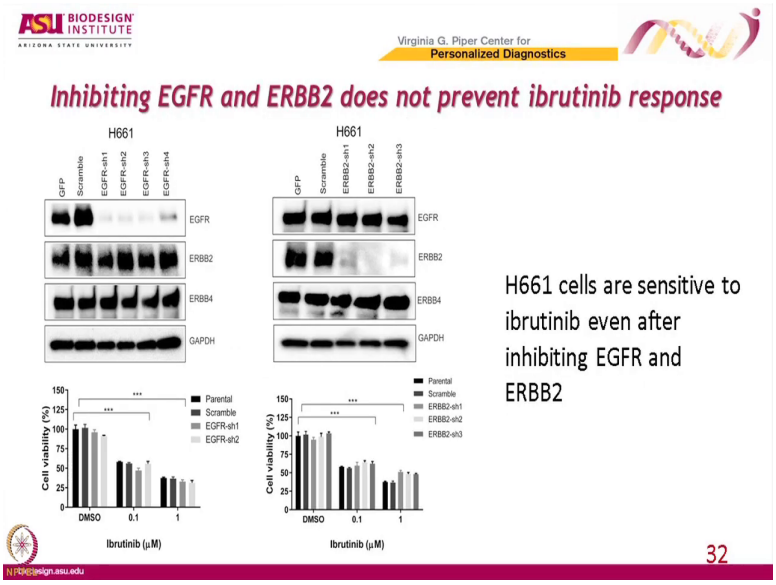
Knock down right. So, you would take out you take these guys out right and still see if you get the effect right.

(Refer Slide Time: 29:35)



So, that is what we did. So, we did shRNA here you can see that this is the level of EGFR using a during shRNA that knocks down EGFR we could significantly reduce EGFR. Notice that these shRNAs do not affect ERBB 2 or ERBB 4 and similarly we had some ERBB 2 shRNAs that knocked out ERBB 2 did not affect EGFR did not affect ERBB 4 and yet and yet despite that despite knocking down EGFR or knocking down ERBB 2 we still saw the drug effect.

(Refer Slide Time: 30:09)



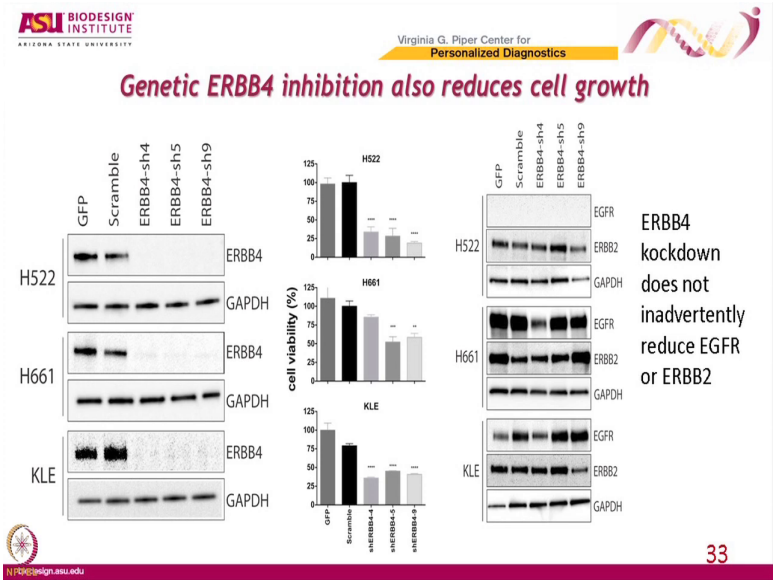
The drugs still inhibited the cells. So, now, we can say that they are sensitive to ibrutinib even if you inhibit EGFR or even if you inhibit ERBB 2 ok. So, so then the question was alright. I am still not convinced how do you know that not can you prove to me that knocking down ERBB 4 is really going to stop cell growth.

So, we are going to a lot of extreme here because people have tried to study ERBB 4 lot and they have not they people have tested it superficially, but they have not really spent a lot of time on it. But so, far the data have not suggested it was oncogenes. So, that is where we wanted to spend some time on it. So, we wanted to ask the question can we knock down ERBB 4 without the drug. How would you go about that?

Student: shRNA.

shRNA right. So, that is what we did.

(Refer Slide Time: 31:08)



So, here is in one of our cell lines 522. This is one of the sensitive cell lines here is an ERBB 4 shRNA clearly knocking down protein right and if you knock down the protein cell division gets reduced. So, it just knocking down ERBB 4 is sufficient to give you cell inhibition. Here is another cell line knock down ERBB 4, once again you see reduced cell growth. Here is yet another cell line knock it down once again you see reduced cell growth.

So, knocking down ERBB 4 by itself using 3 different shRNAs in each case is sufficient to knock down cell growth. So, we felt like we had pretty significantly demonstrated that this cell growth inhibition was due (Refer time: 31:54) to ERBB 4 inhibition. And, this is just to show you that when you knock down ERBB 4, it does not affect EGFR or ERBB 2.

So, that is yet another possible criticism as well you are knocking energy before maybe you are also affecting EGFR or ERBB 2, but we can show you that we actually could not detect your EGFR in these cells. But, if you knock down you do not see any change in ERBB 2, no change in ERBB 2, no change in EGFR no change no change. So, this was really due to EGFR or ERBB 4 yeah.

Student: Is there any other way to knock down to those targets using drugs.

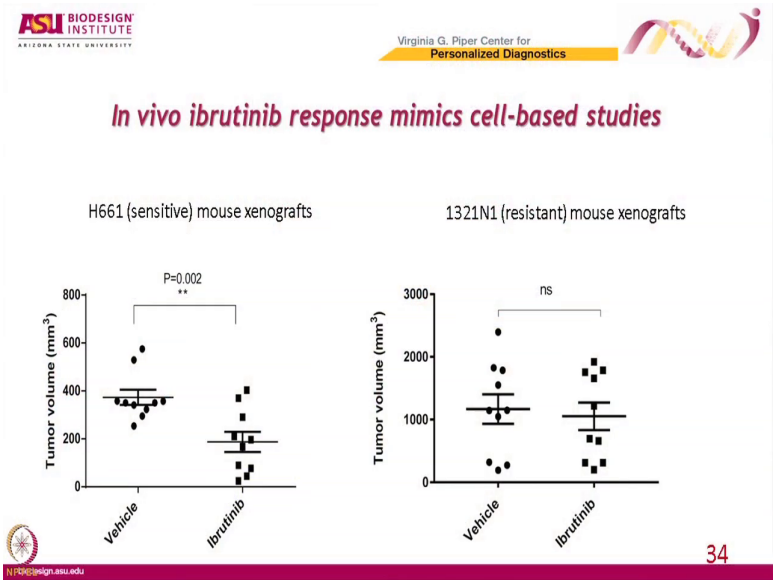
There are not any good drugs right now that we know. I mean obviously we were we think we found one of the first ERBB 4 inhibitor. You could do you could knock down you could block the activity of EGFR or you could have done that, you could have done that yeah.

Student: (Refer time: 32:51).

That would have been another way to do. We actually just did it genetically which was easy, but you we could have gotten the drugs right. And then of course, the other way to take out ERBB 4 would be to do crispr, but that is a very involved process and we did not really need to go that long ok.

So, then kind of the last piece of this piece of this puzzle was asking the question does this matter at all in biology right would it matter in an animal. And so, we took these cells created tumors out of these cell grew tumors out of these cells in mice and then either treated them with a ibrutinib or no ibrutinib and you can see the effect.

(Refer Slide Time: 33:35)



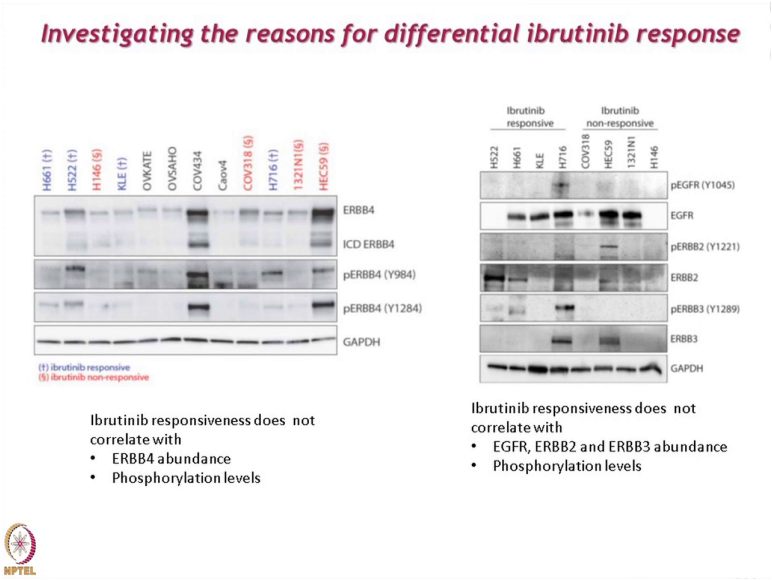
So, this is tumor growth without a ibrutinib and this is tumor growth with a ibrutinib right. So, clearly even in animals this is working and notice that if you take one of the resistant cell lines that is not a ibrutinib responsible that the use of a ibrutinib does not really affect it at all. The difference is really over here where we had a sensitive tumor ok. So, now, you remember when I started all this and I showed you a bunch of cell lines several of the cell lines were sensitive to the drug, but quite a few of them were not sensitive to the drug.

So, the question then becomes why are come why are the non sensitive cell lines resistant right. What makes them resistant? And I think to me that was the crux of the matter because, historically ERBB 4 inhibition had not been a successful cancer or ERBB 4 had not been an obvious oncogene. And, I think part of that reason is because there is a lot of resistant cells

and so when you, when people did expect experiments they sometimes stumbled on these resistant tumors and they saw on a difference as they decided did not matter at alright.

So, we looked at the tumors that we had and we looked to see if we could figure out what was different between the sensitive tumors and their resistant tumors.

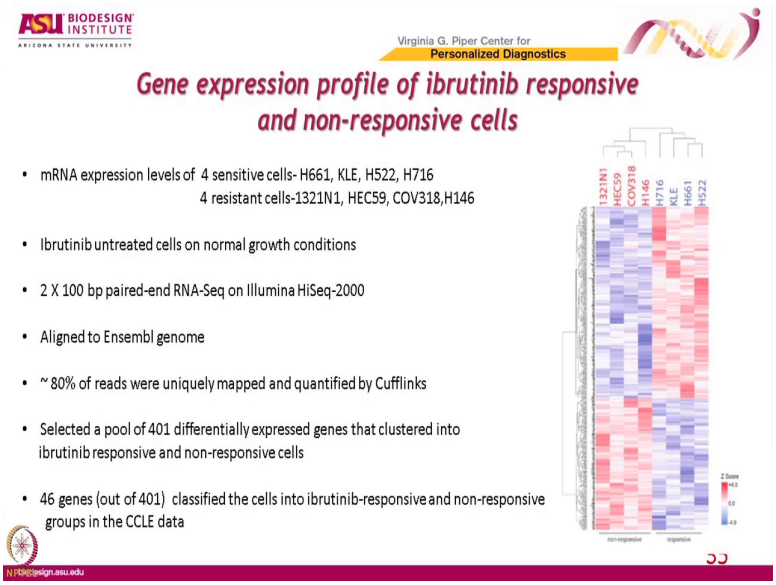
(Refer Slide Time: 34:54)



The responsive tumors are in blue right and then the resistant tumors are in red and so looking at this at ERBB 4 levels you really could not see anything different from ERBB 4; even phosphor ERBB 4 not really an obvious difference. Here is a resistance cell line with very strong phosphor ERBB 4. Here is a sensitive cell line with really strong phosphor ERBB 4 not an obvious correlation there.

And, we looked at two different phosphorylation sides on ERBB 4. So, it did not correlate with abundance or phosphorylation. We also looked to see if the state of the EGFR ERBB 2 or ERBB 3 could also have an effect and we looked at their phosphorylation levels. And, once again you know we could spend some time on this I would not bother comparing responsive to non-responsive there was no obvious difference. So, we were left with this you know unsatisfying situation of having resistant cells and sensitive cells, but not really understanding the difference.

(Refer Slide Time: 36:01)



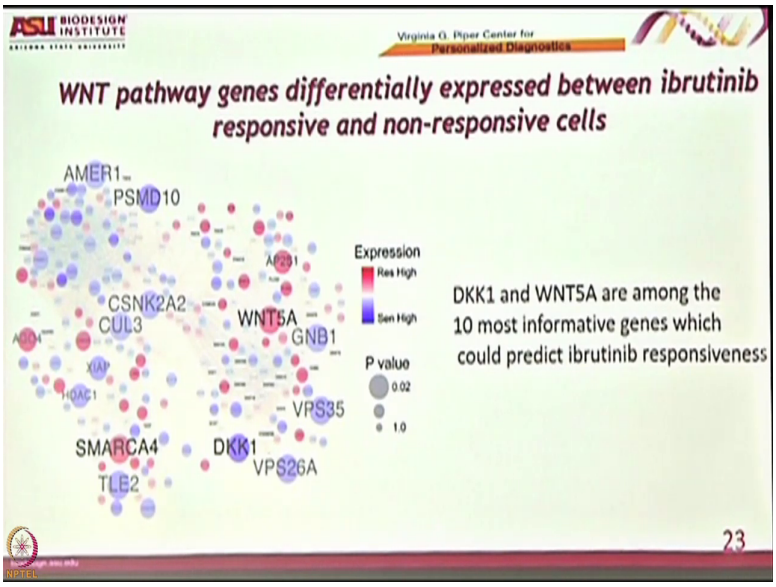
So, we thought we would do a gene expression profile on these cells. So, we looked at we had these 2 groups. We had 3 cells that were sensitive and 4 that were resistant. So, we put them through gene expression and asked is there anything in their gene expression that would

correlate specifically with responsiveness. Could we find a difference right. So, that is what you are looking at here you know.

These are the sensitive cells I get that I get that right sensitive cells resistant cells and right. We are an that did we did them. We did them at untreated cells we did that because we kind of wanted to know what was that baseline. We also did the treated ones, but that gets more complicated. We did high seek sequencing you know assembled them into genes identified what it is responsive and then compared them, compared these results among this population to data that had were these cell lines have been treated by, but have been looked at in the CCLE data.

So, we had our own RNA Seq data and we also looked at the CCLE data. I am not going to go through all of the informatics that we did to kind of sort this out, but in the end what we did was look at which pathways, which gene pathways were best correlated with responsiveness.

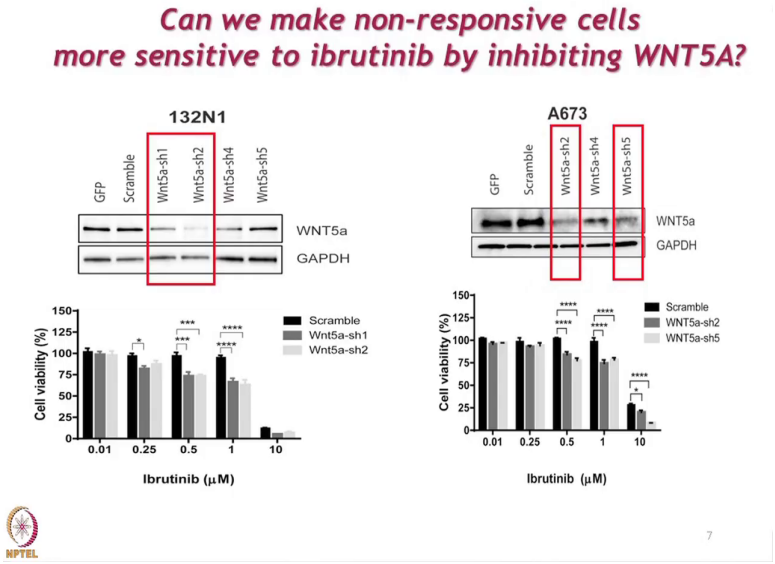
(Refer Slide Time: 37:22)



This is a diagram of gene expression that shows a difference between sensitive versus resistant. So, red means it drives resistance and blue means it drives sensitivity and the bigger the dot the more the effect or actually the more the P value I should say and so what you can see is there is. So, here is the smart gate 4 for example, but we were focusing right here on WNT 5A and DKK 1.

And the reason these two really stood out for us is these are opposing proteins. This guy inhibits that guy. So, they are directly in opposition and they are acting in opposition this guy drives resistance, this guy drives sensitivity. And so, they were among the 10 most predictive genes and we decided to follow them a little bit more closely.

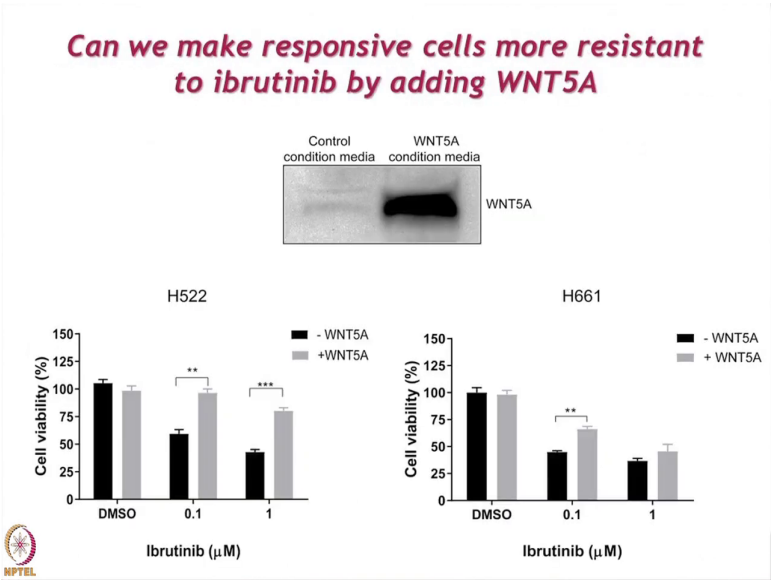
(Refer Slide Time: 38:17)



The one of the question was can you take a non-responsive cell and make it sensitive by blocking WNT 5A. So, our model is that WNT 5A drives resistance. So, the question was could you could you change that? And so, Femina did this work. She took she took the cells, she used a WNT 5A sh shRNA, she tried several of them and she found a couple right there that worked pretty well.

She created cell lines with knockdown WNT 5A and showed that in fact, now they are a little bit sensitive to the drug right. So, this is using scramble shRNA and then these two are both the target as shRNA. So, you can make the cells sensitive by doing that and she did that in two different cell line backgrounds which is heroic right. So, then the flip question is also present. So, here we made the cells more sensitive. So, the questions can we take a sensitive cell and make it resistant by giving it WNT 5A alright and so she did so yeah.

(Refer Slide Time: 39:24)

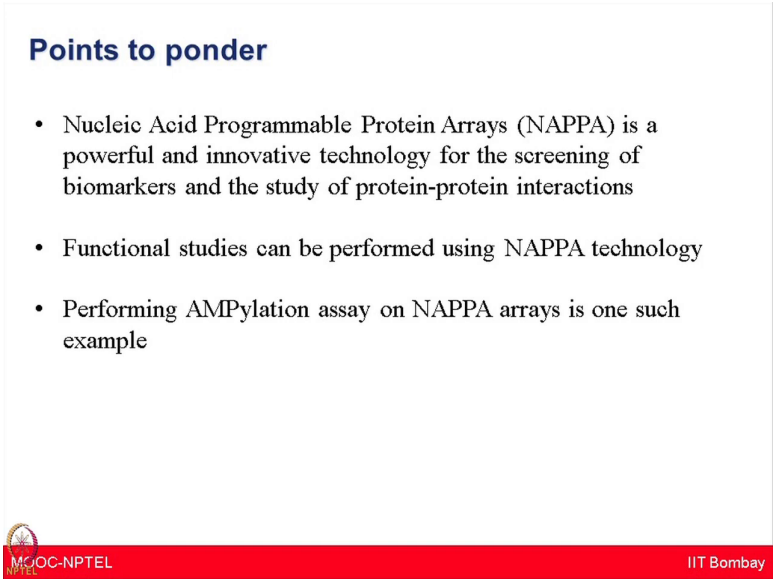


So, she did that experiment. It turns out if you take cells that produce a lot of WNT 5 A. If you take their media just the cell culture media you can there is a lot of WNT 5 A in the media the proteins right there and this is the evidence for that and so if she treats the cells a sensitive cell line with WNT 5 A. So, this is with WNT 5 A and this is without you can see there much less sensitive to drug. So, WNT 5 A does what we predicted it does right.

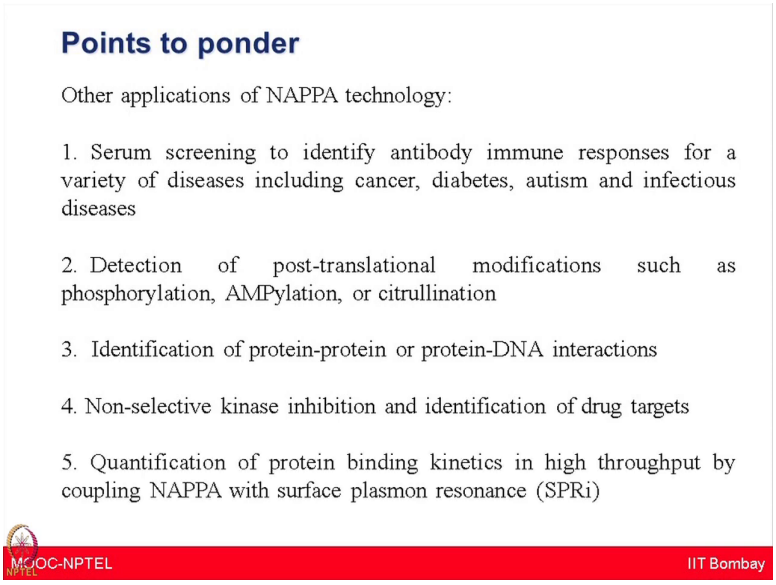
So, that is all I have on that story, but it kind of illustrates for you kind of what you hope that your proteomic studies will do right. They will open up a new idea a new possibility in this case they indicated a new drug that targets a protein that was not previously bought to be related to cancer allowed us to explore that that protein as a possible cancer protein and kind of pick apart a little bit of a story that we think reflects back on the biology and the disease which is what we are really trying to do right.

Our real goal here is to understand disease. It is not just us to use a technology it is to use that technology to study something.

(Refer Slide Time: 40:30)



(Refer Slide Time: 40:46)



So, in conclusions you have learned about how to perform functional studies and specially AMP isolation assays using NAPPA technology. As I mentioned it is telling PDFs are not straightforward you need very sensitive technologies, you need very careful as they design to really try to capture how the post translational modification happens in cells. As a result, NAPPA technology very elegantly offers you a very novel platform to look at high throughput manner how the PTMs can be studied. He also studied about high throughput screening on human studies as well as the varnish drip auto acetylation on NAPPA arrays.

Today were also introduced to the non selective kinase inhibition on arrays and how NAPPA technology could be employed for performing such assays very easily. You are exposed to the concept of identification of drug targets using NAPPA technology in the continuation of

trying to give you the feel of how protein microarrays and the technology associated with microarrays could be utilized for different applications.

In next few lectures we are going to talk to you about different type of array platforms and different clinical applications, how this could be utilized for other biologically relevant problems. You will see how to perform a protein microarray experiment in the laboratory settings. We directly from my proteomics laboratory some of my senior PhD students will show you the various or say the steps performed in doing microarray based experiments.

It will definitely give you much better idea about this technology as well as the intricacies involved in doing the experiments in the laboratory settings. In case if you are planning to apply these technologies in your own research I think these exposures are very valuable and really needed to take your understanding to the actual experiments and try to employ that in your own work.

So, in the upcoming lectures we will use different types of microarray chips. For these experiments as well as some demonstrations will be a given to try to convey you the protocols involved in doing these experiments also the basic principles and the workflow almost remains same whether you use the in vitro transcription translation based protein arrays like NAPPA or you used purified protein arrays like HuProt which will be also showing or you use reverse field arrays variety of these array platforms. The starting materials could be different, but ideally you will see the workflows remains very similar.

But depending on what the objective is you are looking at a very specific potential interactor, you are looking at protein modification; you are looking at a biomarker even a globulin protein or you are looking at some sort of inhibitor assay. Accordingly, your experimental design has to be changed and you have to thoughtfully carefully think about what should be my best controls for giving me answers or the right answers to address these questions.

So, you learn about some of these aspects more in the upcoming lectures. And, I hope you will be then very confident about how to use this one of the very promising technologies for variety of discovery and functional studies in your own work.

Thank you.