Introduction to Proteogenomics

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Lecture - 45
Proteomics: Clinical Applications

Welcome to MOOC course on Introduction to Proteogenomics. Today we have scientist Dr. Suman Thakur from Center for Cellular and Molecular Biology, CCMB Hyderabad. Dr. Thakur will talk to you about mass spectrometry based quantitative proteomics and how it can help the cancer research. He will also talk about why quantitative proteomics is very important, in which way various tools available for doing quantitative proteomics have helped to understand different diseases.

Dr. Thakur will focus on why we are not getting clinically relevant biomarkers for all the cancers and why one drug cannot cure all the cancers. He will talk about different specifications for mass spectrometry based columns. For example: the gradients, the column length and other type of mass spectrometry parameters. How it could be optimized to obtain good results.

He will also talk about why the number of proteins decreases in labelled analysis as compared to the liberal free quantitative analysis. He will then talk about anti-cancer compound screening and how the proteomics, cell biology and animal studies together could only help to find out the clinically relevant information. So, let us welcome Dr. Suman Thakur for today's lecture.

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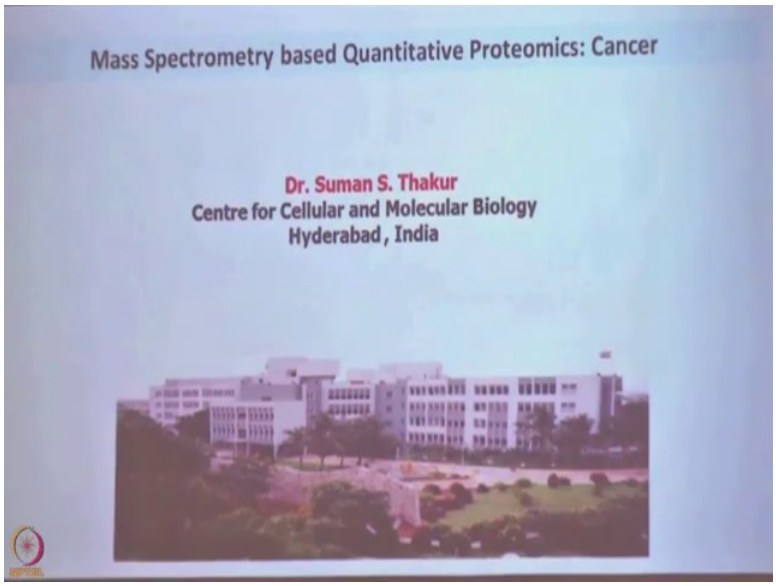

Mass spectrometry based quantitative proteomics in cancer.

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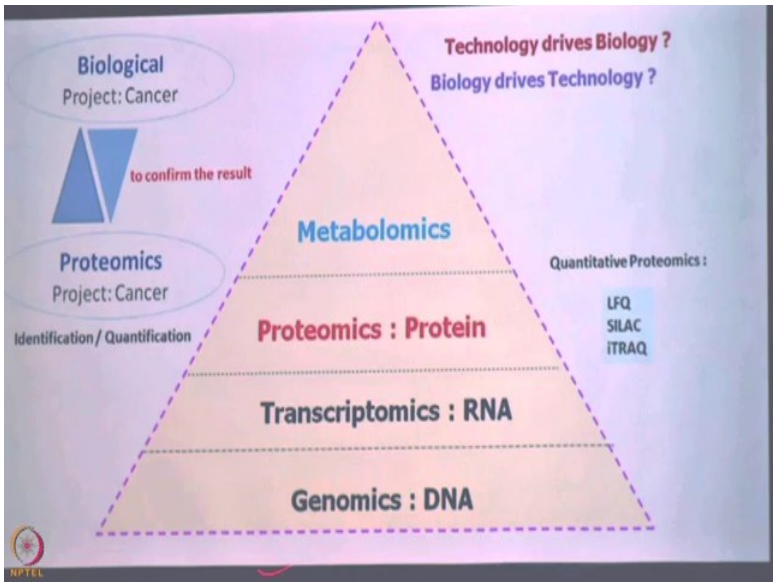

So, anyhow you are well trained and thanks to all the previous speaker who has set the stage and I should just move it. So, all you know proteogenomics; so, genomics is here, proteomics is here, middle transcriptomics, metabolomics for us the all are different subject. But, for body all are happening; billion year, trillion year of evolution has made this. So, this is not a one day anything maybe this is new subject for us.

But what is here? So, biology change thousand year before also question what is life, today is also question what is life? Most probably thousand years later will be also question what is life, what is evolution? But, what change that we have to understand? Technology; so, technology drives biology, you get new technology in your hand or you try to answer different question. If you want to do something in life you have to ask question.

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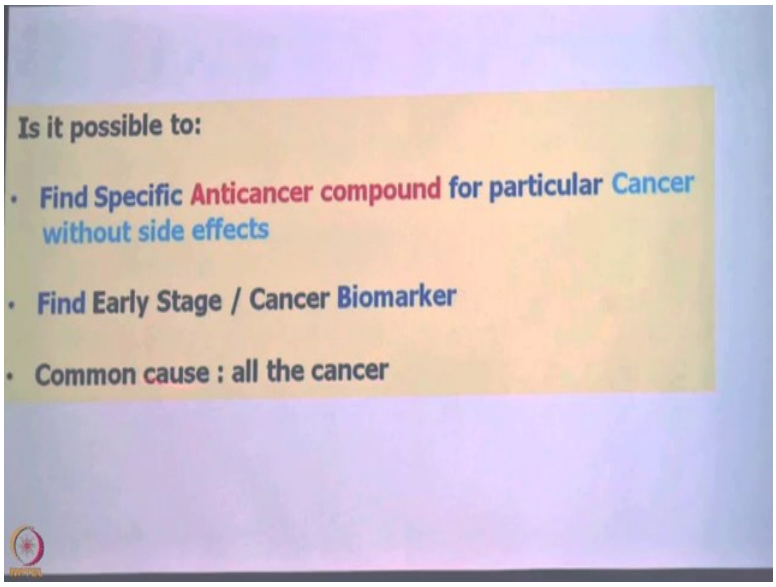

What is the question? Find a specific anti-cancer compound for particular cancer without side effects, is it possible to find a drug without side effect? That is the reason you asked question what is not easily possible. Second find earlier stage and cancer biomarker, cancer is a such a word till you discuss in symposium and lecture hall it is fine. If you see any patient your near and dear you will be second very badly.

So, that is a bad word is a cancer basically is a disease still after so, many years 100 years of research we are not able to cure it, we are still in the middle. So, what you need? Earlier stage every doctor ask earlier stage. But, what is the question? Common cause, there are different organs in the body, different types of cancer are there. If all are there then cancer should be common, this question you can ask and to we try to ask this question and I thought take select 11 or 10 cancer cell liner.

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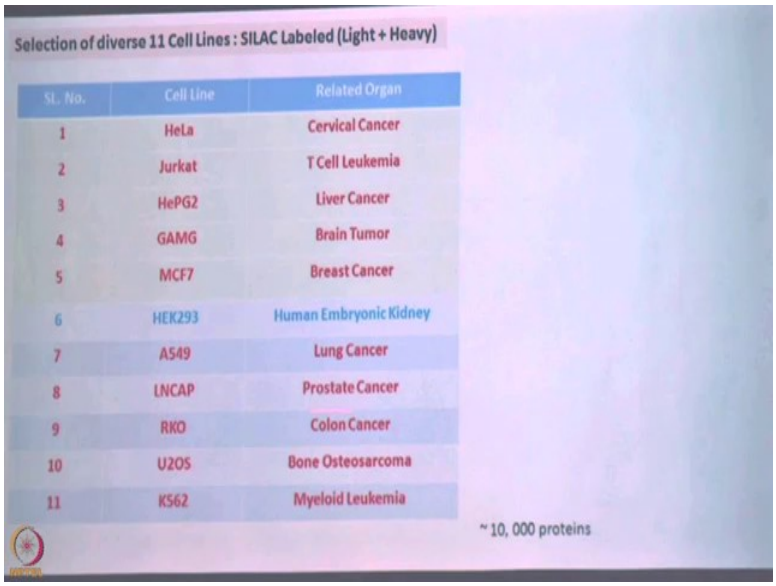

It is very nice to we have to get clear this is the cell line, same thing we are trying to repeat on the tissue also. So, you take HeLa, cervical cancer, Jurkat, leukemia, HePG2 liver cancer, GAMG brain tumor, MCF breast cancer, A549 lung cancer, LNCAP prostate, RKO colon, U2OS bone and K562 again myeloid. So, different type of cancer are there. Why not one drug is there for all the cancer? Impossible origin is different, metastasis different so, that mean it is very complex.

Then you have to take if you want to do anything in the proteomics, we have to select one control and this is non-cancerous cell line. So, we thought we will take this 10 cancerous cell line and very childish way you think, you get 5000 proteins within L 10 or 11 you will have also almost whole human proteome; practically it is not possible. Or, you do different fractionation and you come to 10,000 protein in each cell line is still you will not able to cross 11,000, 12,000 maximum 13,000.

So, what is the matter in; that means, all proteins in all the cancer are same, only quantitation is getting changed. Someplace someone is expressed someplace someone is expressed. So that means, what? Expression level, quantitation. So, finally, what came? Quantitation is important in the experimental and it is happening inside the body. But, how you will do this? This is very simple, we want cancer full proteome that is one or few proteins protein chemistry. We will see some lab is full 20 years has been focused on one protein.

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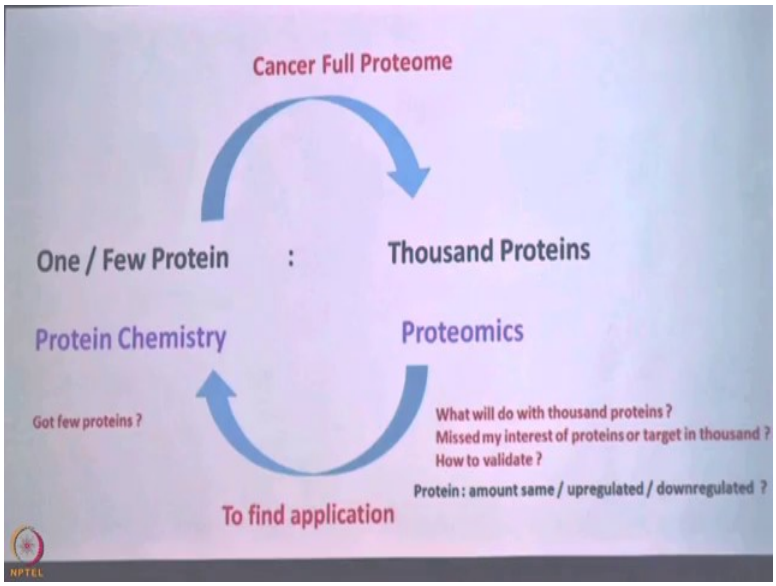

But some lab will not like one protein. Why one protein? We should have thousand protein, but what you will do with thousand proteins. Again what is missed whatever is your interest that you have miss. So, finally, if you want to make a drug or target, you will target on one protein. So, what proteomics has given you? Completely back cycle to find the target and come back on the one protein are make the drug test, same things we are trying also to do.

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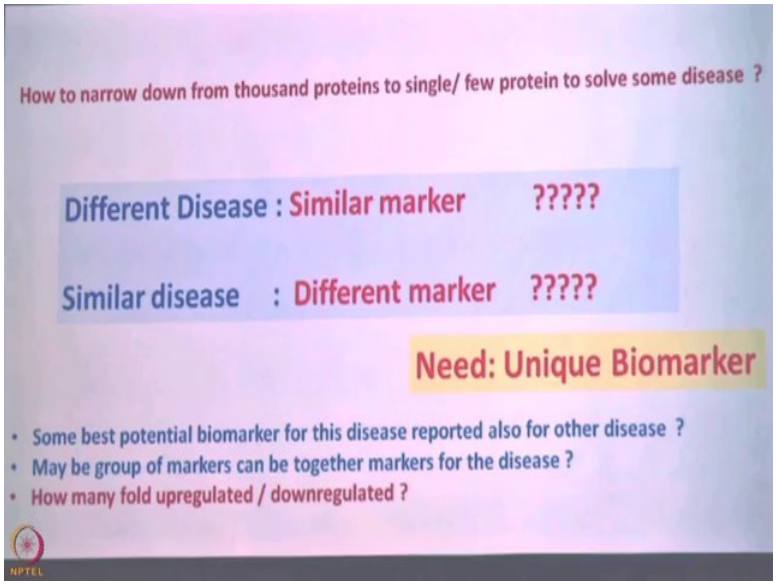

But before that I will go with little bit problem is there. There is nothing everything is good, good, good, good in life, but mostly we talk good, good, good thing. If you go to see us the biomarker things different disease same marker, same disease different marker; all you will get confused and you will end up with the nothing. So, bottom line what we need?

Need unique biomarker that is the whole thing and field should grow it or maybe you need set of biomarker. Just like if someone is telling no this is this disease then again you go to cross check, you go to verification that is thing has to be done. Why all these things is happening?

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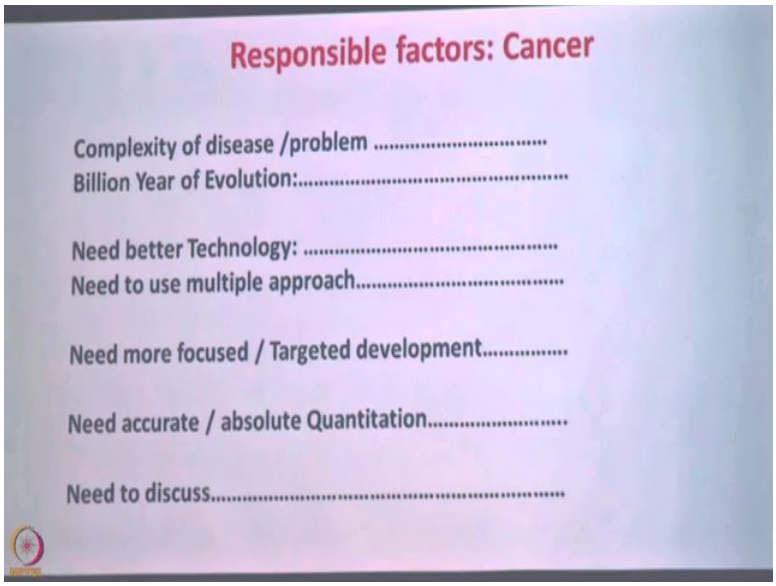

Answer is a billion year of evolution, you cannot easily search find in one day directly sort it. It has to be systematic a study or if you are by luck, you are hitting in the dark it got hit; both are happening together and both example I will show you how we fail, how we pass. We thought instead of 15 centimetre make half a meter column, think little bit weird everyone will tell you crazy, it works you are best; if did not works you still carry with the tag crazy ok.

So now, 15 centimetre 50 centimetre column it is long column, make bead side everyone using 5 micrometre, 3 micrometre, half. What will happen? HPLC pressure will vary increase, to increase the pressure simple you just school knowledge Charles law, Boyle's law. Heat it, down the temperature down that increase the temperature, down the

pressure and somehow you will manage with old HPLC. This 50 centimetre column long run and 5000 protein came that time in one single shot.

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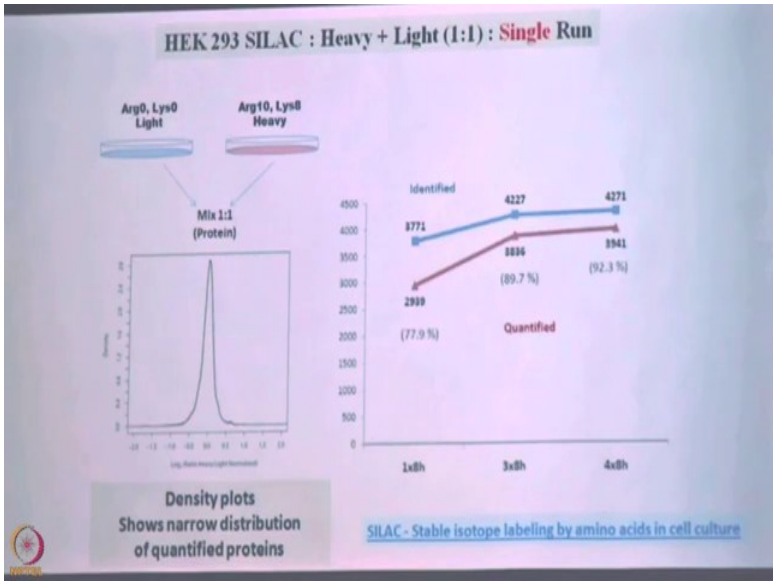

This was just example, same technique we have developed; now recently we have published how long gradient we can use and after 12 hour if you are using the gradient recently published in 2018 there is no use; so, it is saturated. So, need of fixation how long gradient, how long this and that.

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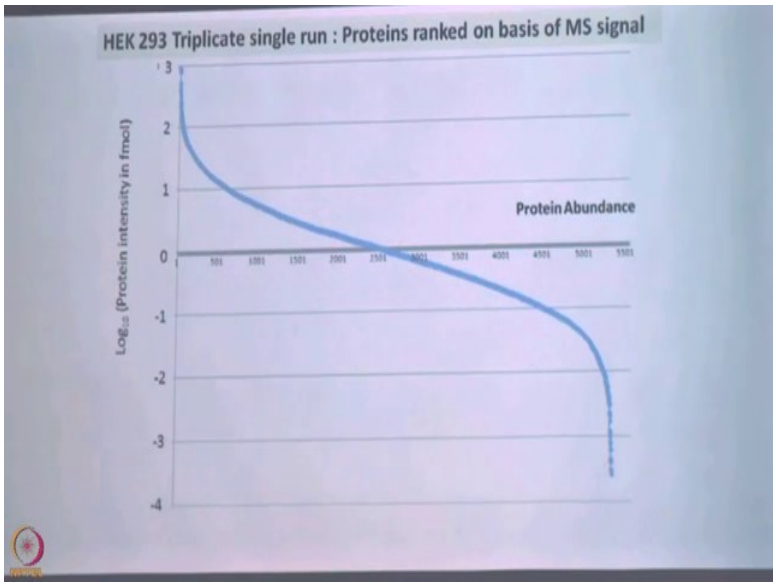

Now, this technique you should use in SILAC that I am going to tell about the cancerous cell. So, this is just introduction; now I show you label free quantitation 5000 protein comes in one run, but when you do SILAC you get less protein. Why?

Because now 2 peak is needed to identify and quantify a protein. So, you increase the complexity; so, if you increase the complexity sensitivity has to be compromise or your protein number has to be compromised. But, we know all the way how to increase the number also. What if in 1 hour you got 3700, make triplicate know then quantify, here bioinformatics you like or dislike bioinformatics you have to use ok. So, now this is the place 1 into 8 hour you got 3,000.

Now, run triplicate you get 4222 because which one peak has come in this run another peak came in this run by bioinformatics you merge and you can increase that. So, this way you are showing, but there is the way triplicate, 4, 5 times, 6 times how many time? This is showing almost in triplicate on 4 almost you are getting close. So, there is no need of going to unnecessary too much ok. You have to keep your temptation and limitation that is also very important otherwise you are doing only one thing.

Now, same thing we did with all these 11 cell line and then we try to find is there any common thing is there or we have to a stop on 11; first we did one then gone 5 then 11. Now, we are planning to have 25 or 50 cell line to get complete idea, but we cannot decide in one it is very costly affair and we are selected in the broad range.

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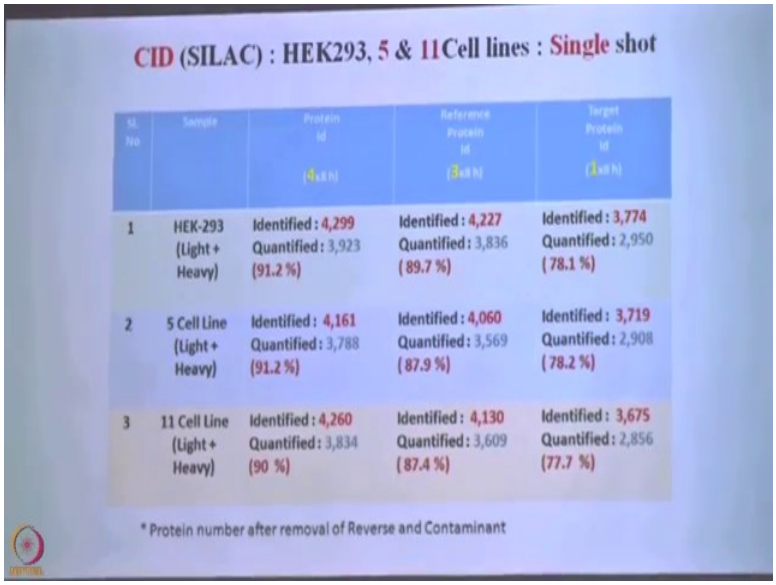

Now, by this if you do deep proteome you will get 10,000 protein in each cell line by fractionation, but we thought we should go single shot also and we should see in one run. There is no comparison between any fractionation, 4 fractionation you might know by bioinformatics 1 run direct result and see, that both approach we have use. Now, you see what happens here, it is single shot; one shot whatever came. This is CID SILAC, you know CID and HCD? You would have heard high energy, today's is the era of HCD ok.

Now, you take HEK 293 light plus heavy ok. So, HEK is there light and heavy, now you are comparing that. There should be theoretically any difference, in quantitation up regulation down regulation? It should not, that is the reason when you quantify identify 4200 and quantify 91 percent in 4. But, when you did in 1 I told you already comes, here you quantified only 78 percent, here 89 percent, here 91 percent. So, what happens when you do multiple run? You are going to quantify more, but now I am increasing 5 cell line.

I am mixing 5 cell line together, then I am trying to see what is happening. One thing you see if one cell line give me 4299 protein, mind tells 5 cell line will give more. Correct or not? Simple thinking, do not think too much; answer is no then we thought 11 cell line will give more, answer is again no. See this pattern is continuing single run is giving less, triplicate is giving more and here you are getting more.

But, when you are increasing the cell line nothing is changing that shows your technology has limitation. Of course, 5 cell line when you put you have more protein 11 cell line when you put you have more protein, but technology has limitation. That means, still our so called well developed, well costly mass spec need to develop less or more?

Student: Very more.

Very more. So, still price will increase, development will continue.

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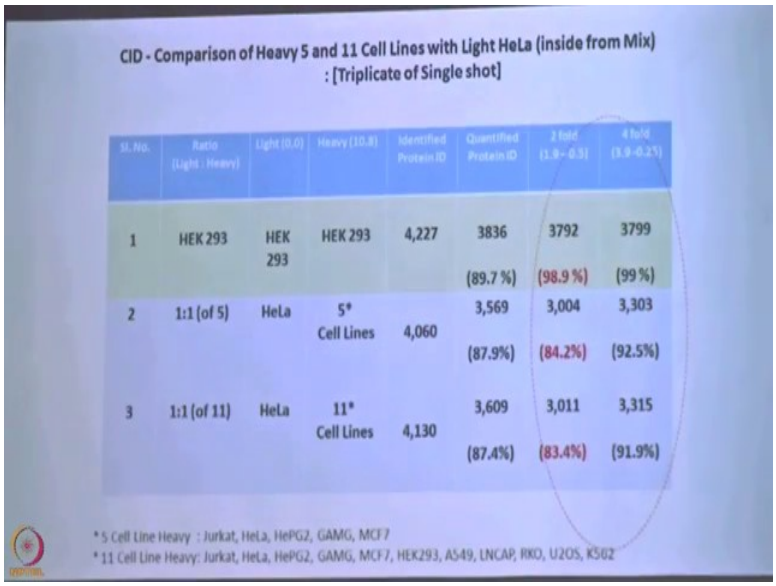

Now, see now this is same CID, here compare HEK 293 versus HEK 293. So, there is theoretically there is no difference ok. When no difference so, what happen in your in quantitation we do not take less than 2 fold ok. See majority of things is falling in this less than 2 fold and whatever it is showing 1 percent that is almost a error ok. Now, I have taken 5 cell line, in 5 cell line I am taking HeLa versus Jurkat, HeLa, HePG2, GAMG, MCF7.

So, this HeLa is inside that 5 cell line, but I am able to quantify and see 2 fold that is falling only 84 percent; that means, others are changing ok. Here is the very less change, here you can see more change; that means, different cell line has different things correct. Now, here I took 11 cell line, it is coming almost same so; that means, because my technology is also coming close and result is also coming close.

So, I cannot comment on that clear, but that is shows me 5 cell line has more protein or different protein compared to that. And, if theoretically same 2 cell line if you compare where is up regulation and down regulation, if you there you get up regulation down regulation; that means, better to stop the experiment ok. So, that is shows your control things are going in right direction, but this HeLa is inside the 5 cell line and 11 cell line.

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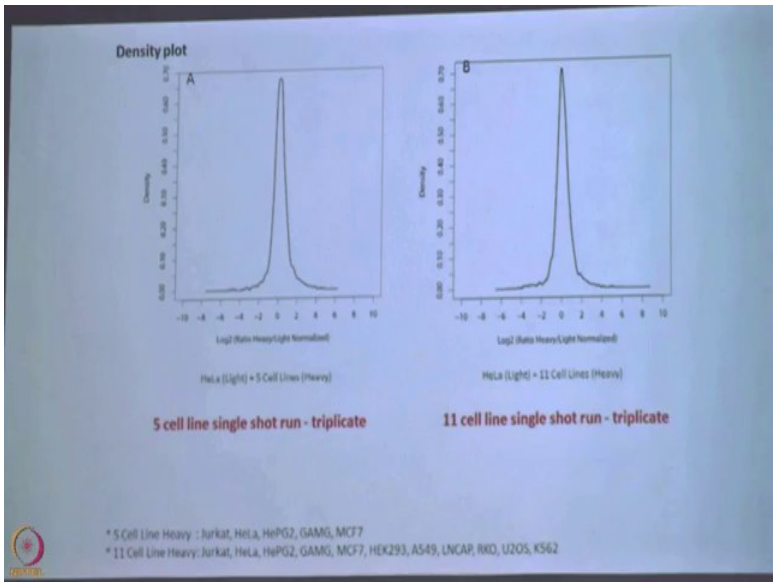

With this you plot, you have learn all the R and all these things today. By plot you can see the 5 cell line is not that much sharp, 11 cell line is more that much sharp; that means, quantitation is better when you have multiple cell line. A chances to find that protein is very high.

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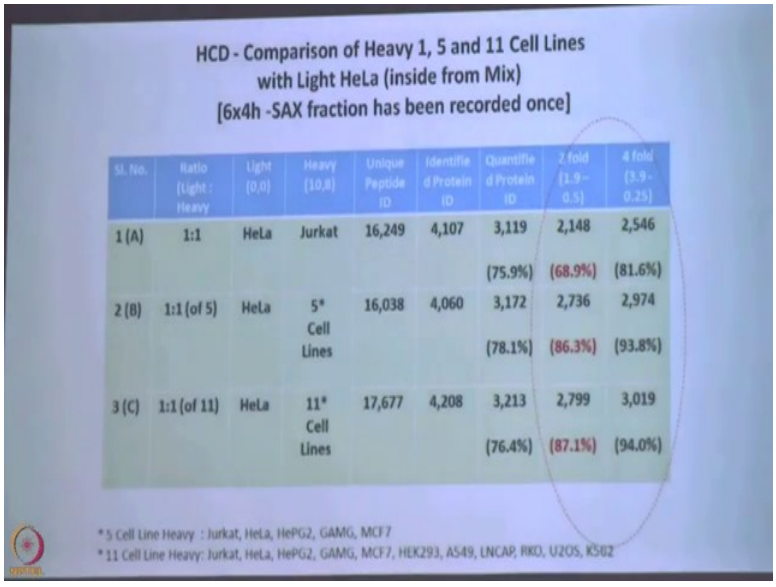

Same things you do with HCD 1 5 11 cell line and you will get different type of things and we are fine here, but this HeLa is again inside that. Now, HeLa compared with

Jurkat you will get only 70 percent, no changes almost. But, HeLa with 5 cell line, HeLa with 11 cell line you are getting more to cover it; now it is different you are comparing.

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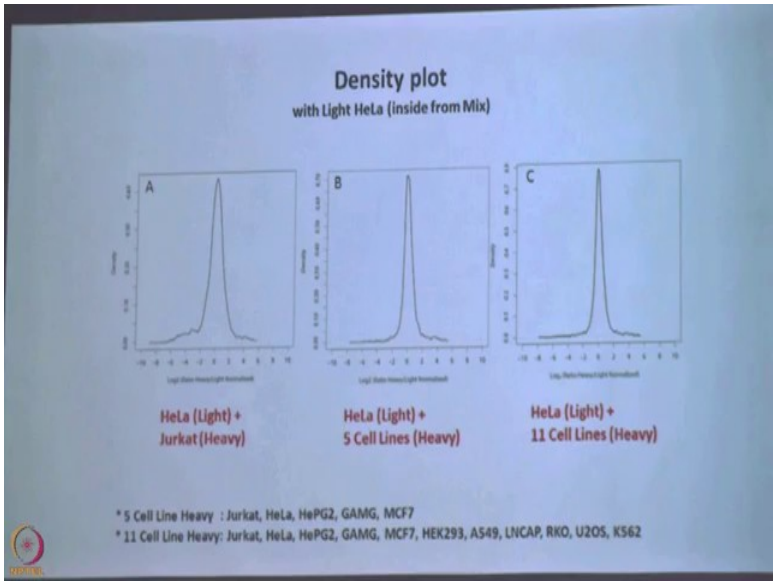

Again you see that HeLa with Jurkat your line, your graph is not density plot is not so sharp, here it is better and 11 cell line better. So, this has given me indication in my 11 cell line or higher a stock I will able to cover more protein of that. If that mix is ready that proper things is ready; that means, I will have more chance to quantify because, in human I cannot label SILAC ok. It is only possible in cell culture ok.

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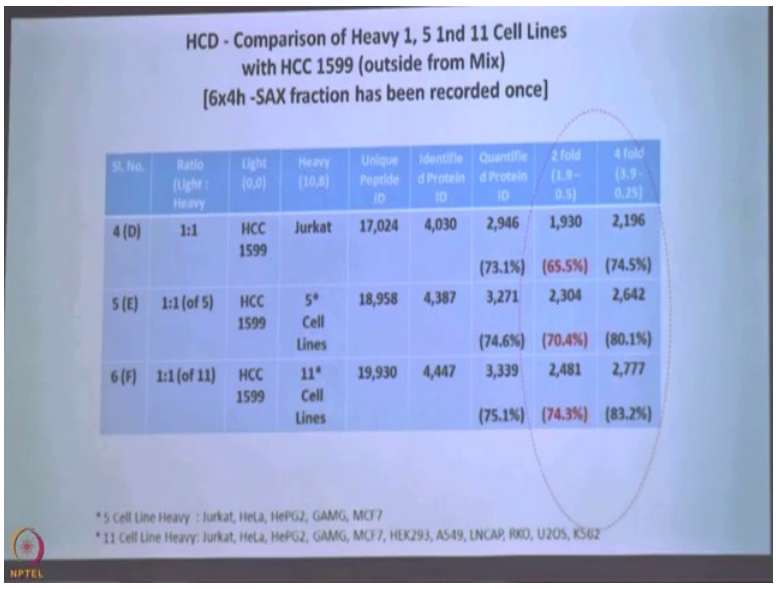

Now, you see here, here we have taken 1 cell line HCC 1599 breast cancer, this is not mix present in the mix of 5 and 11. Now, you see here chances of getting quantification is more or less, because it is not inside you will get different things. So, this concept is coming clearly how you make the mix, do you need a master mix, do you need this. And, whole analysis idea is still we are doing that what common protein is there in all the type of cancer, where it is getting click or where it is getting a start that is still under the way.

Most probably next time I will show you more and better, same things I am trying also to go with the tissue now. When 11 cell line we can take, now we know what is going to happen, now 11 tissue or 20 tissue we are trying to do, all cancerous tissue and we are looking what is the common. And, can we find one common place where all these get trigger that will help, will we do not know what will happen. With this you again say this density plot always 11 cell line is coming better ok, you all have learned R.

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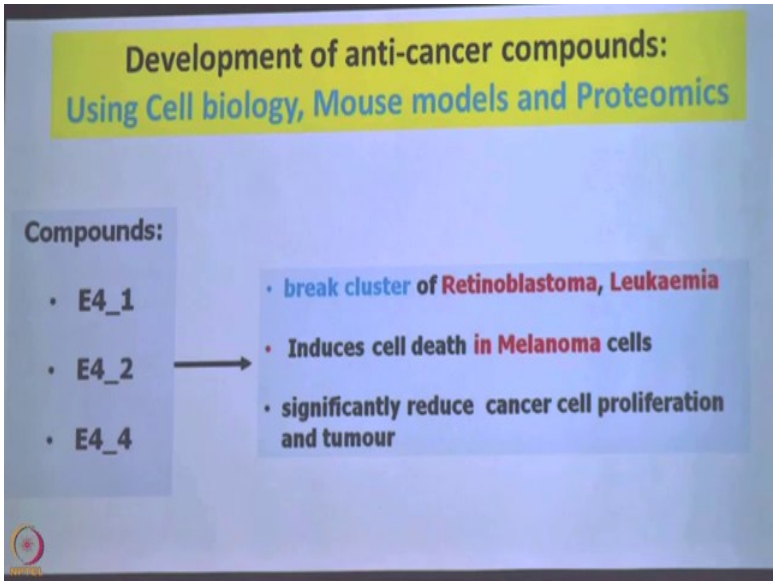

And, with this I will move towards the little bit cancer drug discovery that is the my favourite hard work of PhD student and it is somehow giving result. So, what is there? Development of anti-cancer compound, use cell biology, mouse model and human model you have to go finally, clinical trial. So, that is the what simple cancer means tumor, break the cluster, induce cell death, apoptosis and reduce cancer cell proliferation.

Three things are there target, different compound we have a screen, there are you would heard a company a screen 10,000 15,000 compound library and then come to one target. We thought how can we do a smartly.

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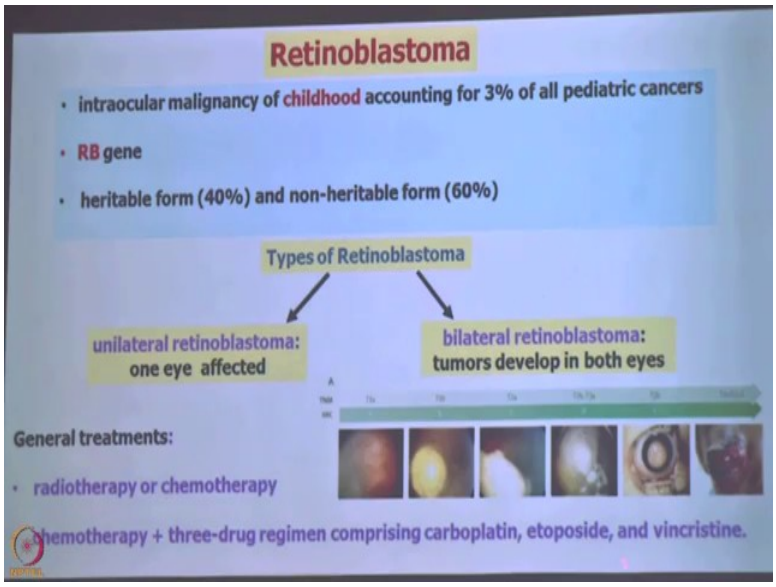

Here this is a childhood cancer, mostly happened in childhood time before 5 year. Why it happens? It is very tough to tell, no one knows. It can happen in 1 eye, it can happen in 2 eye and both the eye can be affected. There is the drug with very high side effect carboplatin, etoposide and vincristine mostly natural towards ok.

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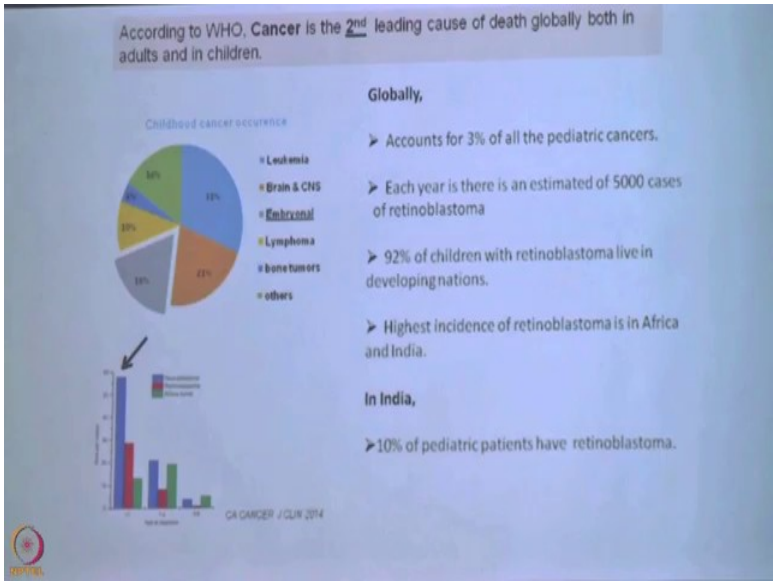

So, we thought nature maybe have something, with this now how to think about the cancer, how much unfortunately this retinoblastoma is very high in India or developing country India, Africa and all this. Why? There is no answer of that, but if you look all these things you will see 10 percent of pediatric patients have retinoblastoma ok.

In that also highest incidence of retinoblastoma is in Africa and India. Why? No one knows. This is the cancer related with the gene, because you are in proteogenomics and this cancer is related with the gene retinoblastoma RB, you would have heard cancer suppressor gene and this is directly link with that ok. So, this is some time hereditary, sometime environmental, but then sometime no one understand why it happen ok.

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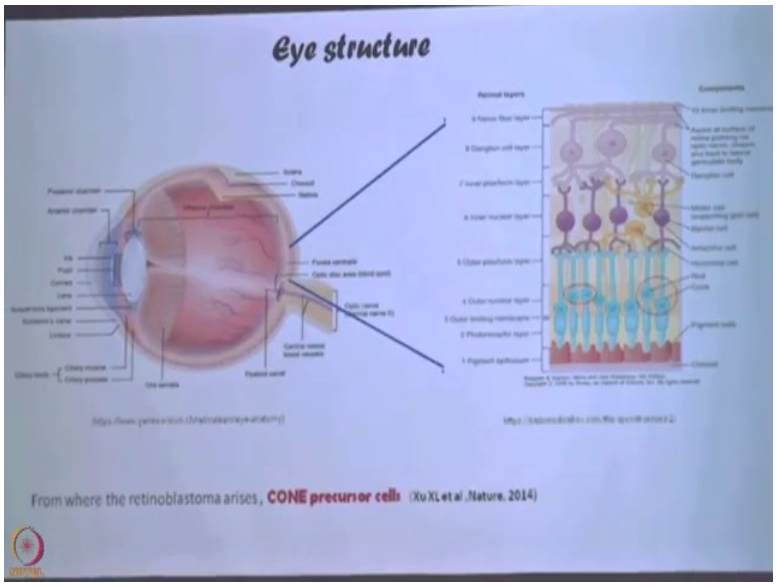

Now, people are a still thinking which place it is just getting a start and recently one in 2014 paper came that they tells it is mostly a starting with the cone precursor cell ok.

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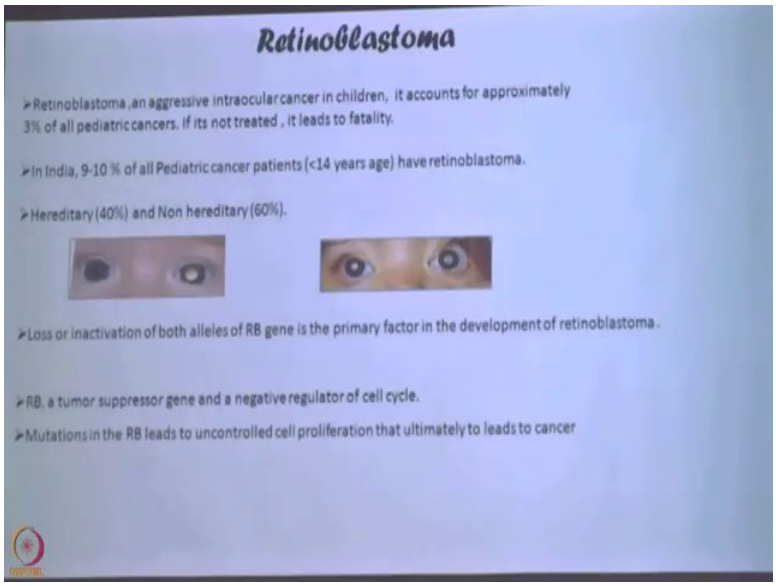

So, this is the little bit evidence has come. So, this is below 14 years says non-hereditary, 60 percent hereditary is 40 percent. So, wonderful model system to a study the cancer where genes genetics is also involved, environment is also involved, but no one has any answer.

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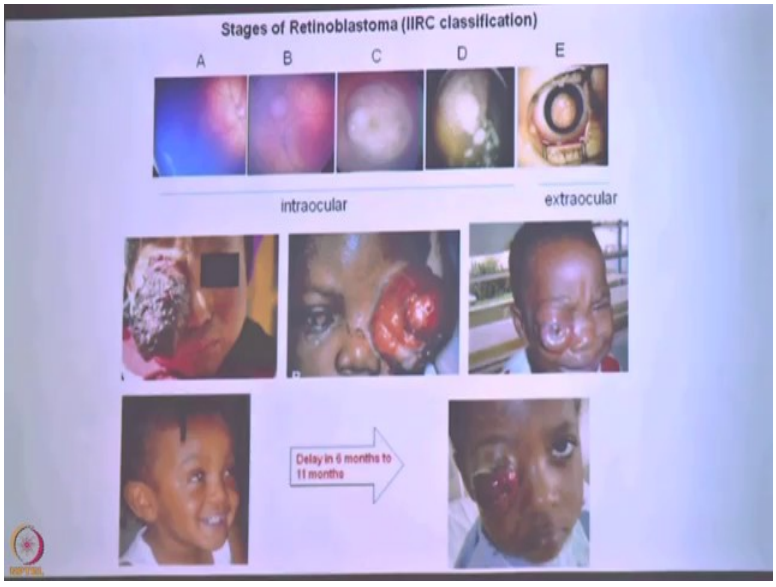

Now, this is terrible make is not good to see too much ok, in 6 months times this become terrible ok.

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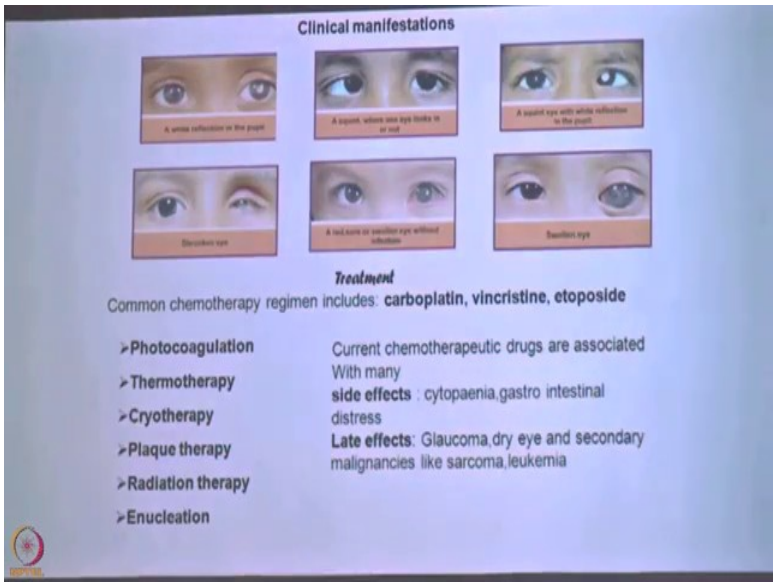

If you did not get treatment then very tough and that is the problem happened in developing country, when you get you see leukocoria you can characterise if a doctor will see a normal person will quickly figure out there is something in the eye. This is the step where you understand there is something leukocoria stable tell. I have mouse glioblastoma mouse in lab we see that directly it ok, but if it get bigger if they get tumor bigger than head size.

Now, you can understand how this happened, if timely there is nothing in nucleation is only that a chances to having metastasis is very very high. So, what you should do? Now, this is the chain if and most of the time what happened children parents not able to take to the hospital, no one takes care it gets different aggressive a stage than it is almost nothing.

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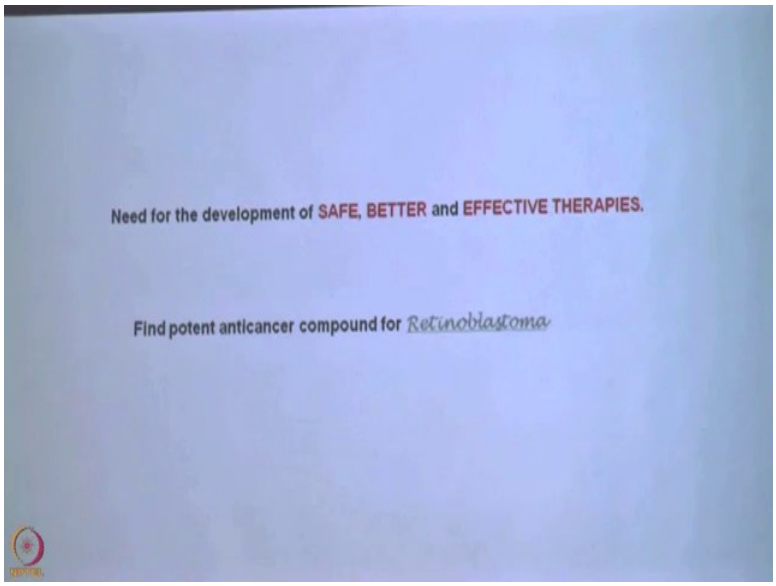

Now so, what we need? We need a need for the development of safe better and effective manner. What you need at this time? You need some drug ok, whatever drug is there that is also not able to cure, it has very side effect.

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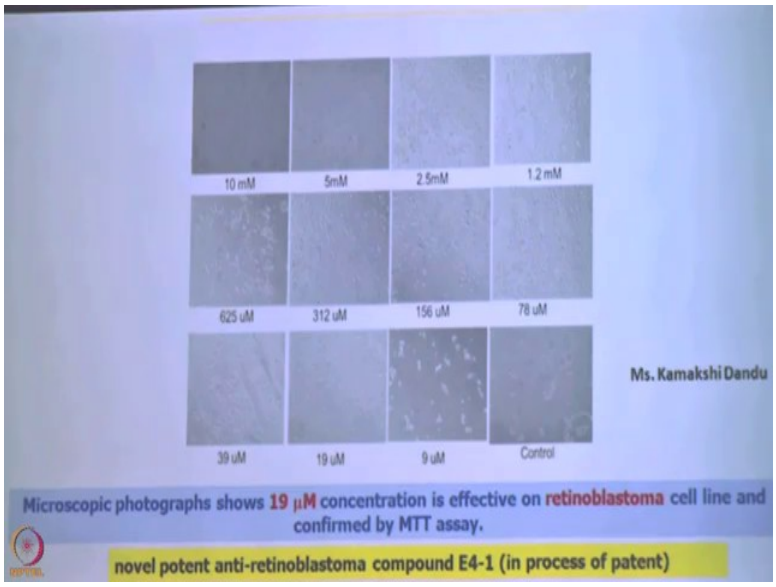

Then this one of the student Kamakshi PhD student has done this work. How? Now, we thought to break the cluster and now idea is that find a drug and find mode of action. In science mode of action is more important. Drug needs patient, mode of action will give

you patient help and to make the another drug better. So now, simple concept break the cluster you say this is cluster forming cell ok.

So now, when you are started giving this control cluster is there, you give we have screens few compounds, several compounds. And, then we came to this compound, this break the cluster, but this is not a big deal. You put Surf excel, Wheel anything it will break the cluster. So, this is nothing conclusive, but indication is there ok.

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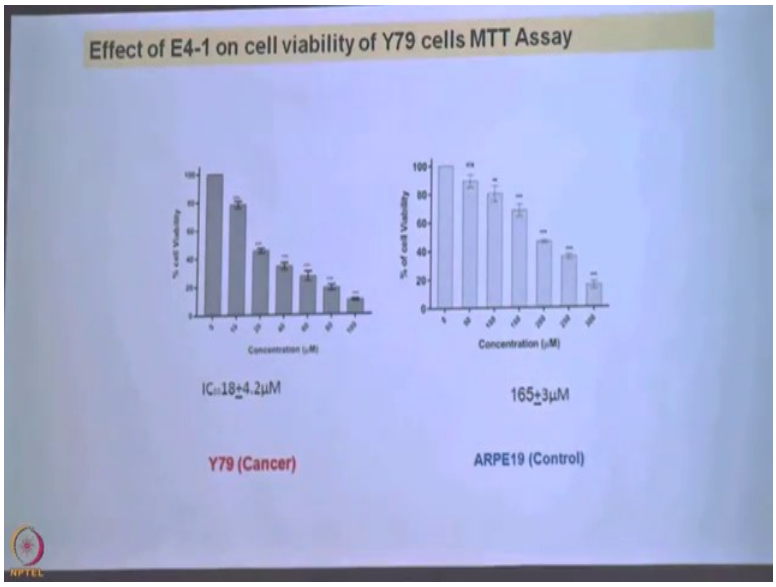

Now, if you got what a biologists will do quickly go and do cytotoxicity ok. So, we did cytotoxicity and somehow we found Y79 cancer retinoblastoma cancer cell line is giving 18 micro molar IC 50. But, if you got 18 micro molar in the cancerous cell, it is also possible this will kill your normal cell ok. If it is killing normal cell then how can this will be drug? First argument you ask yourself and you confirm here. Now, what we did? We took ARP 90, it is also retinal epithelial cell and here you see IC 50 is 165 micro molar; that means, there is it is not killing.

Student: Normal.

Normal cell. We are looking for this, we are looking a compound which has potential to kill only cancerous cell, not to normal cell that is the whole point came here. So now, you see this is our slide, anyhow there is nothing hard to tell fact this experiment we do

did very late. First we did because, we do not have cell line we try to arrange cell line, it took lot of time later we did, but somehow it worked.

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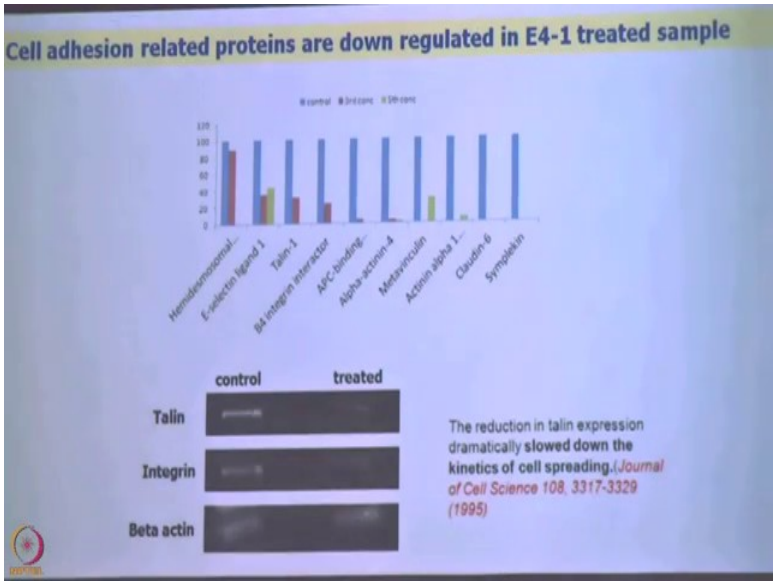

Now, here proteomics played the role. Now, I told you, you put Surf excel also it will work ok. But, when the surf excel concentration will go, it will come again cell closer ok. Now, when this compound this never comes close so, that was one benefit. Now, you took the give the treatment with this compound to different cell both and now you do proteomics. I have shown you how to do it ok. Now, you do proteomics and now you are start comparing what happened after doing proteomics.

When you look the proteomics, what you found cell adhesion related proteins are down regulated in E4, E4 is nothing it is my lab number, room number. So, that is from it. So, now all cell adhesion related protein. So, cell is not getting attached, it is getting detached and cell adhesion protein is getting down regulated; that means, something is going towards the cell adhesion.

No one is going to believe proteomics, if you are not doing antibody or some validation at least reviewer ok. We all believe ok, reviewer will ask you. Now, we have done antibody experiment and we have moved forward.

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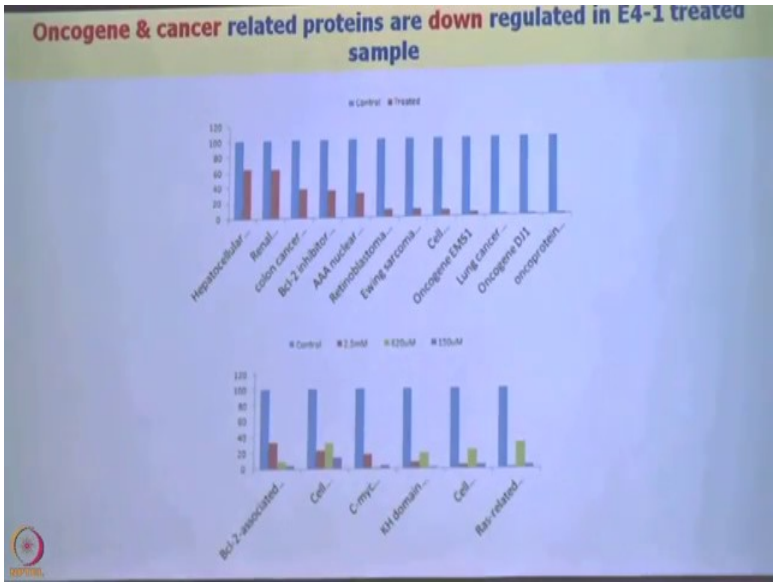

Now, if it is really anti-cancer all oncogene and cancer related protein should be down regulated, perfectly hit ok.

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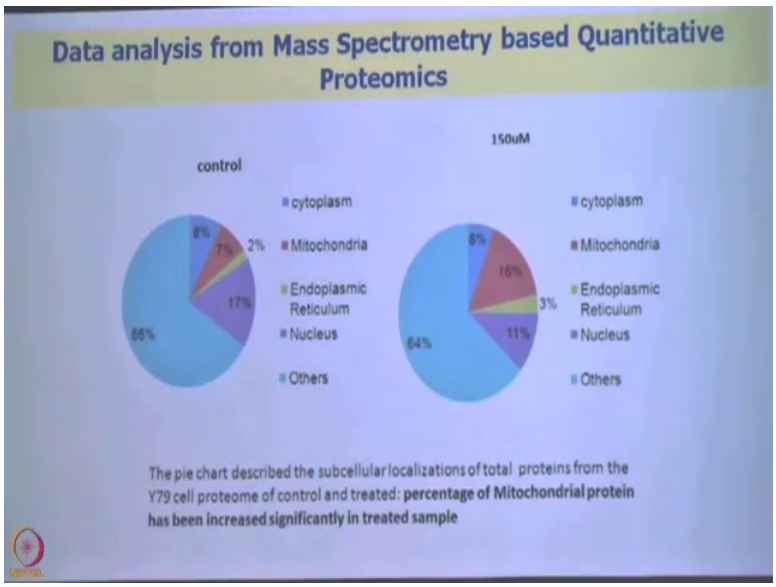

So; that means, it is anti-cancer, now you have to talk about the mechanism. What happens if all mitochondrial protein you will see that is going up regulated in the treatment. So, mechanism in somewhere related to mitochondria. Who is telling proteomics, now this much indication is enough for biologist to kill the or make the project work ok, that is the proteomics played the role.

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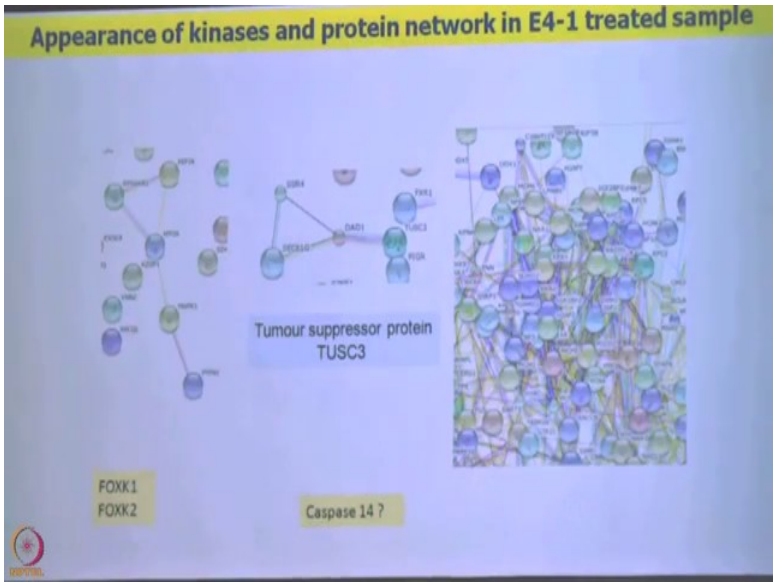

Now, you do whole analysis, cluster, networking all you have learned you know also must do this is very useful. When you do you saw tumor suppressor protein TUSC3 is getting up regulated, that is the logic. Suppressor protein should go up, cancer related protein should go down, but we found this transcription factor and caspase14 came up. So, that we do not have anything's ok, clue about that.

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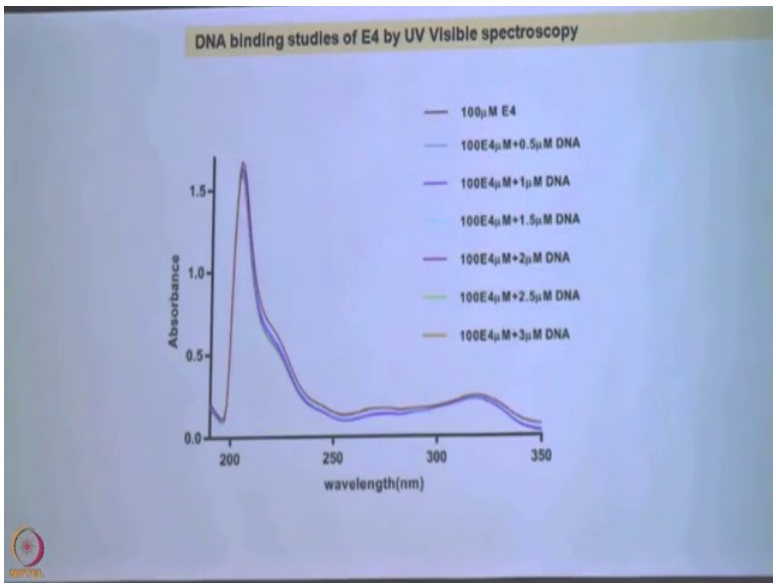

You quickly look this compound is binding with DNA or not, control take a ethidium bromide and just you look it is not binding.

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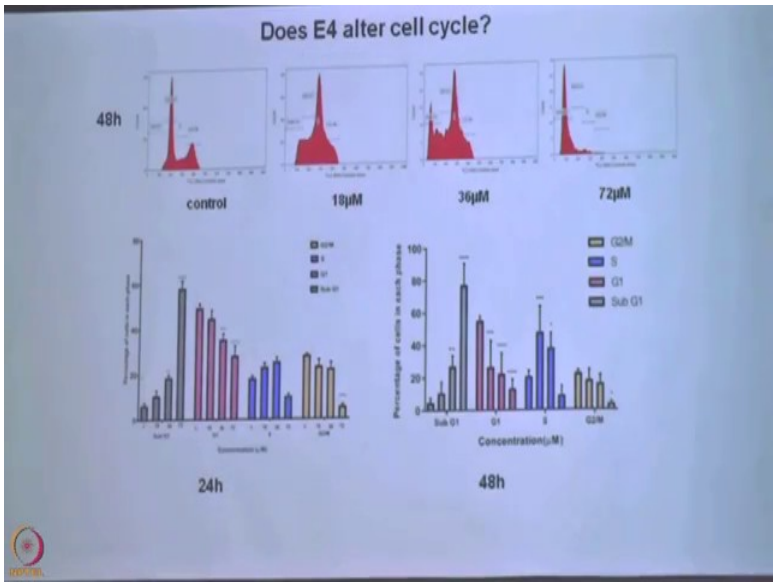

Quickly next experiment Tm, Tm is changing somehow; no need to find quickly mechanism one by one, one by one.

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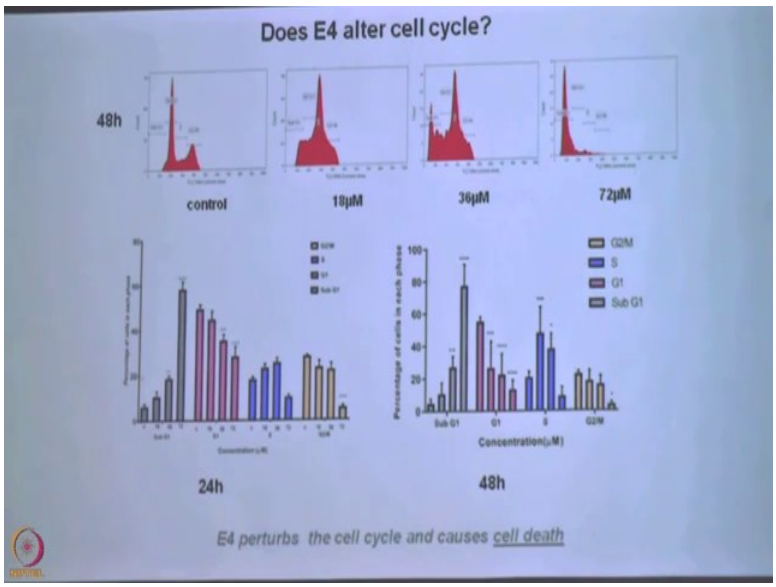

Third you are in CCMB, all are well trained here ok. Now, third quickly look does E4 after alter cell cycle, here we found an little bit very different and strange result. You see this sub G1 population is getting very high with the concentration dose dependency ok; that means, you change the dose IC 50 is 18. If you increase this dose and here you find

the mechanism sub G1 population is getting very high, here you seen same concept thing in 24 and 48 hour result is wonderful.

Something confusion is there, this pattern, this pattern is same; this pattern, this pattern is changing in the S phase, no answer we have ok. This happens; that means, different dose is working at different different time point a different things ok, but this gives sub G 1 population is altering the circle. So, what is the conclusion? E4 perturbs the cell cycle and cause the cell death.

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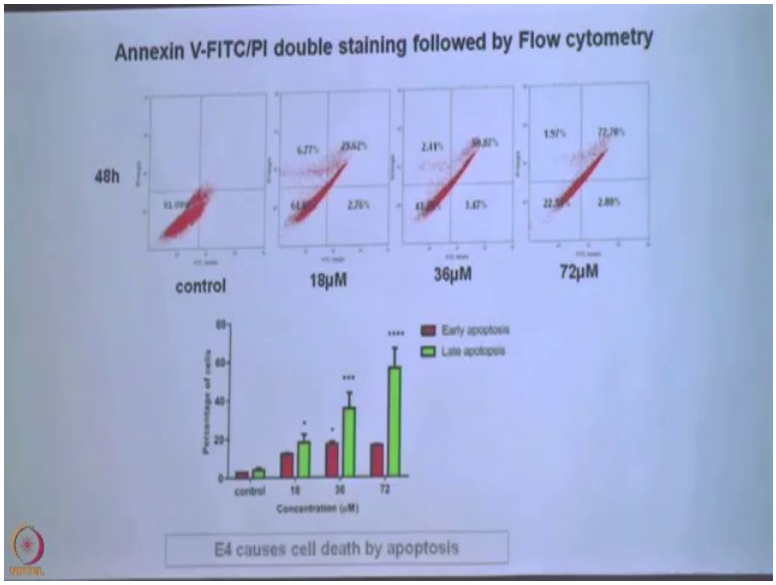

If this we know, then next what we should do? Quickly do apoptosis and quickly we are showing again with the concentrates increasing the concentration what you are doing? You are able to go to late apoptosis more. So, this is working in the time and dose dependent manner that is we come, you can conclude anything more than this.

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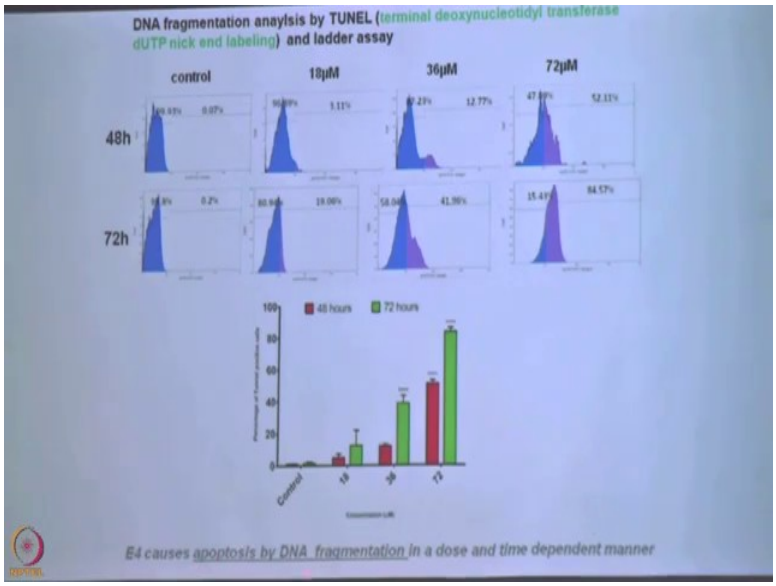

Now, E4 cause cell by apoptosis, now you go another experiment DNA fragmentation, run the gel quickly do it or do tunnel as a little bit fancy and you can see that DNA fragmentation is also going up by higher concentration. So, we are trying to find the mode of action, mode of mechanism and question answer is here. E4 cause apoptosis by DNA fragmentation dose and time dependent manner, even not binding with the that.

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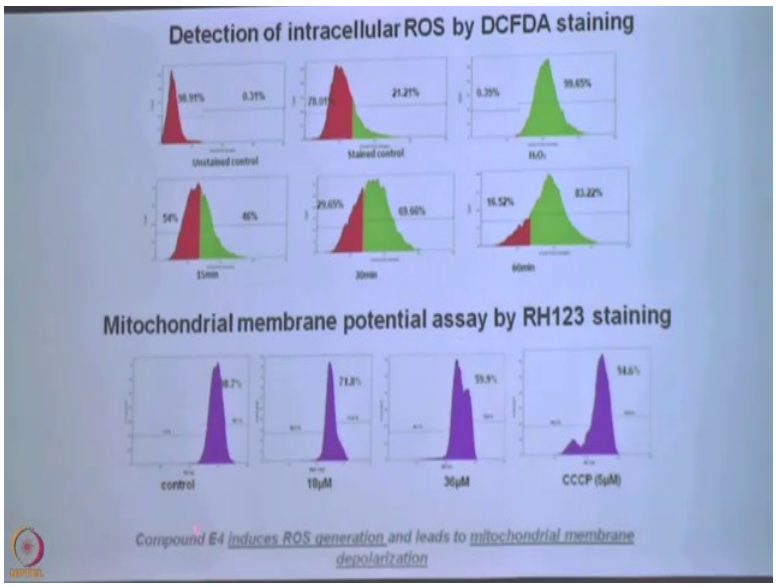

So, now detection of intracellular ROS, we have saw mitochondrial thing, we have saw ROS protein. So, with this we found that this is also making sense and mitochondrial

membrane potential assay we have done; with this we came to that it is something is happening. We are not sure ok, something is happening towards the mitochondrial cell. So, compound E4 induce ROS generation mechanism and leads to mitochondrial membrane depolarization, this indicates that ok. So, what proteomics told mitochondria result came by other biological technique, same or different that is we need.

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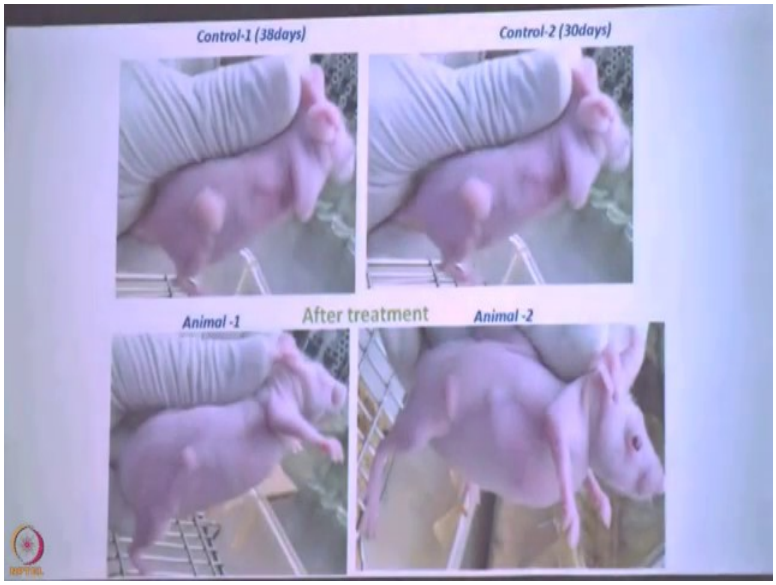

So, with this now no one is going to believe your cell culture only publication ok, if you want to move mouse experiment you have to do. So, this is xenografted mouse, you take the cell line, inject in the mouse, make the tumor, leave the month time to establish, inject the compound. And, if it reduce supervisor will move next stage and a student will go happy another year for move ok. So, like this happens.

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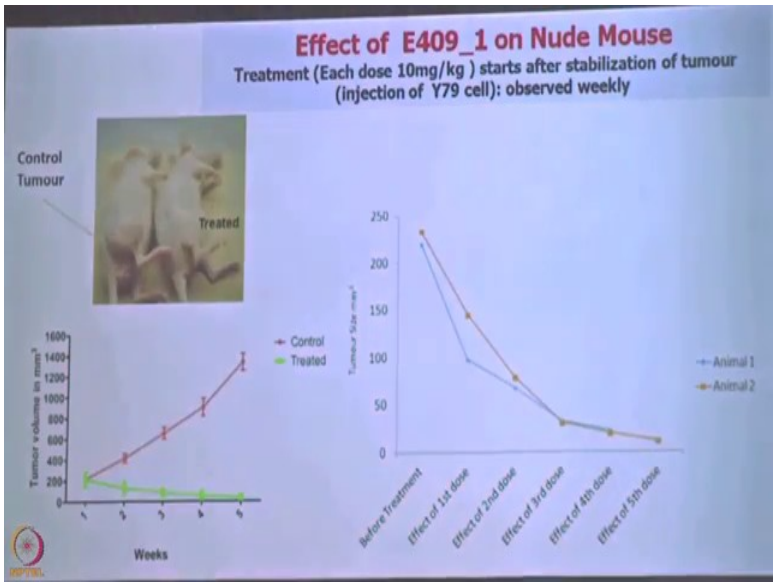

What is the most important things, if you give take control because after 1200 ethical does not permit you to keep the tumor size. And, if you give treatment it is completely demolished. This results gives very nice, even I like this is first experiment you see because experiment you have to plan very well. Give injection only on Monday, student will not feel bad; Monday come inject nicely wait for next Monday and you see the ones how dose dependency go.

So, this proof clear that do not give too much pressure, then they will not think. So, this gives clear that how things are working.

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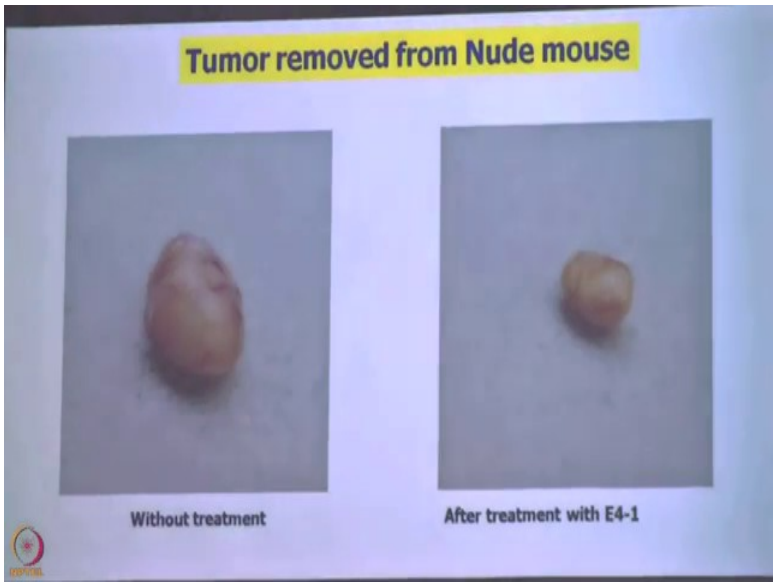

If it is really working, what is the next stage? Again use your proteomics, now we are sitting here, we have this tools technique knowledge.

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We took the tumor from both after treatment that do full proteomics, do histological staining, quickly show where is the apotheosis is happening.

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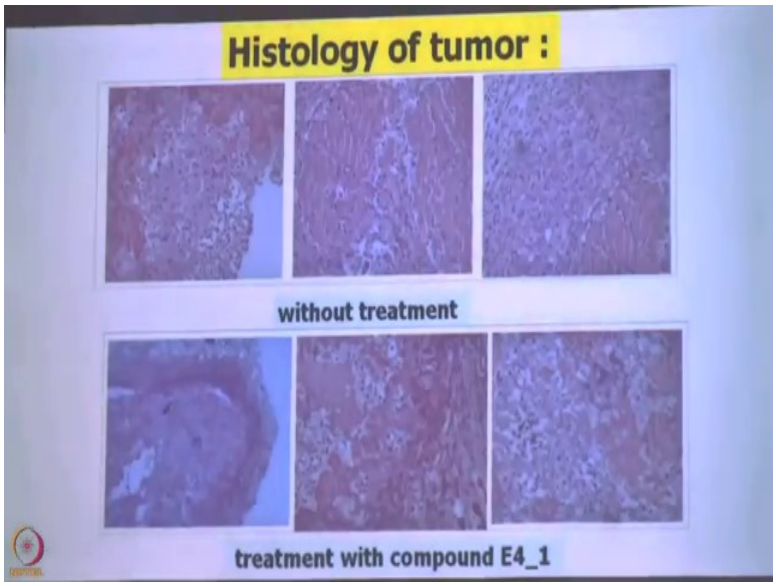

And histology of tumor, thus you can see treatment yeah just this.

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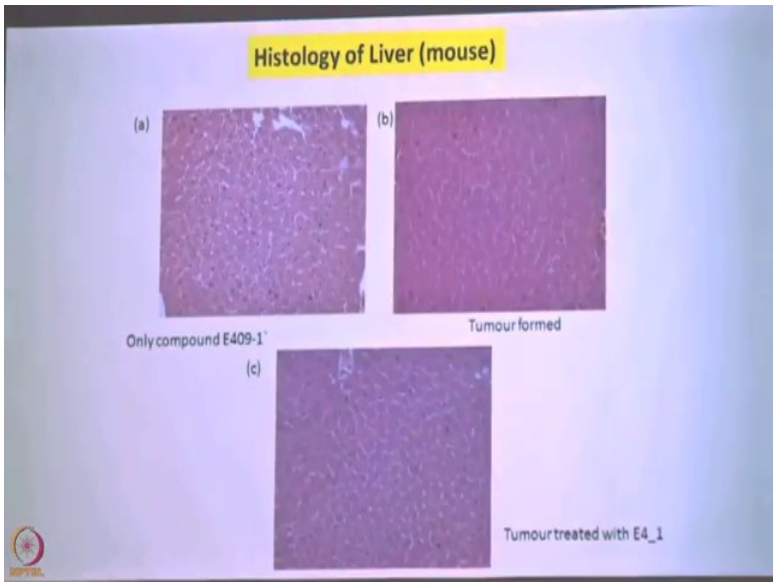

And now I told you one word side effect, there should not be a side effect ok. So now, when histology of liver, we do we do not see any change. Only compound when you inject, when tumor form when tumor form any compound injected we do not see in histology any change and no mouse has died that is the biggest with this.

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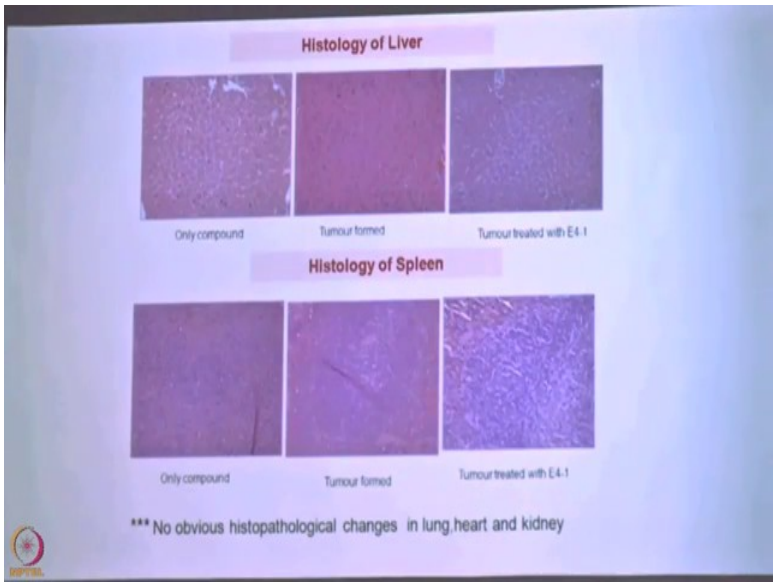

With this you see similar thing in a spleen, you saw similar thing in lung, heart and kidney.

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That is gives the now here two and a half years after hard work of system and paper work finally, we got knock out mouse retinoblastoma where gene is knockout RB- p53-. So, mouse will get automatically retinoblastoma, here we have started treating and we are getting potential very good result. And, I will show you most probably next time whenever we meet.

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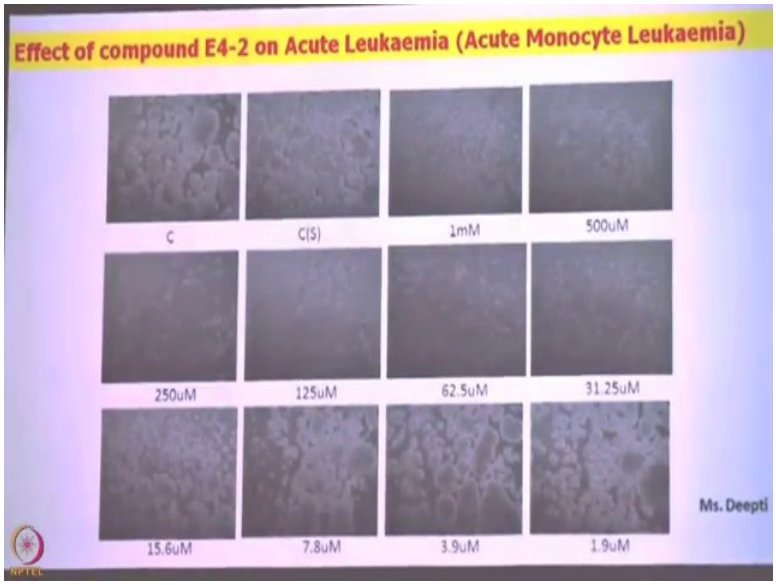

With this I showed you how things will go, similar concept we have used for leukemia.

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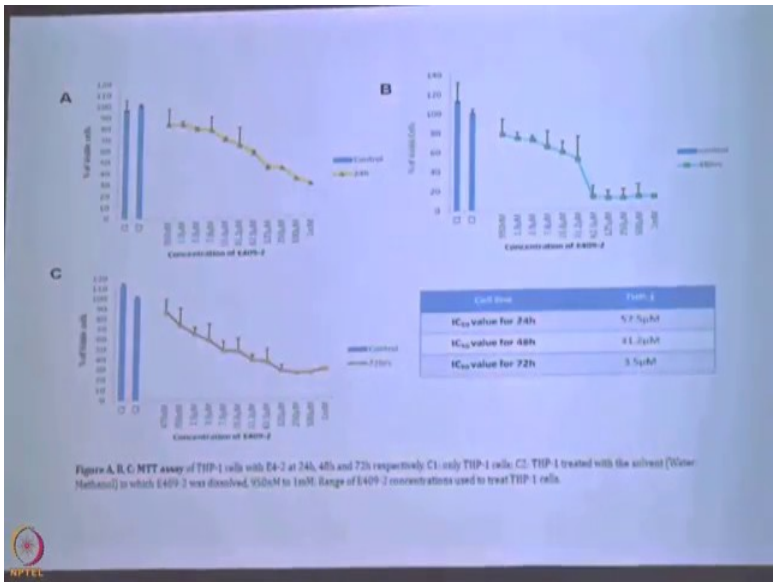

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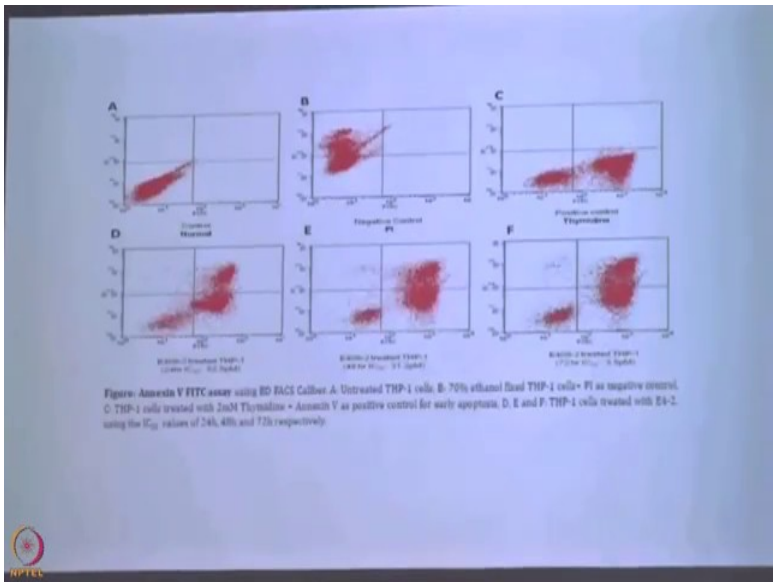

Very nice IC 50 1 1 minute, I will show you the concept is repeating that you got late apoptosis.

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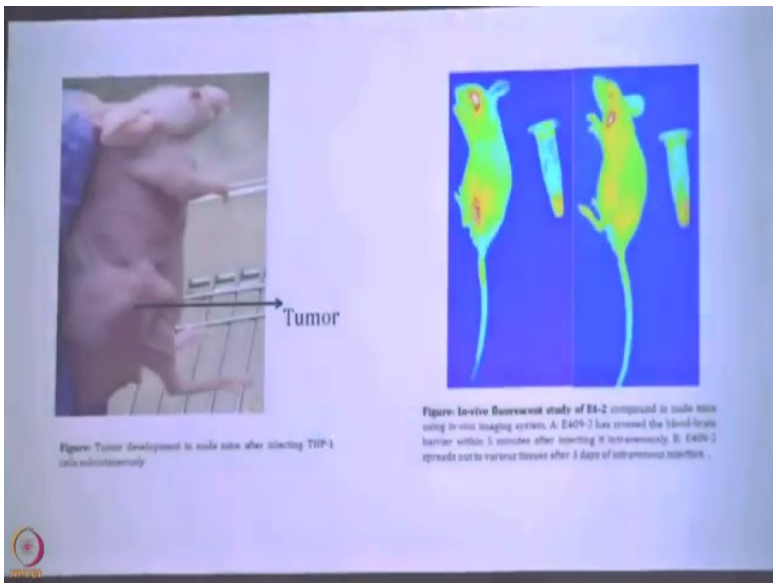

What is the interesting thing here got, when you injected this is another compound, but similar line in 5 minute it reach after injection to brain and if you leave for 45 days this compound get from body out also. So, this is another compound working for leukemia, this is very nice.

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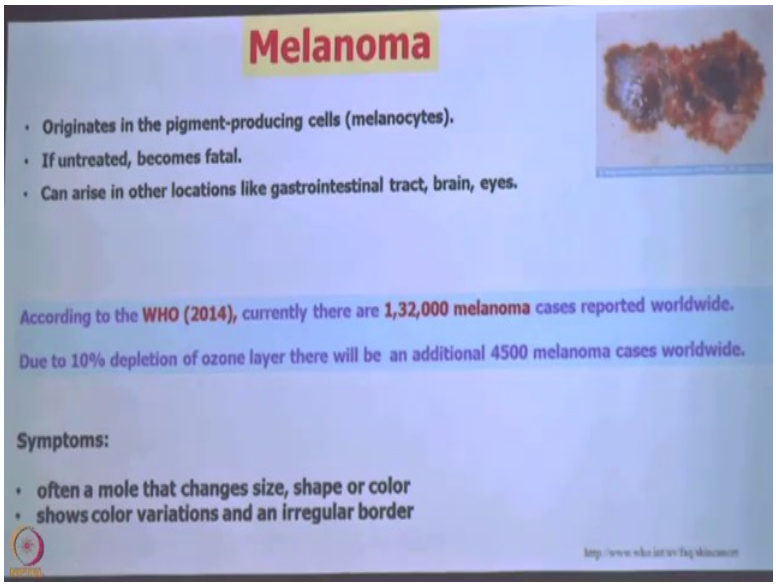

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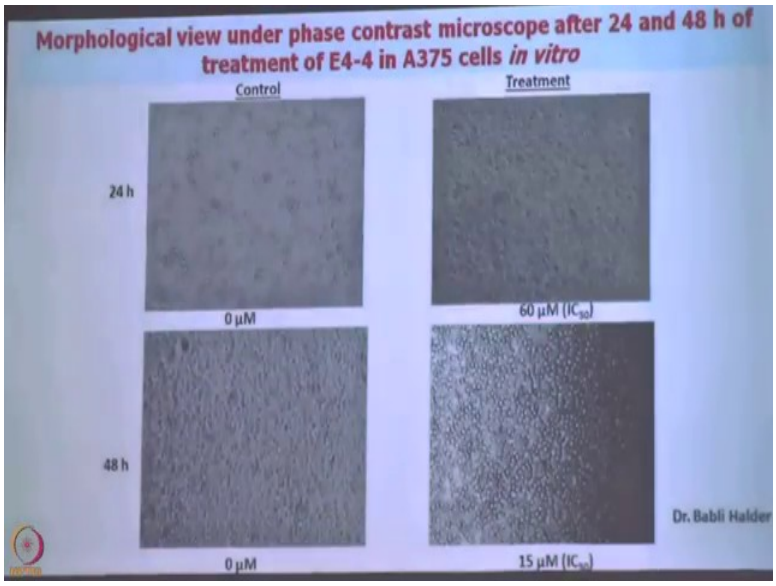

Third is melanoma all you know, what I saw how to come the mechanism. This is the last, here there is no cluster; if it is working there is no cluster.

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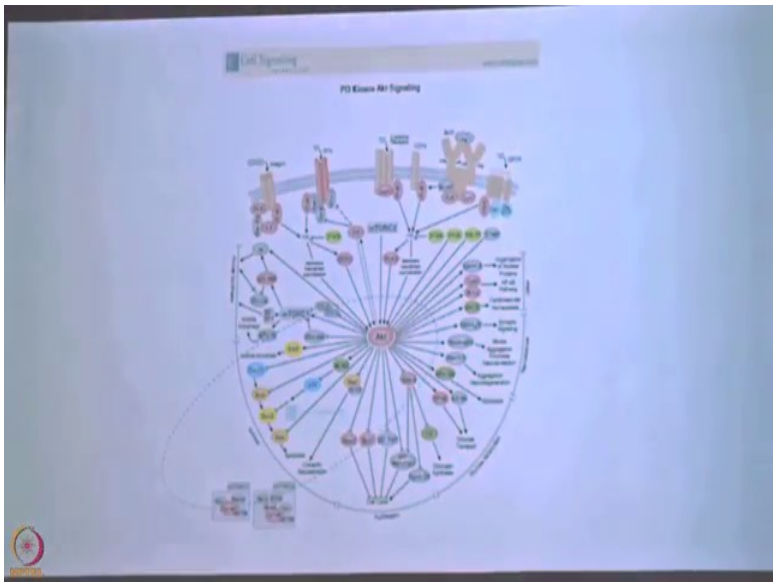

And when cluster is there, still compound is working you find mode of action and mechanism which signalling pathway very simple.

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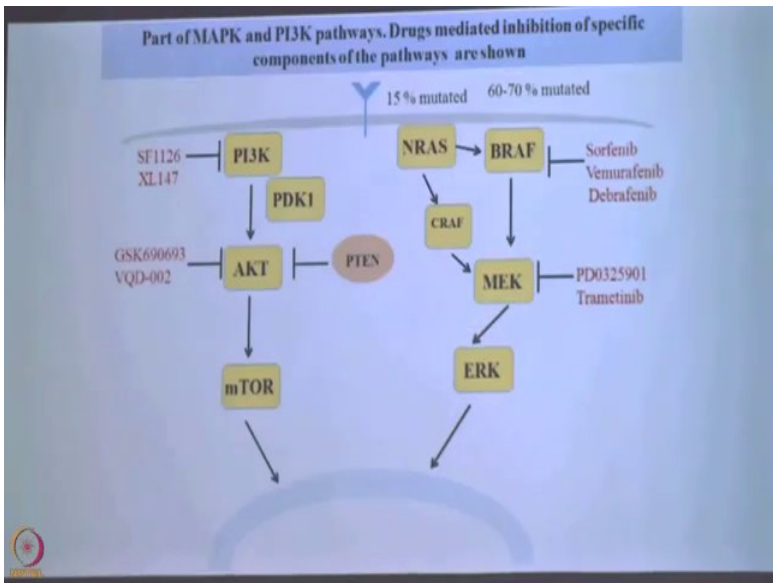

All the known target drug you take, make the slide and even you find this is the target.

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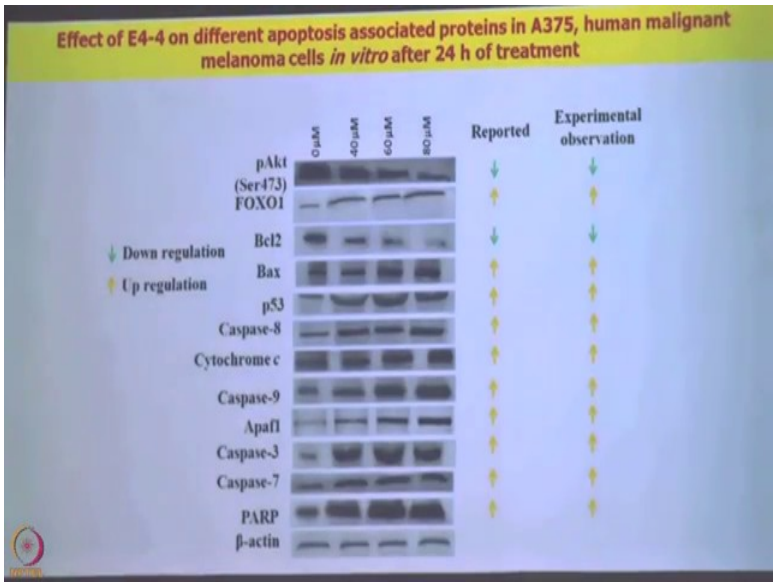

And then you do all your experiment and you will find by antibody ok, this is happening. I told you in biology antibody is very important. Now, you see BCL2 is the down regulated, PAK3 is down regulated. And, if this is really apoptosis all caspases should be down regulated or up regulated?

Student: Up regulated.

Up regulated biology is like this ok, only mass spec is not enough; you need mass spec and parallel this. When you reach this, by this anyone can tell there is the apoptosis, caspase is up. There is something is going.

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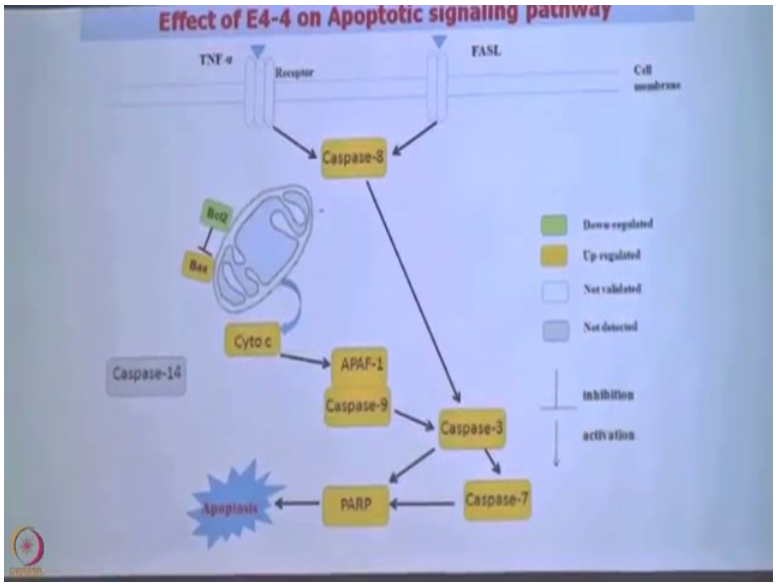

If you have this data you make your models ok, how mechanism is working. When you go to this mechanism, you saw we saw Caspase 8, 3, 7, poly ADP ribose polymerase, Caspase 9 FF, Cyto c, Bcl2, Bax. So, this is the mode of that and mostly it is going towards the Akt signalling pathway ok.

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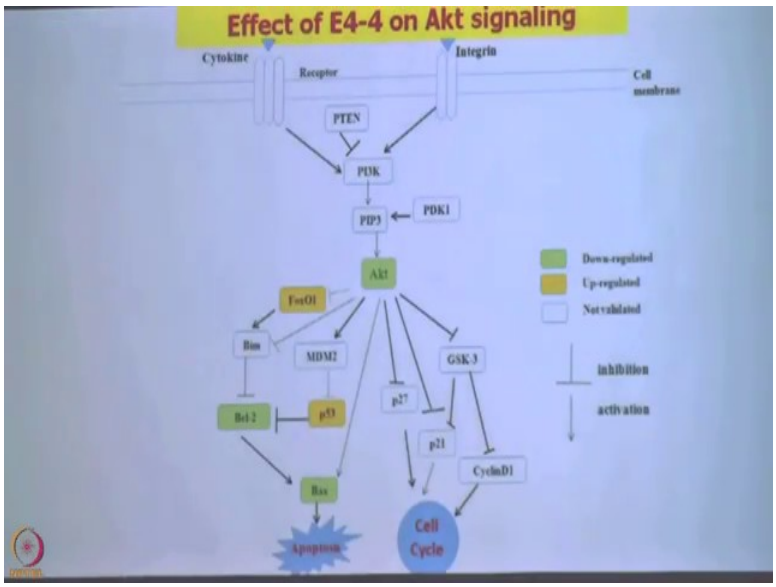

Now, use your knowledge, here experience is experience ok; then you see it is Akt, FoxO1, Bcl 2, p 53, Bax, apoptosis. This is the chain, it is coming down regulated and up

regulated. So finally, we are coming it is towards the Akt signalling pathway. With this thank you all of you, your patience and for time.

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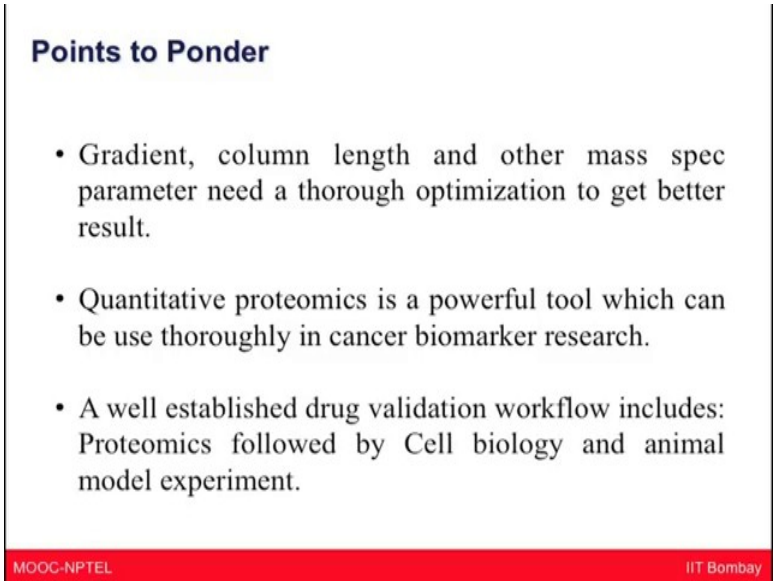

For today's lecture by Dr. Suman Thakur, I hope you got a complete image understanding about why getting a single biomarker for cancer is tough; why many biomarkers have not been able to reach to the transitional work so far in the clinics. Dr. Suman Thakur showed you how multiple cancer cell lines having same protein, but different amount. This is the reason a labelling technique like SILAC or iTRAQ can be helpful.

He also showed a complete overview how cell biology can be used to get the drug screening strategy and enable modern experiment can help in validation. I hope you are gathering different facts from different scientists, clinicians and trying to understand that what are the latest advancements in the field. And also what are the gaps from the clinical as well as its successful translation.

In this way by integration of cell biology, various type of omic based technologies and clinical strategies together only we can made some success in this area. The next lecture is by Dr. David Fenyo, who will talk about predictive analysis.

Thank you.