Steroid Receptor Coactivator, SRC-3, and Prostate Cancer

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Thank you. I would like to take this chance to thank the organizers for inviting me. Dr. Yoshizato-sensei mentioned that in the third session we are going to have a major debate between the frogs and the animals. And I now started to realize why I was invited here, because I was inserted between these two groups to keep peace.

Seriously, as Dr. Seo mentioned, in addition to the steroid hormone receptor, the thyroid hormone receptor, other also play a role in thyroid hormone action. Today I am going to talk about one of them called coactivator.

As shown in this slide, this is how hormone dependent activation of nuclear receptors is regulated. Steroid hormone receptors, is separated into two groups. The first group, called group 1; PR and ER are representative. A second group is the thyroid hormone receptor and the retinoic acid receptor.

In the first group progesterone and glucocorticoid receptor have been shown to be associated with head shock proteins in the cytoplasm and some of them may be in the nucleus. The heat shock protein association presumably to keep them stable by forming the right conformation to receive the hormone.

Upon binding the hormone, there is a major conformation change. It has been determined by our lab that the C-terminal helix 12 is in a loose conformation in the absence of hormone. In the presence of hormone there is a major conformation changes; now helix 12 resides in the protein core. They are not assessable for the protease digestions. Presumably this conformation changes results in the dissociation of the heat shock protein. Without heat shock protein association, this receptor with different conformation can dimerize, bind to DNA, and activate transcription.

In the second group of receptors, like thyroid hormone receptor and retinoic acid receptor, in the absence of hormone it can dimerize. Since they can dimerize, are able to bind to the response element. As mentioned by the previous speaker, when they bind to the response element, they can silence the target genes. When hormone is added the resulting conformation change activates the receptor to be able to stimulate the target gene expression.

For the activation and also silencing, they cannot do it alone; they need cofactors. These cofactors for silencing are called corepressors and for the activation are called coactivators. We are the laboratory that first cloned the steroid hormone receptor coactivator. We called it SRC-1. Subsequently, two other related coactivators also have been cloned. One is by Chambon's group and by Stallcup's group. It is called SRC-2 or Tif2 or GRIP1. The third member, SRC-3, has been cloned by many different groups, six groups in total; they called it in different names: P/CIP, ACTR, RAC3, AIB1, and TRAM1.

All three members have similar conformation, similar protein sequences and similar structure. In addition to be coactivators for the steroid hormone receptors., these three members can also serve as coactivators for other transcription factors, especially SRC-3. SRC-3 can work on many different systems; therefore, SRC-3 must play a very important role in the activation of the target genes of many transcription factors.

SRC-3 is the topic of my talk today. The reason for that SRC-3 has been cloned in the NIH group they call it AIB1, amplified in breast. Amplified in breasts means this gene is amplified in some breast cancer patients. It turns out that amplification may not be as important, instead overexpression of AIB1 is the most important in relating to breast cancer.

SRC-3 is also amplified in ovarian cancer, gastric cancer which was done in Japan. SRC-3 is overexpressed in 40-60% of all breast and gastric cancer patients, and its expression in breast cancer

patients correlates with the tumor size and Her-2/*neu* levels. Both SRC-3 and Her-2/*neu* are equally required for tamoxifen resistant primary breast cancer, which suggests the important of SRC-3 in cancer.

In our lab we are interested in studying prostate cancers. We are asking whether SRC-3 is also overexpressed in prostate cancer. The answer is yes; we have analyzed 134 patient samples. We found that 47% of them have overexpression of SRC-3 in the tumor area. This is roughly falling into the range between breast cancer and gastric cancer.

Next we examined relative "norml" area adjacent to the tumor area. We have analyzed 61 of them, we found only 8.2% of them have overexpression of SRC-3. Therefore this result suggests very clearly that SRC-3 overexpression is also important for prostate cancer as well.

The next question we asked is; whether SRC-3 overexpression correlated with the stage of the cancer. One of the ways to measure the prostate cancer stage is the Gleason Score. If you have a Gleason Score 5, you are in the early stage. If you have a Gleason Score of 9, it means you are at the late stage of prostate cancer. As you can see from here, we have analyzed many different samples at different Gleason Score. There is a general trend between the early stage and low expression of SRC-3 and late stage with high expression of SRC-3.

Similarly, we can also use another way to measure the seriousness of the tumor stage. They call it T2, T3a, T3b, and D1. As shown here, in the T2, early stage, you have only 36% of the patient sample overexpressing SRC-3, but at T3b, the late stage, there are 80% overexpressing SRC-3. In D1 there are only two samples, therefore, the data is not significant.

These two data indicate that the stage of the tumor does correlate with SRC-3 overexpression. The later the stage, the more serious the disease, the more SRC-3 is overexpressed.

In prostate cancer, just like breast cancer, there is a hormone dependent stage and hormone independent stage. The hormone dependent stage, usually you can treat prostate cancer by hormone therapy, but if you develop to the stage which is hormone independent stage, it is usually too late. We analyzed eight patient samples. These samples are very difficult to get. We find 87.5% of them overexpressed SRC-3. From this data we concluded that SRC-3 is overexpressed in prostate cancer patients. SRC-3 overexpression correlates with a poor prognosis of prostate cancer. That is very important because now we maybe able to use SRC-3 as a potential target for late stage prostate cancer treatment. The late stage of prostate cancer is not curable at the present.

Next we asked what is the signal pathway and the underlying mechanism of SRC-3 in tumor progression or tumorigenesis. For that, we tried to over-express and under-express SRC-3 in prostate cells. This turned out to be not as easy. We tried to overexpress SRC-3 constitutively, but we failed to do so. We only generated a stable cell line, which express very low levels of SRC-3. So we resorted to use an inducible system.

This inducible system was developed in our group. We have used two vector systems, one is inducible regulator, the other one is a target. This regulator under the control of the CMV promoter contains the Gal4 DNA binding domain and the activation domain of p65.

In between we put in a PR ligand binding domain. But this PR ligand binding domain cannot bind to progesterone because 19 amino acids have been deleted. Therefore, this protein cannot be active since it cannot be regulated by any steroid hormones inside the cell.

However, this PR ligand binding domain deletion mutant can bind to RU486, the antagonist of progesterone. RU486 can bind to this regulator and thus activate the regulator. In the presence of RU486 the regulator is active and it can bind to the target gene promoters to turn on the target genes, in this case SRC-3. We also use a HA tag at the N-terminal, so we can trace the newly synthesized SRC3.

Using this method we finally succeeded in generating several different cell lines. As shown here, using the HA tag which detects only the exogenous SRC-3, we find that in the absence of RU486 they do

have a low level of leakage expression.

In the presence of RU486, as you can see, there is a major induction of SRC-3 expression in this stable LNCaP cell line. So we are using #6, #12, and #48 for our studies. #6 we also call HG5, and #12 we also call HG11.

When we examined the expression level with SRC-3 antibodies that can measure total exogenous and endogenous SRC-3, you can see there is a major induction. It is at least a 5- to 6-fold induction of the SRC-3 as compared to the parental cells.

Now with this stable cell line we can start to ask, what will it happen when you induce overexpression of SRC-3 by RU486?

We first asked whether thymidine incorporation is increased when SRC-3 is over expressed. The answer is yes, as shown here there is a major increase of ^{3}H thymidine incorporation, suggesting that overexpression of SRC-3 can cause proliferation of these cells by increasing DNA synthesis.

We also asked what is the signal pathway of SRC-3 induction on cell proliferation? So we used the inhibitor called Wortmannin this is an inhibitor for the PI (3) kinase. As shown here, PI (3) kinase can inhibit the thymindine incorporation, suggesting that PI (3) kinase plays a very important role in SRC-3 induced growth of LNCaP cells.

What is PI (3) kinase? PI (3) kinase is the signal for the growth factor. When growth factor binds to its receptor, it can stimulate PI (3) kinase. PI(3) kinase phosphrylates a lipid, phosphotidylinositol, from 4 or 4,5 diphosphate to become 3,4 or 3,4,5 triphosphate. This lipid is associated with cell membrane, recruiting PDK and Akt into the membrane. When they are recruited into the membrane, the PDK can activate the Akt by phosphorylation in two sites. Akt is a major player in cell growth. Akt can go through many different pathways to decrease apoptosis and increases survival, proliferation, and growth. Therefore, Akt plays a very important role in cell growth.

We asked whether overexpression of SRC-3 increase the Akt pathway. As shown here, the Akt expression in parental cell does not increase in the presence of RU486, but when we induced the SRC-3 expression in our stable cells harboring SRC-3 expression system, we found the total Akt increases tremendously.

Subsequently, when this total Akt increases, we also detected phosphorylation of Akt, which is the active form of Akt, also increases. This increase does not occur in the parental LNCaP cell line. As expected the increased phosphorylation of Akt occurs at both sites as shown in this figure. This result suggests that SRC-3 overexpression, indeed, can induce the expression of total Akt and also the active from of Akt. Thus SRC-3 plays a role in the PI (3) kinase and Akt pathway. This may be the reason why we think that the overexpression of SRC-3 may be related to tumor growth.

There is another question we asked about cell growth. As you know, in addition to proliferation and DNA synthesis, tumor cell growth also plays a very important role in tumorigenesis. So we asked the question; does SRC-3 have an effect on cell growth?

As is shown here, control parental LNCaP cell line treated with RU or without RU, the cell size is not different as shown by forward scattering analysis. There is no increase in the size of the parental cell. However, treatment of HG11 cells with RU486, the cell size increased, suggesting that in addition to thymidine incorporation SRC-3 can also increase the cell size and cell growth.

The size increase is true not just for LNCaP cell, but it is also true for the PC-3 cell. As shown here, the size is much bigger in SRC-3 expression line.

So overexpression of SRC-3 can induce ^{3}H-thymidine incorporation and also increase cell growth. How about the effect of underexpression of SRC-3 does on cell growth? For this purpose we used siRNA to inhibit the SRC-3 expression. First, we showed that siRNA is very specific. As shown here siRNA to SRC-3 inhibits only SRC-3 and not SRC-2 expression. In contrast, siRNA to SRC-2 inhibit SRC-2 but not

SRC-3 expression. So this result suggests that siRNA inhibition is very specific.

We next examined whether down regulation of SRC-3 by siRNA increases cell growth using forward scattering analysis. As you can see here, SRC-3 siRNA can inhibit the synthesis of SRC-3 and also inhibit the cell growth. So these results in combination suggest that SRC-3 plays a very important role in cell growth.

Next question we asked was what is the pathway? As I have mentioned, Akt plays a very important role in cell growth. The increased cell growth can go through three different pathways. It goes through the GSK3 pathway, mTOR pathway and MAP kinase cascade. So we asked what pathway is important for the SRC-3 increased cell growth.

In order to do that, we used three inhibitors. One is the LY compound, which inhibits PI (3) kinase and Rapamycin which inhibits the mTOR pathway and PD compound which inhibit MAP kinase pathway.

As shown here, induction of Akt is inhibited by the PI (3) kinase inhibitor LY compound as expected. When you treat cells with Rapamycin to inhibit downstream mTOR, it has no effect on the induction of Akt as well as phosphorylation GSK3. Similarly, MAP kinase inhibitor PD compound has no effect on Akt induction.

But when you are looking at mTOR induced phosphorylation of $p70^{S6K}$ protein, which is an active form of $p70^{S6K}$, it is induced in the presence of SRC-3 and this induction is blocked by both LY compound and the Rapamycin compound, suggesting this pathway plays a very important role in SRC-3 induced cell growth.

In regarding to cell size as shown here, RU486 increases their size, as I have shown you before. When we treated cells with RU486 in the presence of LY compound to inhibits PI (3) kinase, SRC-3 increased cell size is completely blocked.

Similarly, Rapamycin treatment, which inhibits mTOR, also blocks the cell size increase suggesting that mTOR pathway plays a role in SRC-3 induced cell growth. In contrast PD98059, which inhibits the MAP kinase pathway, had no effect, suggesting that this pathway is not important for cell growth induced by SRC-3.

These results clearly indicate that SRC-3 increases total Akt phosphorylation and by the mTOR pathway to activate $p70^{S6K}$ and S6 activity which in turn to increase the cell size. However, I have to emphasize that we can rule out the other two pathways at the present.

In conclusion, I think low level SRC-3 expression increased LNCaP DNA synthesis and cell growth. Active Akt is increased in SRC-3 overexpressed cells suggesting it may play a role in SRC-3 induced cell proliferation.

These results suggest a possibility that endocrine disrupters may target cofactors, and these cofactors can be used as a marker for some of the interrupters. Therefore one can work on disruptor interfering with the coactivator or corepressor functions, instead of working on the receptor directly.

Finally, I would like to acknowledge the people who contributed to this work. This work was done in collaboration with the Sophia Y Thai's laboratory and carried out by Drs. Ge Zhou, Yoshihiro Hashimoto. Patient sample was provided by Drs. Thomas Wheeler and Xin Lei, the pathologist. I will stop here and answer questions. Thank you.

Q&A

Seo: Thank you very much, Dr. Tsai. We have several minutes for discussion.

Q: Thank you very much for your presentation. Presumably artificial overexpression of SRC-3 may be induced, for instance mice, may induce epigenetically susceptible for prostate cancer or other region. Do you have some evidence?

Tsai: We do not have evidence, right now. We are generating mouse with over expression of SRC-3 in transgenic animal using prostate specific promoter and we are hopefully looking for that. We do see it in one of the animals we generated, unfortunately it is not causing a tumor in the prostate but causing tumors in the other areas, which suggests this promoter is not completely specific.

Q: Yes, that sometimes happens when you do this. Do you know the reason why?

Tsai: We do not know, because maybe the insertion site of SRC-3 transgene makes a difference, we are generating other transgenic nice and hopefully we will be able to answer that question.

Q: Very interesting.

Tsai: Thank you.

Q: I forgot actually in your talk, are those patients with SRC-3 overexpression responsive to androgen?

Tsai: As you know, most of the patients you can get your hands on are in the early stage and they are the hormone dependent cancers. Yes, when you treat with castration or treat with antagonists or surgically remove, tumors in these patients usually regress. But a few years later, tumors most likely will come back.

So, this is a very important question; do these SRC overexpressing patients, their tumors start to grow earlier after hormone therapy? Currently, we are trying to correlate with that. Because all of the experiments we have done so far are using *in situ* hybridization it is very laborious and it is not very easy to do, so we are trying to develop an antibody specific for SRC-3 so we can do immunostaining.

If we can do it using the antibody immunostaining and we can use SRC-3 as a marker for prognosis, we can then correlate with the outcome for the patient.

Q: Another question is Akt induced in prostate cancer?

Tsai: Yes, we have not correlated SRC overexpression with Akt yet, but Akt has been shown to be overexpressed in quite a lot of patient samples.

Seo: A short question, please.

Q: A related question: you look at the LNCaP cells, actually that is known as an androgen dependent growth cell line. Actually, does this SRC-3 overexpression have any effect on androgen dependent growth?

Tsai: good question! We have shown, not just using the LNCaP cell, we have used D145 as well as PC-3 cells. They are androgen independent cell lines, and we can duplicate the same results. With or without androgen, these cells are larger when SRC-3 is expressed. In these cases the cell growth does not depend on androgen. The SRC-3 alone will do it.

Q: The LNCaP cell has some dependency in terms of growth on the androgen, right? So my question is…

Tsai: Proliferation is dependent on androgen, yes, but growth is not.

Q: Oh, I see.

Seo: OK, thank you very much, Dr.Tsai.

Tsai: By growth I mean size.