

Thyroid Hormone Receptor Mutations and the Development of Thyroid Cancer

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Good afternoon. First of all I would like to thank the organizers and the Ministry of the Environment for the kind invitation to speak at this meeting and I am very happy to be here.

What I am going to talk about today is outlined in this slide. After a brief introduction I will then review several representative studies to show a close association of thyroid hormone receptor mutations with human cancer. Then, I will present our unique mouse model of thyroid carcinogenesis, and we designate this mouse as TR β ^{PV/PV} mouse. Then, I will discuss our genomic data of thyroid carcinogenesis, and at the end I will summarize what we have learned about thyroid carcinogenesis from TR β ^{PV/PV} mice.

Here is shown schematically the comparisons of thyroid hormone receptor isoforms. Prof. Seo and the speakers ahead of me have already discussed TR isoforms. Therefore, I will not spend more time on this slide, but just to mention that mutations of the TR β gene are known to cause a genetic syndrome of resistance to thyroid hormone.

However, whether the mutations of the TR β gene also cause other human diseases including cancer are not known at present. However, over the years there have been studies to suggest that mutations of the TR β gene could play a role in human cancer. For example, loss of the heterozygosity of the 3p21-p25 was found in up to 100% of small cell lung cancers. Please be reminded that the 3p21-p25 is where the TR β gene is located.

Loss of the heterozygosity of the same region was also identified in up to 64% of nonfamilial renal clear cell carcinoma. A loss of the heterozygosity of the similar region was also found in 75% of invasive breast cancer carcinomas. And recently, inactivation of the TR β gene by hypermethylation was found in 11 out of 11 cases of primary breast cancers.

So, these studies raise the possibility that the TR β gene could act as a tumor suppressor. Furthermore, in collaboration with other investigators, we have found a close association of TR β mutations with several types of human cancer. In this study, mutations of the TR β gene were found in 76% of hepatocellular carcinoma. In another study, mutations for TR β gene were found in 31% of renal clear cell carcinomas. And recently, it is very exciting for us to discover that mutations of the TR β gene were also identified in 94% of papillary thyroid carcinoma but only in 8% of adenoma.

So, these studies, however, I want to point out that the mutations of the TR β gene were derived from somatic mutations. The TR β mutants identified in these tumors are functionally impaired. Many of them have lost the T3 binding activity, and also many of them have lost transcriptional activity, and some of them have abnormal DNA binding activity, and many of them exhibit dominant negative activities.

However, in all these studies, how the mutations of the TR β are involved in the development of these tumors was not elucidated. Therefore, clearly there is a need to have mouse models to study the role of TR β mutants in cancer.

Towards this end we have created a unique mouse model that can be used to study thyroid carcinogenesis. These mice harbor a mutation in the TR β gene and the mutation, we call this mutation PV. PV was identified in a patient at the National Institute of Health, USA. This patient has one mutated TR β gene, and this patient suffers from the genetic syndromes of resistance to thyroid hormone. So what kind of mutation does PV have?

PV has a mutation at the C-terminal region of TR β , and this is due to a C-insertion at codon 448. This insertion leads to a frameshift mutation in the C-terminal 14 amino acids. As a result, as indicated here, the mutant sequence is different from the wild-type.

This slide shows the functional consequence of the PV mutation. PV has completely lost T3 binding activity and PV has completely lost transcriptional activity; importantly, PV exhibits potent dominant negative activities.

Please note that these functional consequences of PV mutation are similar to the TR β mutants identified in human tumors that I just talked about.

Here is shown schematically how we had targeted the PV mutation to the TR β gene locus. The PV mutation was introduced in Exon 10 downstream of the poly A tail we place NeoR gene flanked by two loxP sequences. After homologous recombination, the PV mutation was introduced in Exon 10.

However, preliminary experiments indicate that expression of this NeoR gene, which is important for screening, interferes with the expression of PV genes. Therefore, we deleted this NeoR gene *in vivo* by crossing this mouse with transgenic mice expressing prerecombination. The resulting offspring of the cross, which do not have the NeoR genes but still retain the PV gene were designated as TR β PV mouse.

After extensive characterization of the phenotype, we found that the homozygous TR $\beta^{PV/PV}$ mice exhibit severe dysfunction of the pituitary thyroid axis. In other words, the negative feedback loop of the pituitary thyroid axis was severely impaired such that these mice have elevated circulating levels of thyroid hormones, but the 10-to 15-fold increase of thyroid hormone cannot suppress the synthesis and secretion of TSH.

So these mice have 400-to 500-fold increase in TSH levels. These mice also have enlarged thyroid glands extensive papillary hyperplasia. These mice do not survive well as they age.

Here is shown that at the age of 14 months, only 20% of mice survive. In contrast, more than 95% of mice of the heterozygous TR β PV mice survive at the same age. We only observed two deaths, but these two dead mice did not have any abnormality in the thyroid. Here is shown that no deaths occurred for the wild-type mice.

Upon evaluation, we found that these dying mice developed thyroid carcinoma. Morphological examination indicates that these mice as they age, beginning at the age of five months, in addition to papillary hyperplasia, the thyroid developed capsular invasion. Soon after that these mice also developed vascular invasion and anaplasia. Beginning at the age of nine months, these mice developed metastases to the lung and to the heart, but please note, not to the lymph nodes.

Most of the metastases exhibit follicular morphology, but we also observed in some cases these mice developed an anaplastic morphology.

Here is summarized the histological progression of thyroid neoplasia in 5-14 month old TR $\beta^{PV/PV}$ mice. All the mice we have examined developed hyperplasia, 91% of mice developed capsular invasion, 74% of mice developed vascular invasion, 35% of mice developed anaplasia, and 30% of mice developed metastases.

These data indicate that thyroid carcinogenesis progresses through pathological changes. I would like to point out that as far as we know our mouse is the first mouse model of thyroid cancer which exhibits such a high percentage of metastasis.

In the next two slides, I would like to show you examples of the pathological changes in these mice. Here is shown the example of capsular invasion of the thyroid gland. Here is shown the vascular invasion; the tumor cells have invaded the vascular system. Here is shown the example of anaplasia, and here is shown an example of metastatic thyroid carcinoma lesion in lung. This particular one exhibits follicular patterns.

Here is shown an example of papillary hyperplasia, and at higher magnification here are shown the

spindle cell anaplasia, and here is shown another example of metastatic and anaplastic carcinoma in the lung. Here is shown metastasis in the heart.

Based on the morphological patterns and the metastatic features, we concluded that the thyroid tumor that develops in our TR $\beta^{PV/PV}$ mice is follicular carcinoma. We are very excited about our mice because these mutant mice exhibit the full spectrum of carcinogenesis from hyperplasia to distant organ metastasis. What this means is that these mice provide an unusual opportunity to study alterations in gene regulation during tumor progression and metastasis.

We have started to use microarrays to profile genomic changes during tumor progression and metastasis. The cDNA microarray we have been using consists of 22,000 genes. In the next few slides I would like to share with you our unpublished data from the comparison of mRNA expression between the wild-type and mutant mice at age six months.

Here is shown that among 22,000 genes, 200 genes were up-regulated ranging from 2-to 17-fold and 95 genes were down-regulated ranging from 2-to 20-fold. Among the up-regulated genes, 55% of the genes were named genes and 45% of the genes were unnamed genes. One example is that cyclin D1 was up-regulated 8-fold. I believe that you all know that cyclin D1 is a very important cell cycle regulator gene and cyclin D1 was found to overexpress in many cancers, including thyroid cancers.

Among the down-regulated genes, 64% of the genes were named genes and 36% of the genes were unnamed genes. We then classified the named genes which have known biological function into groups so that we have some understanding about the cellular pathways that are associated with thyroid carcinogenesis.

However, due to the limitation of time I cannot show you all the genes in various functional groups. Based on the gene profile, we found that these cyclin pathways were activated and repressed during thyroid carcinogenesis.

Here is shown that the TSH cyclin pathway was up-regulated, so did the TGF- β pathway, PKC, Notch, NF-kB, TNF α /INF γ , TGF α /EGF and Ras. We found that Bcl2 and PPAR γ pathways were down-regulated. So these tables clearly indicate that complex cellular pathways are associated with thyroid carcinogenesis.

The critical question is how the expression of PV affects some or all of these pathways. Based on the array data, we have begun to sort out these complex pathways and to understand their possible link to the actions of PV. We decided to look at the PPAR γ pathway first. What is PPAR?

PPARs are peroxisome proliferators-activated receptors. They are members of the nuclear receptor superfamily. Like the other members of the receptor superfamily, they have the amino terminal AF domain, C-DNA binding domain, and a ligand binding domain. There are three major subtypes. PPAR α is mainly involved in lipid metabolism; PPAR β is mainly involved in development and embryo implantation; and PPAR γ is mainly involved in adipocyte differentiation. However, it is very exciting that recently it was discovered that PPAR γ could be involved in carcinogenesis.

One of the reasons we decided to look at PPAR γ is because recently there has been a lot of interest in understanding the role of PPAR γ in thyroid carcinogenesis since the publication of this *Science* paper by Kroll et al with the title "Pax8-PPAR γ Fusion Oncogene in Human Thyroid Carcinoma," you already have heard about pax8.

These authors showed that expression of pax8-PPAR γ and here is shown the fusion gene, pax8 and PPAR γ mRNA and protein in 5/8 thyroid follicular carcinomas but not in 20 follicular adenomas, 10 papillary carcinomas, or 10 multinodular hyperplasias. So, it is very unique for follicular carcinoma.

These authors also found that expression of pax8-PPAR γ inhibited thiazolidinedione-induced transactivation by PPAR γ in a dominant negative manner. This is the ligand for PPAR γ . They proposed that PPAR γ plays an oncogenic role in thyrofollicular carcinoma.

As I have already indicated, our TR $\beta^{PV/PV}$ mice already developed follicular carcinoma. Therefore, we decided to evaluate whether the expression of PPAR γ was changed in the thyroid of our mice.

Here is shown the expression of PPAR γ , expression in six wild-type mice and here is shown the reduced expression of PPAR γ in our mutant mice. Here is shown the control after quantification, and normalization. We found that expression of PPAR γ was reduced in our TR $\beta^{PV/PV}$ mice.

These results indicate that PV could act to repress the expression of PPAR γ . However, we also believe that it is possible that PV could also act to interfere with the transcriptional activity of PPAR γ . Indeed, we found that PV represses the ligand dependent transcriptional activity of PPAR γ .

In this experiment, we used a primary thyrocyte from the wild-type mice. This bar shows that in the presence of ligand troglitazone, the transcriptional activity of PPAR γ was activated as compared with the basal. Cotransfection of PV abolished the ligand dependent transcriptional activity of PPAR γ to the basal level.

Taken together, our data indicate that PV has a dual functional role on PPAR γ , one, to repress the expression; two to repress the ligand dependent transcriptional activity on PPAR γ . Therefore it is important for me to point out that during thyroid carcinogenesis the expression and activity of PPAR γ remain low.

At the present time, the precise role of PPAR γ in thyroid carcinogenesis is not clear. However, activation of PPAR γ by ligands has been shown to inhibit growth and to induce apoptosis of human breast, colon, and prostate cancers *in vitro* and *in vivo*.

In several human thyroid cancer cell lines, ligands for PPAR γ have been shown to inhibit growth and induce apoptosis. So, the current thinking is that PPAR γ could act as a tumor suppressor gene in thyroid follicular cells. Based on this finding and our own data, the door is open for us to use our TR $\beta^{PV/PV}$ mice to further understand the role of PPAR γ in thyroid carcinogenesis.

We are currently considering the possibility of treating our mice with PPAR γ agonist to see if the activation of the signaling pathway of PPAR γ could alter the course of tumor progression in our mice.

This brings me to the last point of today's presentation; what have we learned about thyroid carcinogenesis from TR $\beta^{PV/PV}$ mice?

We have learned that thyroid carcinogenesis progresses with sequential capsular invasion, vascular invasion, anaplasia, and eventually metastasis. We have also learned that the collaboration of PPAR γ and PV-mediated signaling pathways may be critical in thyroid carcinogenesis. We also learned that thyroid carcinogenesis is a multi-genetic event.

In a very broad sense, the mutation of the TR β gene could be considered as a thyroid disrupter. Our data indicates that such disruption could lead to cancer.

In conclusion, we believe that the TR $\beta^{PV/PV}$ mouse is a valuable model for elucidating the molecular genetic basis of thyroid carcinogenesis, and that TR $\beta^{PV/PV}$ mice can be used to obtain signature genes for diagnosis. We also believe that TR $\beta^{PV/PV}$ mice can potentially be used for testing drugs and designing treatment strategies.

I would like to thank my coworkers from my own laboratory. Hideo Suzuki and Hao Ying have done most of the work I talked about today, and also from my own laboratory, Masahiro Kaneshige and Kumiko Kaneshige prepared the TR β^{PV} mouse. Mark Willingham from Wake Forest University has done all of the histological analyses, and Robert Walker and Paul Meltzer from the National Human Genome Research Institute have helped with array analysis.

Thank you very much for your attention and I welcome your comments.