Molecular Profiling of Genes Showing Altered Expression in the Livers of Rats Treated with Non-Genotoxic Carcinogens for 28 Days

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Thank you. The first slide, please.

Today's presentation data are not about the endocrine disruption issue. I present here toxicogenomic profiles of gene expression induced by non-genotoxic carcinogens using rats. Next slide, please.

In order to elucidate common gene clusters in response to Ames(-) non-genotoxic carcinogens in rats and then identify the genes that can serve as early biomarkers of carcinogenic potentials for new chemicals, we performed global gene expression analysis using high-density microarrays in the livers of rats treated with representative non-genotoxic liver carcinogens for 28 days. Next slide, please.

As animals, we used 6-week-old SD:IGS rats. The feeding study was performed for 28 days. As non-genotoxic carcinogens, 600 ppm-phenobarbital, 600 ppm-thioacetamide, or 2% diethylhexylphthalate (DEHP) was fed with the diet. As a non-carcinogen, a hepatotoxic dose of acetaminophen was administered. Next slide, please.

Total RNA was isolated from liver tissue. The numbers of animals were three in duplicate for use in probe hybridization. For the probe array, an Affymetrix Gene Chip, Rat Genome U34A array was used. In this chip, 4,500 known and 1,700 unknown genes and 800 EST sequences were included. Next slide, please.

In the normal rat livers, a total of 2,700 of 7,000 genes are expressed. This number is the same as the reported number expressed in human livers. Next slide, please.

This slide shows the number of up-regulated genes in the livers of rats treated with each chemical. Both acetaminophen and phenobarbital induce only about 40 genes. On the other hand, thioacetamide induced more than ten times as many genes. DEHP induced 179 genes, somewhere between phenobarbital and thioacetamide. Next slide, please.

This is down-regulated genes. Similar to the up-regulated genes, both acetaminophen and phenobarbital down-regulated a small number of genes, but the number was higher than up-regulated genes. Thioacetamide down-regulated many genes, with the greatest numbers among the chemicals. DEHP also down-regulated many genes, the number being higher than up-regulated genes. Next slide, please.

These are the functional clusters of up- or down-regulated genes by acetaminophen-treatment. A major functional cluster of up-regulated genes is for metabolism, and for down-regulated genes, metabolism, cell-signaling, and communication genes are involved. Next slide, please.

Phenobarbital is a famous enzyme inducer to cause centrilobular liver cell hypertrophy. As in the acetaminophen case, the major functional cluster of up-regulated genes are those for metabolism, and down-regulated genes are for cell-signaling, metabolism and cell organism defense — a more diverse range. Next slide, please.

Thioacetamide causes liver cell regeneration which will lead to liver cirrhosis by cellular damage due to oxidative stress. Functional clusters of both up-regulated and down-regulated genes are very diverse. Next slide, please.

DEHP is a famous peroxisome proliferator to induce β-oxidation in the liver cells. The major cluster of up-regulated genes is those for metabolism; this includes β-oxidation enzymes. The functional clusters of down-regulated genes are diverse with this chemical. Next slide, please.

This slide shows the up-regulated genes, and the down-regulated genes consistent with non-genotoxic carcinogens. Genes do not change their expression level when acetaminophen treatment was selected. As a result, 5 of up- and 7 of down-regulated genes are selected.

We performed real time RT-PCR analysis at the time point of 28 days and 1 year of feeding study. The blue background ones are up-regulated genes and the yellow background panels are those for down-regulated genes. In the case of up-regulated genes, two genes showed suppressed expression with DEHP-treatment, but thioacetamide did not change the expression level. In the case of down-regulated genes, the controls decreased their expression level at 1 year, so this may be an aging-related effect. Next slide, please.

On the other hand, we had one EST gene that showed an inverse expression pattern from 28th day to 1 year. At 28th day, the control level is high, and the carcinogen treated groups are down-regulated. But at 1 year, the non-genotoxic carcinogen treated groups are up-regulated. Next slide, please.

In conclusion, at 28th day, the major functional cluster of up-regulated genes in response to non-genotoxic carcinogens was classified as metabolism. On the other hand, the functional clusters of down-regulated genes were found to be diverse. Therefore, this diversity may determine the phenotype of biological response induced by each chemical. Among those genes whose expression levels were unchanged by acetaminophen, a total of 12 genes showed similar expression patterns between non-genotoxic carcinogens. At 1 year, many of the selected genes decreased their expression level in all treatment and/or control groups, and they did not show common expression patterns to non-genotoxic carcinogens. EST-1 showed an inverse expression pattern from 28th day to 1 year, and this change may reflect the altered cellular function caused by long-term exposure to non-genotoxic carcinogens. Thank you for your attention.

Q&A

Inoue: Thank you very much, Dr. Shibutani. So we have some time to have questions and comments. Please.

Sone: I am Sone from National Institute for Environmental Studies. Various types of chips such as micro-array, glass array and Affymetrix are currently vailable. What is your reason for selecting this chip?

Shibutani: You want to know why I selected Affymetrix?

Sone: Yes, that's correct.

Shibutani: I probably selected it because it contained a large number of genes.

Sone: That means Affymetrix currently offers the best reproducibility when profiling toxicity of the substance…

Shibutani: Reproducibility is good. There are also three chips for rats that include about 21,000 genes.

Nohara: Nohara from National Institute for Environmental Studies. You really did a great job of summarizing effects. What I would like to know is, when you profiled the liver in its entirety after long-term exposure, I think that the results are significantly influenced by changes in immunocytes and hemocytes from the blood. Do you think this would be valid in deciding if there is a connection with onset of cancer, for example, profiling by dividing heptocytes only?

Shibutani: Although I think it would be difficult for heptocytes only, using technologies such as micro-dissection, I think probably in the very near future they will develop methods of specifically cutting out such parts.

Nohara: Are you studying other effects?

Shibutani: Yes, we are.

Nohara: Thank you very much.

Aoki: I am sorry to have so many questions coming from the same organization, but my name is Aoki from National Institute for Environmental Studies. You have observed various genetic manifestation patterns haven't you? When however observed from the perspective of manifestation of toxicity for example, if organized from the perspective of whether enzyme protein or functional protein actually exists by what sort of organelle, it seems that it would be easier to organize from the perspective of toxicity. What is your opinion concerning this?

Shibutani: You are correct that the experiments have not been organized in that way, and you are correct that toxicological profiling needs to be conducted.

Aoiki: Next you looked at what came after the repeated administration test I suppose.

Shibutani: Yes.

Aoki: That means that rather than the effect on an extremely specific target, we are seeing genes manifested as the result of various secondary effects. So what would you call it, when observed from the perspective of histological variation and its correspondence, it would be interesting if viewed from such a perspective. I therefore think the perspective of organization of each organelle is one way of looking at it. What do you think?

Shibutani: I think so too.

Aoki: Thank you very much.

Inoue: Any other questions? I have a question to you and also to Timothy Zacharewski. You mentioned — can I make sure — that 28 days is

enough to see the fixation of the so-called carcinogenicity, or what?

Shibutani: We do not know. From our data we cannot say anything.

Inoue: If you fix or if you define the specific profiling for non-genotoxic carcinogenicity, then can you define the fixation point by the 28th? What is your prospect?

Shibutani: If you pile up the data to make informatics, you can predict some carcinogenicity genetic potential related gene cluster, I think.

Inoue: OK, thank you very much. And Dr. Timothy Zacharewski. You said that treatment increases the variance of expression. What is your opinion for the least time interval to see the chemical effect?

Zacharewski: We have done some other studies with the uterotrophic assay for *in vivo* and we see responses within two hours and probably even earlier. If you are looking at cells as fast as you can get the RNA out, you can probably see some sort of a response.

Inoue: Thank you very much. No questions, OK? Thank you very much.