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Chapter V

HOW DNA MICROARRAY TECHNOLOGY CONTRIBUTES TO THE RETINOID EVALUATIONS

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ABSTRACT

More than a quarter of a century has passed since retinoic acid (vitamin A acid) was found to differentiate human promyelocytic leukemia cell line HL-60 into granulocytes. As the pleiotropic biological activities of retinoic acid reveals, it has been applied to treatments of many kinds of diseases, including acute promyelocytic leukemia. According to the expansion of its application, many compounds that have the equivalent effect of retinoic acid (retinoids) have been developed. Retinoids are thought to exert its biological activities through the binding to retinoic acid receptors (RARs) and/or retinoid X receptors (RXRs). Both RARs and RXRs are members of the nuclear hormone receptors, and control the expression of dozens of genes by binding to upstream controlling sequences as an RAR homodimer or an RAR-RXR heterodimer.

Candidate compounds of retinoid have been screened by the biological systems based on their activities, *i.e.* the differentiation assay with HL-60 or with other acute promyelocyte derived cell lines, or the reporter assay with exogenetically expressed RAR and/or RXR. According to the accumulation of the application of retinoids to divergent

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diseases, there appear compounds, whose activities are not able to be explained by the simple binding to RAR/RXR homo- or hetero-dimer. Transcriptome analyses, which utilize the DNA microarray and bioinformatics, were emerged in the last decade and have been successfully applied to reveal yet unforeseen aspects of a variety of biological phenomena. Since retinoids exert their effects by controlling the gene expressions, we have been trying to apply this relatively new technique to re-evaluate retinoids developed by the HL-60 cell differentiation assay-based screening. We have already shown that there were potential subclasses of retinoid synergists (RXR agonists), and that there were different effects on the modulation of PI3-kinase/Akt pathway between all-*trans* retinoic acid (natural retinoid) and Am80 (tamibarotene; a novel clinically approved retinoid). In this chapter, we describe the re-analyses of these previous works with the different bioinformatics techniques first, and report our recent achievement to characterize a widely used cell line, HL-60. Two sublines, HL-60WT (parent line) and HL-60S (retinoid sensitive line) are deposited in the Japanese Collection of Research Bioresources (JCRB). They were characterized and distinguished by the gene network analyses and genomics. We discuss how DNA microarray analyses, in combination with bioinformatics and gene network analyses, will help to evaluate retinoids and to reveal underlying mechanistic differences.

INTRODUCTION

Breitman *et al* reported that all-*trans* retinoic acid (ATRA; vitamin A acid) induced the differentiation of the human promyelocytic leukemia cell line HL-60 into granulocytes in 1980 (Breitman *et al*). More than a quarter of a century has passed since then, and the applications of ATRA as a chemotherapeutic drug have been expanding every year, especially in the fields of oncology and dermatology. One of the successful applications of ATRA is the differentiation therapy in acute promyelocytic leukemia (APL). High complete remission (CR) rates were achieved in APL with ATRA treatment in most cases. The rate of CR by ATRA treatments is much higher than that achieved by the conventional chemotherapy (Zhang *et al*). According to the increase of possibilities of wider clinical applications, a number of compounds have been developed that have the equivalent biological or clinical effects of ATRA. Such compounds are called retinoids. For example, Shudo's group has reported the novel retinoids continuously (Figure 1). Most of them show the retinoidal activities alone (*i.e.* Am80 in Figure 1). However, some of them show their activities only when they are used with, for example, Am80 (*i.e.* HX630, TZ335, PA024). They enhance the activities of Am80 synergistically, thus they are called retinoid synergists. And there also are the compounds which are antagonistic to retinoids (retinoid antagonist; *i.e.* TD550, BlBn, LE540). They have been mostly screened by the differentiation potency of HL-60 cells by morphological observation under the microscope and nitro-blue tetrazolium (NBT) reduction assay, because this differentiation system is easy to assay and its results are reliable and reproducible. Because of these facts, this system is widely used for the screening of retinoids worldwide. However, since it has also been revealed that retinoids have pleiotropic biological activities, such as cell differentiation, proliferation, apoptosis, and embryonic development, to foresee the unexpected effect of retinoids is very difficult by observing the differentiation potency of HL-60 cells alone.

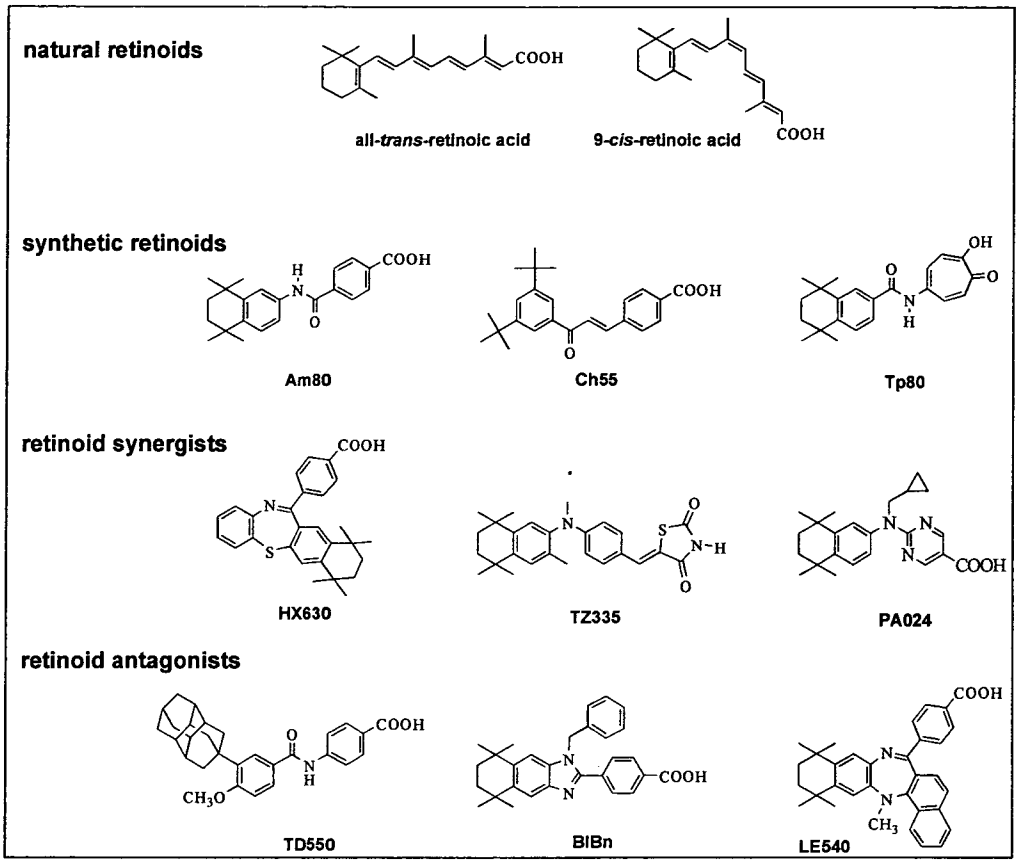

Figure 1. Structures of retinoids, retinoid synergists, and retinoid antagonists.

The fundamental mechanism underlying the retinoidal activities has been revealed by three groups independently with molecular biological analyses (Petkovich *et al*, Giguere *et al*) and biochemical analysis (Hashimoto *et al*). They found that retinoids exert their biological functions by binding to the receptor, retinoic acid receptor (RAR), which belongs to the nuclear hormone receptor superfamily. Since the first reports were published in 1987, the members of RAR and their relating receptor (retinoid X receptor; RXR) increased, and three RARs (RARα, RARβ, and RARγ) and three RXRs (RXRα, RXRβ, and RXRγ) are basically known today. Same as other nuclear hormone receptors, RARs bind to the specific DNA sequences locating upstream of the target genes as homo-dimer or hetero-dimer with RXRs and control the expressions of target genes upon the binding of retinoids to the receptors. ATRA is the natural ligand of RARs, and 9-*cis* retinoid acid (9cRA), the structural isomer of ATRA, binds to both RARs and RXRs. Mechanistic studies of retinoidal activities have revealed that retinoids bind to RARs and retinoid synergists bind solely to RXRs. Thus, retinoids are thought to be able to induce the activities same as or close to that of ATRA, and retinoid synergists exert their synergism by the simultaneous binding of retinoids to RAR and retinoid synergists to RXRs (Kagechika).

Considering that retinoids exert their biological activities through the binding to these receptors, it is reasonable to evaluate candidate compounds based on their target gene induction potency. One of case is the so-called "reporter assay". In this system, an

expression plasmid, which constitutively expresses one of the RARs, and a reporter plasmid, which harbors an RAR binding motif upstream of reporter gene such as luciferase, are cotransfected into a certain cell line, *i.e.* Hela or COS7. Then candidate compounds are added to the culture medium and their transactivation ability is evaluated by measuring the reporter gene activity. The "reporter assay" is also done easily and is popular as the screening method. The reaction is conducted in the cultured cell, however major apparatus are provided by exogenetical expressions and the selection of the combination of RAR and its binding motif is totally dependent on the assay design, thus it should be considered as a kind of *in vitro* assay system.

More than five hundred genes have been reported as retinoid regulated genes (Balmer & Blomhoff). Not all of them are direct RAR targets and even some of them might be regulated independent of the RAR pathway. Thus, if the behavior of these genes are looked at simultaneously, it might be possible to evaluate the retinoidal activity of candidate compounds as a whole. Measuring such a wide range of different gene expressions by the reporter assay system is not realistic, because it's impossible to prepare hundreds of different reporters. However, if one can use endogenous retinoid target genes as reporters, it might be possible to conduct such measurements. Moreover, because such measurements are based on the physiological cellular apparatus, one can characterize candidate compounds under a more natural environment than that of the reporter assay system. The emergence of DNA microarray technology in the last decade has made this type of analysis plausible. Tens of thousands of gene expressions are able to be measured by using DNA microarray at only one measurement. After the analysis of this robust gene expression data with the help of statistics and bioinformatics, one can visualize the effects of chemical stimuli on cells or characterize the differences of chemicals. As the DNA microarray measures the gene expression globally and the statistical and bioinformatic analyses are done without a preconceived idea, there might be a chance to find unforeseen characteristics of chemicals. The progress of the DNA microarray production process has reduced the cost and made this new technique familiar now. However, there have been only a limited number of reports, including by us (Ishida *et al* 2003, Ishida *et al* 2004), which utilized the DNA microarray technique to evaluate the retinoidal activities of chemicals so far. In our cases, we showed that there were potential subclasses of retinoid synergists and that there were different effects on the modulation of PI3-kinase/Akt pathway between ATRA and Am80 by DNA microarray analyses.

The MicroArray Quality Control (MAQS) project has reported recently that the DNA microarray data is reproducible and comparable among different formats and laboratories (Shi *et al*). This result would promote the usage of DNA microarray data for the approval process of new drugs (Frueh). Taking these circumstances into account, we will report our recent two advances as the case studies of the applications of DNA microarray technology and bioinformatic analyses for the retinoid study; 1) we re-analyzed our classification data of retinoids and retinoid synergists (Ishida *et al* 2003) by Principal Component Analysis (PCA). PCA revealed more subtle classification of these chemicals and gave clearer insights of their differences, including ATRA vs. Am80 (Ishida *et al* 2004). 2) we compared two publicly available HL-60 sublines on the basis of global gene expression data applied by gene network analysis and genomics. Both cases clearly indicated that the DNA microarray is also robust technology in the field of retinoid study.

CASE STUDY I

Re-Classification of Retinoids and Retinoid Synergists by PCA Analysis

We started this case study attempting to classify two synthetic retinoids (Am80 and Tp80) and three retinoid synergists (HX630, TZ335, and PA024) alone or in combination with Am80 into one of three classes, ATRA-like, 9cRA-like, and control-like classes by comparing gene expression patterns in HL-60 cells treated with these retinoids and/or retinoid synergists to those with ATRA or 9cRA or the vehicle (control).

To accomplish this attempt, we thought the methodology used for the molecular phenotyping of human tumors was applicable. This method, called Class Prediction Analysis, is designed to predict the class of uncharacterized samples ("test set") by analyzing the expression pattern of a group of genes ("predictors"), that were selected based on the ability to discriminate classes exist among the well characterized sample ("training set") in advance. In the application of Class Prediction Analysis for the discrimination of tumor samples (e.g. Alizadeh *et al*, West *et al*), genes which distinguish tumor samples from normal samples most in the training set were used as a marker for the diagnosis of samples in the test set. So, if we were able to select a group of genes which distinguishes ATRA treated-, 9cRA treated-, and control samples, it would be possible to classify synthetic retinoids and retinoid synergists by Class Prediction Analysis using such genes as predictors.

For this purpose, HL-60 cells were treated with ATRA, 9cRA, and the vehicle (ethanol) for 72 hours or 96 hours as a training set and harvested at the end of the culture period. Total RNA was prepared from each cell lysate, converted into double strand cDNA, and amplified and labeled with biotinylated ribonucleotide analogue by *in vitro* transcription reaction with T7 RNA polymerase to get labeled cRNA. Obtained cRNA was subjected to human genome U95A GeneChip (Affymetrix) for the measurement of global expressions of 12,626 genes. A subset of genes to set up the predictor genes for the Class Prediction Analysis was selected by testing the reproducibility between replicate measurements and the fold change induced by ATRA and 9cRA of each gene. Number of the selected genes at this stage was 333. Next, the predictor genes were identified by examining the power of genes to discriminate each class from others individually, ranking them on their power, and picking top fifty genes up. These procedures were conducted by the "Class Predictor" tool in GeneSpring (Agilent Technologies). In parallel, HL-60 cells were treated with synthetic retinoids and retinoid synergists with or without Am80. Labeled cRNA was prepared from total RNA same as the training set. Gene expressions were measured by U95A GeneChip and the obtained data were imported into GeneSpring for the classification by Class Predictor tool using the predictor genes. Expression pattern of each test sample was plotted in the fifty-dimensional space with those of test samples and the number of nearest neighbors of each test sample was statistically counted among the training set samples. Each test sample was classified into the class of training set with highest probability as the neighbors.

Mechanistically speaking, retinoids are thought to exert their activities through the binding to RARs. Retinoid synergists bind to RXRs, although their bindings alone induce no significant biological response. However, once retinoids and retinoid synergists bind to RAR-RXR heterodimer simultaneously, retinoid synergists enhance the activities of retinoids. This

synergism is illustrated in the NBT reduction assay performed at the same time when the Class Prediction Analysis was conducted (Table 1). In this case, HL-60 cells were treated retinoids and retinoid synergists with or without Am80 for forty-eight hours to clarify the effects of retinoids and/or retinoid synergists. This time period is shorter than that used routinely for the retinoid screening (ninety-six hours). In this assay, ATRA as well as retinoids induced the differentiation in less than one third of HL-60 cells. On the contrary, 9cRA and the retinoid synergists with Am80 induced the differentiation in more than half of the cells. It is noteworthy that the concentrations of Am80 and retinoid synergists assayed in combination were one tenth of that of retinoids or retinoid synergists assayed alone. As expected, retinoid synergists did not induce significant differentiation by itself. Based on this result, the expectation of the classification of these compounds is as follows: retinoids = ATRA-like class, retinoid synergists = control-like class, and retinoid synergists with Am80 = 9cRA-like class.

Table 1. Effects and predicted class of retinoids and/or retinoid synergists

retinoid treatment	NBT positive cells (%)	predicted class
control	0.0	NA*
100 nM ATRA	26.2	NA
100 nM 9cRA	96.0	NA
100 nM Am80	30.0	ATRA
100 nM TP80	8.5	ATRA
100 nM PA024	6.3	control
100 nM TZ335	8.2	control
100 nM HX630	0.0	control
10 nM Am80 + 10 nM PA024	92.1	9cRA
10 nM Am80 + 10 nM TZ335	68.8	ATRA
10 nM Am80 + 10 nM HX630	53.0	ATRA

*NA : not aplicable

As is shown in Table 1, the Class Prediction Analysis classified retinoids and retinoid synergists alone as expected. However, the Class Prediction Analysis strikingly classified retinoid synergists with Am80 cases into two different classes. PA024 with Am80 belonged to 9cRA-like class as expected. On the contrary, both HX630 and TZ335 with Am80 were classified as ATRA-like class, which was beyond the expectation. This result indicated that the Class Prediction Analysis, which was originally used for the prediction of patients' tumor status, was applicable to the classification of chemicals and clearly showed the robustness of the DNA microarray technique with the combination of the statistics and bioinformatics. When we reported this result, we used the hierarchical clustering program, "Gene Tree", for the visualization of the result. This time we used PCA for the visualization and found more subtle classifications than we got by the Class Prediction Analysis.

PCA is able to decompose the higher dimensional space into two- or three- dimensional space by finding the principal component vectors, which explain the variance of the sample. While conducting the Class Prediction Analysis, each test sample was plotted in the fifty-

dimensional space (which was equal to the number of selected predictor genes) based on the expression pattern of the predictor genes. Visualization of the scatter plot of such data is very difficult. After the analysis of expression data of test cases with those of training cases by PCA, the variances of all cases are able to be plotted in the two-dimensional space, in which significance of PCA component 1 is on the X-axis and significance of PCA component 2 is on the Y-axis. Thus, all the samples are plotted in the two-dimensional space, which makes it much easier to visualize the data. The result is shown in Figure 2. Each dot represents each retinoid and/or retinoid synergist treatment for seventy-two or ninety-six hours exposure or the control. Same was the case of the Class Prediction Analysis as in Table 1, the treatments that induced similar effects on the gene expressions were plotted close to each other and made classes. Based on the dots of control samples, ATRA samples, and 9cRA samples, dots in the fourth quadrant were the control-like class, dots in the second quadrant were ATRA like class, and dots in the third quadrant were 9cRA-like class. Samples of retinoid synergist alone were plotted close to the control samples (control-like class), and samples treated with Am80 alone plotted close to the ATRA-treated samples (ATRA-like class). As expected from the Class Prediction Analysis, samples treated with PA024 and Am80 was close to 9cRA-treated samples (9cRA-like class) and samples treated with either HX630 or TZ335 together with Am80 were plotted close to ATRA-treated samples (ATRA-like class).

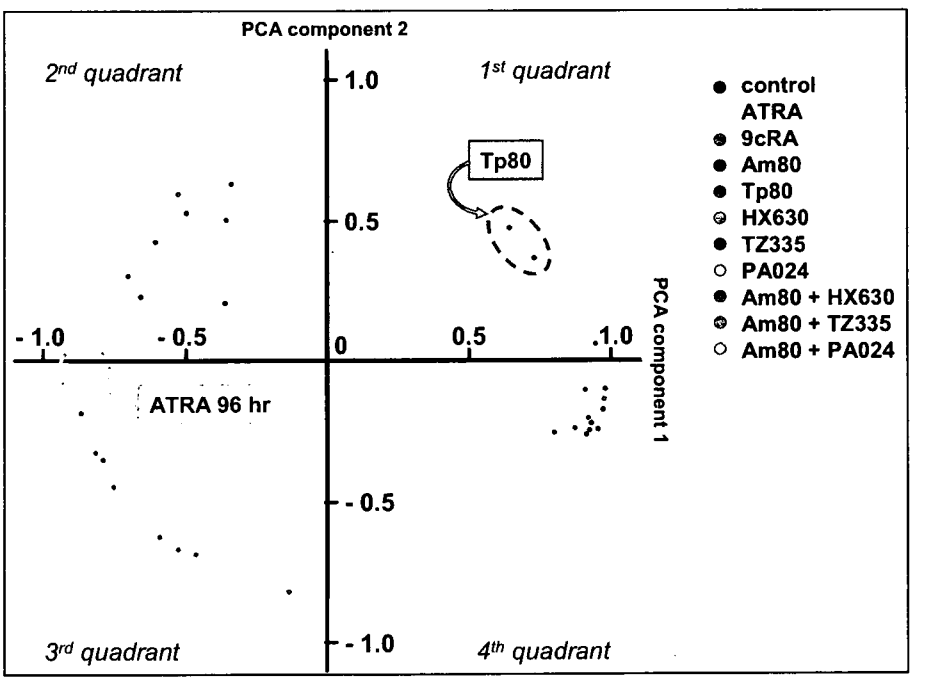

Figure 2. Principal Component Analysis of retinoid, retinoid synergists and their combinations. The results of the Class Prediction Analysis (Ishida 2003) were visualized by PCA. Each condition was plotted on the first and second principal components, which were calculated based on the expressions of fifty predictor genes selected by the Class Prediction Analysis.

Besides these expected findings, two new insights of retinoid activities were revealed by this plot. First insight was that Tp80 treated samples were plotted in the first quadrant, which

was between the control-like class (fourth quadrant) and ATRA-like class (second quadrant). As Am80 was expectedly classified as ATRA-like class, this result, that Tp80, another retinoid agonist, was plotted in between the control-like class and ATRA-like class, suggested the unique characteristics of Tp80. The Class Prediction Analysis was not able to reveal such slight differences, in which Tp80 was classified as ATRA-like class. And the Gene Tree, which makes clusters hierarchically based on the expression patterns of the fifty genes could also not distinguish Am80 and Tp80 clearly (Ishida *et al* 2003). As mentioned in the original report (Ebisawa *et al*), Tp80 and relating tropolone derivatives have partial agonistic characteristics based on the two observations; 1) these compounds induced partial induction of HL-60 cell differentiation at the concentration of 1 X 10^{-7} M, while Am80 induced more than 80 % differentiation of cells at the same condition and 2) their differentiation induction activities were enhanced by retinoid synergist such as HX630 up to the same extent obtained by Am80 and HX630. The plot (Figure 2) showed that Tp80 does not have complete retinoidal activity like Am80 and indicated its characteristics as a partial agonist. Second insight was derived from the result that one of the ATRA treated samples was plotted in the third quadrant, where samples belonging to 9cRA-like class should have been plotted. The sample plotted in this quadrant was the one treated with ATRA for ninety-six hours. This suggested that ATRA could induce the gene expression similar to 9cRA with the prolonged exposure like ninety-six hours. The fact that all of the Am80 treated samples were plotted in the second quadrant suggested that the long exposure with ATRA but not Am80 evoked the 9cRA like responses, and there were differences between ATRA and Am80. We already reported the different effects on PI3-kinase/Akt pathway between ATRA and Am80 treatments (Ishida *et al* 2004). This study was conducted based upon our experimental observations, such as these two compounds differently suppressed the growth of HL-60 cells, and clinical observations, such as the side-effects induced by ATRA were usually severer than that induced by Am80. As Am80 had been approved for the treatment of the acute promyelocytic leukemia patients who relapsed after the first treatment of ATRA, finding the differences between the two was important. We compared the genes induced by these two compounds in time dependent manner. After the filtering and clustering of the global gene expression data, we found two components of the PI3-kinase/Akt signal transduction pathway, *phosphoinositide-3-kinase, β-catalytic subunit* and *ribosomal protein S6 kinase polypeptide 1*, which are related to the regulation of cell proliferation and apoptosis, were differently modulated by the two retioids. ATRA treatment specifically suppressed the expressions of these genes, which coincided with the suppressive effects of ATRA on the HL-60 cell proliferation. This study was done based on the biological and clinical observations, however the PCA analysis done this time (Figure 2) clearly suggested the existence of such types of differences between ATRA and Am80.

In this case study, we analyzed our classification data of retinoids and retinoid synergists from a different point of view. The Class Prediction Analysis was powerful enough to classify retinoids and/or retinoid synergists into several groups and to find unexpected classification of the samples treated with retinoid synergists (HX630, TZ335) with Am80 based on only one measurement of each sample. According to our previous report (Ishida *et al* 2003), it would be possible to do such classification if we measured the apoptosis and the cell growth suppression induced by these compounds and compared them to each other.

However, such experiments are tedious to do, and to find what kind of molecular or cellular biological assay is adequate for the discrimination of the compounds is usually not easy. Actually, we selected these assays because we expected that they would give the different results among the samples treated with retinoid synergists and Am80 by the careful analyses of the gene expression data obtained by DNA microarray analysis. Moreover, our new analyses described in this case study clearly revealed the existence of more subtle differences among these retinoids and retinoid synergists. These results clearly indicated the combination of the DNA microarray technology and bioinformatics is really robust and possible to reveal unforeseen characteristics of chemicals.

CASE STUDY II

Molecular Characterization of the Two HL-60 Sublines

HL-60 cells are widely used for the studies of retinoids and other varieties of research subjects, and several sublines of HL-60 cells are known. We are interested in the differences between the one we are using and the others, because there is no such comparison reported though the existence of the differences among the sublines has been recognized widely. And we expected such a comparison might become a model to study the differences existing among the APL patients. HL-60 cells we are using are deposited as HL-60S in Japanese Collection of Research Bioresources Cell Bank. There is another HL-60 cell line deposited in the same cell bank, which is thought to be closer to the original HL-60 cells. We considered that to compare our cells, HL-60S, to this cell line, designated as HL-60WT hereafter, is a good case for the study of bioinformatical analyses, because the origin of these cells were the same, thus their biological fundamentals were expected to be similar. This closeness of the samples to be compared is important to conduct the global gene expression comparison, because the comparison of the samples whose expressions were completely different is hard to interpret. In this case study, we analyzed the differences of these two HL-60 sublines by gene network analysis and genomics and supported the results by conventional cellular and molecular biological experiments.

Characterization of Differences between HL-60WT and HL-60S by Gene Network Analyses

To characterize HL-60WT cells and HL-60S cells, genes differentially expressed in HL-60WT and HL-60S were analyzed by DNA microarray. Global gene expressions in six total RNA preparations from each subline were compared by human genome U95 GeneChip (Affymetrix), and genes which were differently expressed with statistical significance were selected. The number of such genes was 1,598. Then, the genes whose expressions were different more than three fold between them were selected to focus the highly differentially expressed ones. There were 141 such genes left among the 1,598 genes. We tried to find an unforeseen relationship which is able to characterize the differences of these two sublines

with the help of bioinformatics. For this purpose, we put the gene list which contains all 141 genes into Ingenuity Pathway Analysis (IPA, Ingenuity Systems). IPA is a tool that suggests the network most relevant to the experimental data set together with its biological functions, based on the knowledge database gathered by curating the published articles manually at Ingenuity Systems. One of the networks which IPA suggested is depicted in Figure 3 and the genes involved in this network are listed in Table 2. The biological functions of this network predicted by IPA were "cellular movement", "hematological system development and function", and "immune response". We were interested in the function, "hematological system development and function", and thus further analyzed the genes involved in this network. We found that four genes whose expressions were lower in HL-60S (symbols with green color in Figure 3) were encoding the members of primary granule proteins or its candidates (Theilgaard-Monch *et al*) (Table 2). Besides the genes in Table 2, we also found *alpha-1-defensin*, *alpha-4-defensin*, *azurocidin 1*, and *cystatin F* were involved among the

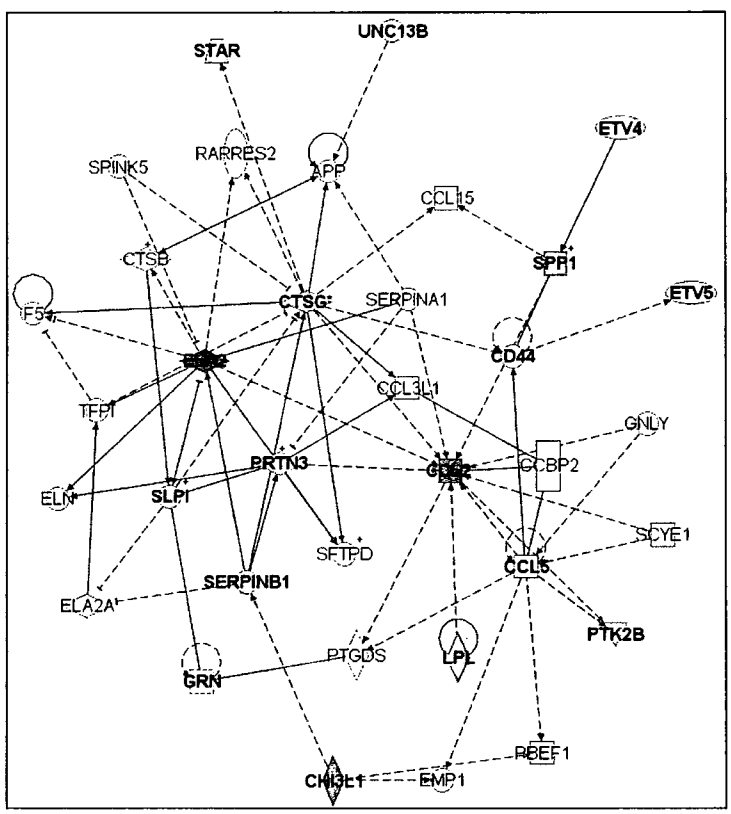

Figure 3. The network extracted by Ingenuity Pathway Analysis among the genes differentially expressed in HL-60WT cells and HL-60S cells. The list of genes whose expressions were different more than three fold between HL-60WT cells and HL-60S cells were analyzed by Ingenuity Pathway Analysis (Ingenuity Systems, Inc.). The symbols of the genes whose expressions were higher in HL-60S cells than HL-60WT cells were colored with red, and those that were lower in HL-60S cells than HL-60WT cells were colored with green. The symbols without color were the genes added by Ingenuity Pathway Analysis to produce this network. Lines in the network indicated the direct interactions and the dotted lines indicated the indirect interactions.

Table 2. List of genes involved in the network 1

Acc. No.	gene title	gene symbol	chromosomal location	signal ratio log_2(S/WT)	primary granule
M28225	chemokine (C-C motif) ligand 2	CCL2	chr17q11.2-q21.1	6.3	
M15856	lipoprotein lipase	LPL	chr8p22	4.45	
AF052124	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	chr4q21-q25	4.04	
M21121	chemokine (C-C motif) ligand 5	CCL5	chr17q11.2-q12	2.28	
X96381	ets variant gene 5 (ets-related	ETV5	chr3q28	1.94	
D12765	ets variant gene 4 (E1A enhancer binding protein, E1AF)	ETV4	chr17q21	1.72	
M59040	CD44 antigen (homing function and Indian blood group system)	CD44	chr11p13	1.66	
U02020	pre-B-cell colony enhancing factor 1	PBEF1	chr7q22.3	0.77	
U43522	PTK2B protein tyrosine kinase 2 beta	PTK2B	chr8p21.1	-1.6	
AF055008	granulin	GRN	chr17q21.32	-1.94	
AF020202	unc-13 homolog B (C. elegans)	UNC13B	chr9p12-p11	-2.94	
U17280	steroidogenic acute regulator	STAR	chr8p11.2	-3.47	
X55668	proteinase 3 (serine proteinase, neutrophil, Wegener granulomatosis	PRTN3	chr19p13.3	-3.47	yes
M93056	serpin peptidase inhibitor, clade B (ovalbumin), member 1	SERPINB1	chr6p25	-3.84	yes
X04470	secretory leukocyte peptidase inhibitor	SLPI	chr20q12	-4.06	
M16117	cathepsin G	CTSG	chr14q11.2	-4.64	yes
Y08374	chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1	chr1q32.1	-7.77	
M34379	elastase 2, neutrophil	ELA2	chr19p13.3	-10.64	yes

selected genes, whose expressions were lower in HL-60S cells. In total, there were eight genes found among 141 genes. The number of primary granule proteins or its candidates listed by Theilgaard-Monch was eleven. The incidence that eight out of eleven genes were found in the list was quite high. Granule proteins are sequentially synthesized according to the progress of the granulocytic differentiation of promyelocyte accompanying the formation of primary, secondary, and tertiary granules. Theilgaard-Monch *et al.* analyzed the transcription program of terminal granulocyte differentiation by DNA microarray, and reported the genes coding the primary granule proteins were expressed solely in promyelocyte, and their expressions were downregulated during the later differentiation stages. The clearly different expression pattern of the genes encoding the primary granule proteins between HL-60WT cells and HL-60S cells indicated that these two sublines were not at the same stage of the granulocytic differentiation process. HL-60WT cells should be at the promyelocyte stage, because the primary granule relating genes were expressed in these cells. We did not know if HL-60S cells were at earlier or later stage than at the promyelocyte stage, because the expressions of primary granule relating protein genes were lower at either stage. However, if HL-60S cells were at the later stage of granulocytic differentiation process, they tend to differentiate more than HL-60WT and vice versa. To confirm this hypothesis, HL-60WT cells and HL-60S cells were treated with seven retinoids for ninety-six hours at different concentrations serially. Differentiation induced by retinoids was measured by NBT reduction assays in triplicate and the result is depicted in Figure 4. Responses of HL-60WT and HL-60S to retinoids were strikingly different. It was clearly shown that HL-60S cells were much more sensitive to all the retinoids tested than HL-60WT cells. HL-60S cells were almost thousand times more sensitive than HL-60WT cells in all cases. This result supported

the idea that HL-60S cells were at the stage a little bit closer to granulocytes than HL-60S cells, to which HL-60 cells are terminally differentiated by retinoids. If this were the case, HL-60S cells would have lost the differentiation potency towards other lineage, because the previous reports indicated that HL-60 cells have the pluripotency of the differentiation, *e.g.* they differentiate into granulocytes by retinoids, into macrophage by phorbol esters and so on (reviewed in Collins). Our analysis showed that HL-60S cells were less sensitive to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) than HL-60WT cells (Ishida *et al.* unpublished results). These results might support the idea that HL-60WT cells and HL-60S cells are at the different stages of granulocyte maturation.

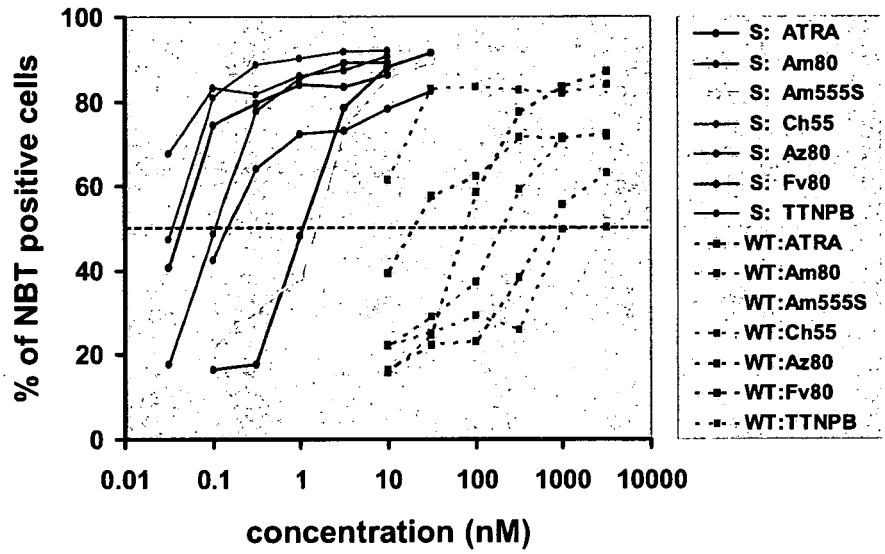

Figure 4. The different responses to a variety of retinoids between HL-60WT and HL-60S. HL-60WT cells or HL-60S cells were exposed to the serial dilutions of a variety of retinoids for 96 hours. Percentage of the differentiated cells was determined by NBT reduction assay. All assays were done in triplicate.

Characterization of Differences between HL-60WT and HL-60S by Genomics

There was no indication which was able to explain the mechanism underlying the different responses to the retinoids of the two sublines. Since the major receptor for the retinoids in HL-60 cells is RARα, we first checked its cDNA sequences expressed in both cells and found no mutation in their ORFs (data not shown). Next, we analyzed the expression levels of RARα mRNA and its heterodimer partner RXRα mRNA in both cells. The relative expression levels of both genes in either subline were measured with the five independently prepared total RNAs and the averaged values of them were depicted in Figure 5 after the normalization by those of corresponding β-actin gene expressions. The expression levels of both genes did not differ much in both sublines. Thus we analyzed again the DNA microarray data by expanding the gene list to include all 1,598 genes. To visualize the fine

meaning of this huge gene list, we plotted expression data of these 1,598 genes according to their chromosome locations. We found that plotted genes tended to be localized on several specific regions of certain chromosomes. The differences of expressions in HL-60WT vs. HL-60S were around two fold in the most gene cases. It is known that the chromosome copy number and the gene expression of corresponding region correlate with each other (Pollack *et al*). We compared the gene expression patterns to the result of the chromosome copy number measurements by array-based Comparative Genomic Hybridization (aCGH), and found these expression differences correlated well to the differences of the copy number of the corresponding region of chromosomes (data are available on request). One of the interesting regions was that of chromosome 5 (Figure 6). Two genes, Cdk7 and cyclin H, important for the control of RARα functions, locate on this chromosome. Cdk7 and cyclin H forms a complex (CAK kinase) with the assembly factor MAT1. CAK kinase is a component of the general transcription factor TFIIH, which has been shown to associate with RARα and to phosphorylate it at Ser-77 (Rochette-Egly *et al*). This phosphorylation controls the transcription activities (Rochette-Egly *et al*) and the stability of RARα protein (Kopf *et al*). Kopf *et al* indicated that the phosphorylation of RARα at Ser-77 inhibited the degradation of RARα through the ubiquitin-proteasome pathway. Higher expressions of Cdk7 and cyclin H in HL-60S should have produced more CAK kinase in HL-60S than in HL-60WT. These incidents were interesting because both Cdk7 and cyclin H were expressed 2.9 fold higher in HL-60S than in HL-60WT by DNA microarray measurements. These differences were confirmed by Western blot analyses (Figure 7). Protein level of MAT-1, another component of CAK kinase, was also measured at the same time, and found higher expression in HL-60S cells. These facts suggested that more CAK complex exists in HL-60S cells than in HL-60WT cells and phosphorylates RARα at higher degree, which protects its degradation by ubiquitin-proteasome pathway. Considering these facts together, RARα protein level was

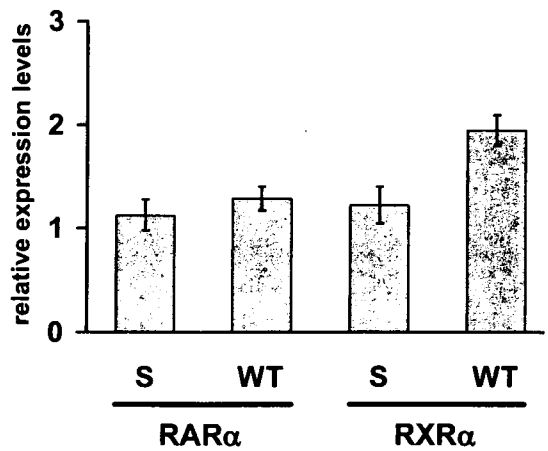

Figure 5. Expressions of RARα and RXRα mRNA in HL-60WT cells and HL-60S cells. Expression of each gene mRNA was measured by real-time PCR with the specific probe and depicted after the normalization by the expression level of β-actin gene. All measurements were done with the five independently prepared total RNAs in duplicate. The averaged values were shown with the error bars indicating the standard deviation of the five measurements.

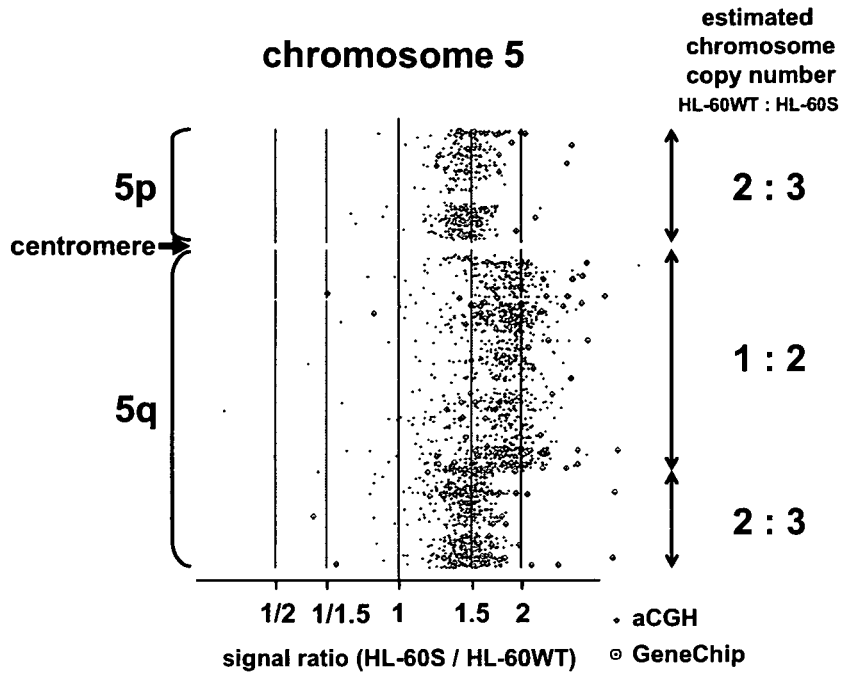

Figure 6. Comparison of chromosome copy number and gene expression on chromosome 5. Local chromosome copy number ratio (HL-60S / HL-60WT: blue dot) and gene expression signal ratio (HL-60S / HL-60WT: red dot) were plotted according to the locations of the probes on chromosome 5. Local chromosome copy number ratio was measured by Human Genome CGH 44B array-based Comparative Genomic Hybridization array (Agilent Technologies). Gene expression was measured by human genome U95A GeneChip (Affymetrix). Locations of probes on the chromosome 5 were determined based on the information provided by the manufacturer.

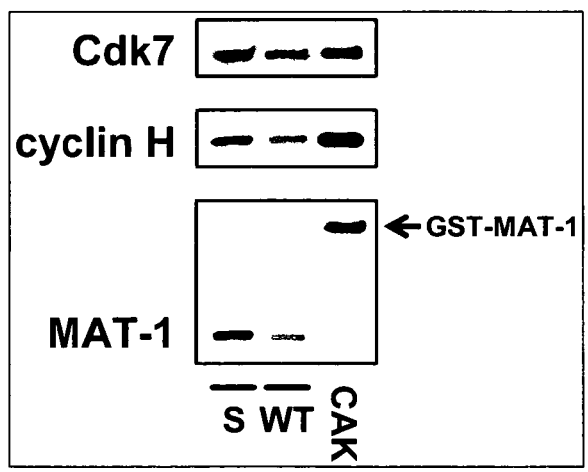

Figure 7. Protein expression levels of Cdk7, cyclin H, and MAT-1, that are the component of CAK complex. Baculovirus expressed Cdk7 (6His-tagged full-length), cyclin H (full-length), and MAT-1 (GST-tagged full-length) were blotted on the same membrane as the controls (CAK).

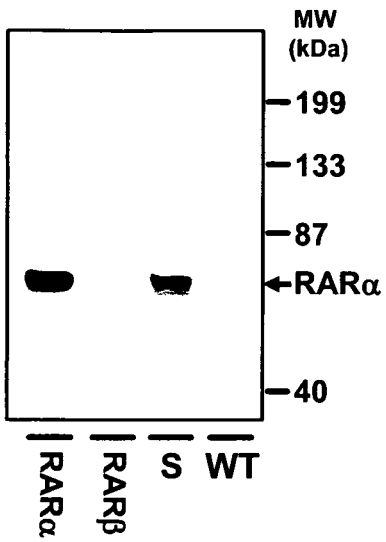

Figure 8. Protein expression level of RARα in HL-60WT and HL-60S cells. Recombinant full-length RARα and RARβ expressed in the baculovirus system were blotted on the same membrane to confirm the specificity of the antibody.

expected to be higher in HL-60S cells than HL-60WT cells. Thus, we measured the protein expression level of RARα in the two sublines and, as expected, found magnificent differences (Figure 8). The facts that RNA levels of RARα in both cells were almost the same (Figure 5) and that the protein levels were different (Figure 8) were indicating that the RARα protein degradation by proteasome is differently controlled in the two cell lines because of their different CAK kinase activities. This difference might be one of the major causes to explain the different responses to retinoids of the two sublines. If these were the case, when proteasome activity in HL-60WT cells is inhibited by its inhibitor, the degradation of RARα is reduced and HL-60WT cells should become more sensitive to retinoid induced differentiation. To prove this hypothesis, HL-60WT cells were pretreated with MG132, a proteasome specific inhibitor, overnight, washed, and then exposed to three retinoids, Am80, Ch55, and TTNPB, at different concentrations for four days, and differentiated cells were counted by NBT reduction assay. As shown in Figure 9, the treatment of MG132 significantly enhanced the induction of HL-60WT cell differentiation by retinoids. Such enhancement was not observed when HL-60WT cells were pretreated with z-VAD-fmk, a caspase inhibitor. These results strongly suggested that the lower expression level of Cdk7 and cyclin H in HL-60WT leads the lower phosphorylation status of RARα, which causes the more degradation of RARα. As aresult, HL-60WT has a smaller amount of RARα and it requires a higher concentration of retinoid to get the same extent of the differentiation. These mechanistic explanations look plausible generally. However, when we took a closer look at the results of proteasome inhibitor experiments (Figure 9), we found effects of the inhibitor were not uniform to the three retinoids. The pretreatments of HL-60WT cells with MG132 recovered the differentiation potency well in the cases of Am80 and Ch55. On the contrary, relatively lower differentiation was achieved when pretreated cells were exposed to TTNPB. TTNPB showed the strongest differentiation inducing activity in HL-60S cells, however not

so active in HL60WT cells (Figure 4). The inhibition of proteasome degradation pathway should have

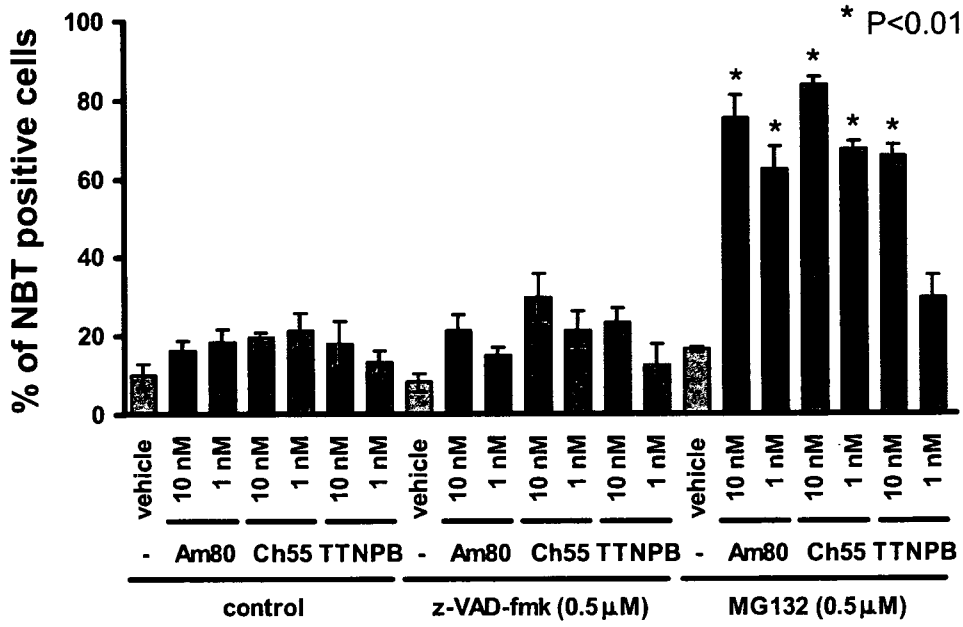

Figure 9. Effects of proteasome inhibitor MG132 on the differentiation of HL-60WT cells treated with three retinoids. HL-60WT cells were pretreated with MG132 overnight, then cultured with Am80, Ch55, or TTNPB for 96 hours and the differentiated cells were measured by NBT reduction assay. Caspase inhibitor, z-VAD-fmk was used as the negative control. All the measurements were done in triplicate. The averaged values were shown with the error bars indicating the standard deviation of the three measurements. * indicates the percentage of NBT positive cells was statistically different ($P < 0.01$) comparing to the corresponding control.

elevated the RARα protein level in HL-60WT cells, which was enough for HL-60WT cells to respond to either Am80 or Ch55, but not enough to TTNPB. According to Rochette-Egly *et al*, phosphorylation of Ser-77 by CAK kinase also controls the transcription activation abilities of RARα (Rochette-Egly *et al*). The treatment of proteasome inhibitor should have restored only the RARα protein level. It should have changed nothing on the phosphorylation status of restored RARα protein. It might be possible that the ligand binding activities of RARα to individual retinoids were modulated by the phosphorylation status of Ser-77 differently. This is a very interesting point to be elucidated in the future.

CONCLUSION

Over a decade has passed since the introduction of DNA microarray technology into the molecular biological field. The application fields of this technology are expanding year by year. The MicroArray Quality Control (MAQC) consortium has reported its reproducibility and compatibility among different platforms and different laboratories recently (Shi *et al*).

The series of articles reported by MAQS consortium should be thought to be a milestone for the DNA microarray technology, because it clearly showed the usefulness of this technology not only in the research fields but also for the drug development and approval. More and more will this technology be used in drug-development and regulatory decision making in the near future (Frueh). On the other hand, only a few articles were reported that apply the DNA microarray technology to the development and evaluation of retinoids. We have reported how this technology is useful for the evaluations of retinoids (Ishida *et al* 2003, Ishida *et al* 2004). In our previous reports, we analyzed retinoids and retinoid synergists by the bioinformatics, including the Class Prediction Analysis, with the DNA microarray data of retinoids and/or retinoid synergists, and were able to reveal the characteristics of retinoids and retinoid synergist that had been difficult to find by the conventional cellular and molecular biological analyses. We have extended this study in Case Study I in this chapter by re-analyzing the data from the different point of view. This time we employed the Principal Component Analysis, which is suitable to reveal the fine structures of complex data set through the decomposition of the higher dimensional data space into two- or three-dimensional spaces by finding the principal component vectors. This analysis was able to reproduce the classification of the retinoids and the retinoid synergists as reported before, and moreover was able to find more precise characteristics of ATRA and Tp80 (Figure 2). In Case Study II, we analyzed the differences of global gene expressions existing in the two publicly available HL-60 sublines, HL-60WT and HL-60S, with the help of the gene network analysis by IPA (Ingenuity Systems) and genomics. One of the gene networks focused on by the IPA analysis (Figure 3) was relating "hematological system development and function". Actually, eight out of eleven primary granule protein coding genes, whose expressions were strictly controlled during the granulocyte development stage, were found among the genes differently expressed (> 3 fold) in these two sublines. Expressions of all the eight genes were higher in HL-60WT cells than in HL-60S cells, suggesting that these two sublines were at the different stage of granulocyte maturation process. The differentiation assays with retinoids, which induce differentiation into granulocyte lineage, and with phorbol ester, which induces the differentiation into macrophage lineage, were indicating that HL-60WT cells were closer to promyelocytes and HL-60S cells were closer to granulocytes. One of the possible causes of different sensitivities to retinoids shown in these differentiation assays, *i.e.* HL-60S cells were a thousand times more sensitive to all seven retinoids tested than HL-60WT cells, was revealed by analyzing the DNA microarray data with the assistance of genomics. All the genes expressed differently with statistical significance were plotted according to the corresponding chromosome location. They were not randomly distributed, on the contrary, they were located on the certain specific chromosome regions. This expression pattern was precisely correlated to the chromosome copy number obtained by array-based Comparative Genomic Hybridization. We found Cdk7 and cyclin H, which are the member of CAK complex, among such regions on chromosome 5 (Figure 6). Cdk7 and cyclin H were expressed more than two fold higher in HL-60S cells than in HL-60WT cells, together with MAT-1, another component of CAK complex (Figure 7). CAK complex phosphorylates RARα, which inhibits the degradation by proteasome and changes its transcriptional activation activity. The protein level of RARα in HL-60S cells were much higher than in HL-60WT cells (Figure 8), indicating that the different phosphorylation of RARα by CAK complex would induce the difference of

degradation by proteasome. The results that treatment HL-60WT cells with the proteasome inhibitor restored the responsibilities to retinoids supported this idea (Figure 9). The different phosphorylation status due to the different amount of CAK complex in either sublines might also induce the different responses to the individual retinoids (see the different restoration magnitude of Am80, Ch55 vs. TTNPB in Figure 9). Although there are still several points left for further analyses, Case Study II showed the incorporation of DNA microarray data analysis with the gene network analysis and genomics was able to help to understand the molecular mechanism which induced the differences of the two HL-60 sublines.

DNA microarray has become one of the daily analytical tools for the molecular or cellular biologists these days, as the cost of the DNA microarray has decreased. And according to the expansion of its use, the procedure of analysis, from the RNA preparation and the probe labeling to the scanning of DNA microarray, has been quite established. Once a sufficient amount of DNA microarrays were in hand, the measurements of global gene expressions would be easily done these days. However, DNA microarray analysis produces a huge amount of data, which most molecular or cellular biologists have never dealt with. As often happens, scientists get lost when they are handed such a huge amount of data. Because of such experiences, DNA microarray analyses had not become popular in earlier days. However, as shown in the two Case Studies reported in this chapter, DNA microarray analysis is useful and robust enough to produce new and unforeseen findings, which were difficult to obtain by other conventional analyses. Both case studies were conducted with careful experimental planning and with persevering analyses using bioinformatics, such as Class Prediction Analysis, PCA or gene network analysis, and genomics. More and more new analysis tools are continuously introduced for the DNA microarray data treatment. We believe this type of well organized study with the help of bioinformatics would contribute to the development of novel retinoids and to the understanding of their biological characteristics.

ACKNOWLEDGEMENTS

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MATERIALS AND METHODS

Retinoids

ATRA were purchased from Sigma Chemicals Co. (St.Louis, MO). Am80, Am555S, Ch55, Az80, Fv80, and TTNPB were synthesized at The University of Tokyo. All retinoids were dissolved in ethanol.