

future. In the following overview, we will try to focus on the current role of genomic techniques in translational cancer research. In the first part of this paper, we will summarize the most important published studies for frequent tumor entities. Furthermore, the individual methods and their clinical relevance will be critically reviewed. In the second part of the manuscript, we will deal with issues of reliability and reproducibility of reported genomic array data.

BREAST CANCER

In 2000, a Norwegian – American Cooperative Group performed an analysis of breast cancer cell lines and tumor tissue based on DNA microarrays [Perou 2000]. They could classify the tumors into subtypes distinguished by their different gene expression profiles using the “hierarchical clustering” methodology based on 1753 genes independent from the histological classification. The tumors were subdivided into an ER+(estrogen-receptor-positive)/luminal like, a basal-epithelium-like group, an ERB-B2-(erythroblastic leukemia viral oncogene homolog 2) group and a normal-breast-like group. The same group published within the following year, a further investigation on this subject [Sorlie 2001]. This time, in a larger number of tumors, at least six subtypes could be differentiated on the basis of their gene expression patterns using an intrinsic set of 457 genes (Table 1). The previous (ER+)/luminal-like group could be subdivided into three prognostic groups. In addition, a list of

256 genes related to clinical outcome was developed using a special supervised data-analysis (SAM: significance analysis of microarray, this technique will be explained more detailed in the second part of this article) derived from a clinical data set of 76 carcinomas, in which sufficient data were available. This 256-gene-“predictor” was correlated with clinical outcome in a cohort of 49 patients with locally-advanced breast cancer uniformly treated in a prospective clinical investigation. The basal-like subtype was associated with poorer prognosis regarding overall as well as relapse-free survival (Table 1). Interestingly, significant differences could be observed in the clinical outcome between two different estrogen-receptor-positive groups. West and co-workers could demonstrate the potential usefulness of DNA microarray analysis to discriminate different breast cancer patients on the basis of their ER (estrogen-receptor) status (Table 2) [West 2001]. A 100-gene “predictor” to estimate estrogen receptor status was developed analyzing a set of 38 samples (the “training set”). Then in a second step, the “predictor” was validated in a set of nine independent tissue samples (the “test set”) (Table 3). In some samples, the “predictor” could adequately predict clinical ER-status. Five samples with conflicting results of immunohistochemistry and immunoblotting data regarding the ER-status were included within this “test set”. In some of these samples, the predictive probability of the “predictor” was found to be lower. This could reflect the heterogeneity of expression

Table 1. Breast Cancer Microarray Classification by Sorlie - Based on a Intrinsic Set of 457 Genes

Correlation of microarray classification with overall survival prognosis (Sorlie 2001)	
(n=49; p<0,01)	
Subtype	Prognosis
ER+/luminal like Typ A	good
ER+/luminal like Typ B	intermediate
ER+/luminal like Typ C	intermediate
Basal like	poor
ERB-B2	poor
Normal like	intermediate

The estrogen receptor positive ER+/luminal like group is subdivided into three subtypes. Correlation with overall survival reveals a poor prognosis for the Basal like and ERB-B2 group. Interestingly different prognosis for patients was found within the three estrogen receptor positive (ER+) groups.

Table 2. Class Prediction Studies Regarding ER-Status in Breast Cancer

Author	Patients	Technique	Statistical method	Number of genes of predictor	Training set	Test set	correct prediction (%)
West (2001)	48	cDNA microarray	Bayesian regression	100	38	9	100
Grubberger (2001)	58	cDNA microarray	Artificial Neural Network	100	47	11	100

“Predictors” for estrogen receptor status based on microarray data were established by to different groups in 2001. Both “predictors” include 100 genes. After developing the “predictor” in a set of samples and corresponding clinical data (Training-set) both groups could validate their “predictor” in independent set of samples and clinical data (Test set) with high accuracy.

¹ **Hierarchical clustering:** A hierarchical clustering is a sequence of partitions in which each partition is nested into the next partition in the sequence

within the individual tumor. Furthermore, impressive data regarding an analysis of estrogen-receptor status by calculating gene expression profiles were published by a

Table 3. Top 5 Ranked Genes for Prediction ER-status

Rank	West 2001	gruvberger 2001
1	Trefoil factor 1 (ps2)	Estrogen Receptor 1
2	Estrogen receptor	Trefoil factor 3
3	Cytochrome P450	GATA Binding protein 3
4	Trefoil factor 3	ESTs
5	Estrogen like growth factor	Calgranulin A

West and Gruvberger established in 2001 independently "predictors" for estrogen-receptor status in breast cancer based on microarray data. The five genes with strongest correlation of expression and ER-status of the 100 gene "predictors" by West and Gruvberger are listed in this table. Both "predictors" show similarities. Beside the estrogen receptor itself the trefoil factor 3 is found within the five top ranked genes in both studies.

Swedish group within the same year (Table 2) [Gruvberger 2001]. A 100-gene "predictor" for ER status was developed in a "trainings set" of 47 tumors based on microarray data using "artificial neural network"² (Table 3). The "predictor" was validated in 11 independent samples of a "test set". All 11 tumors could be classified correctly by this 100-gene marker. Interestingly, even without the top level discriminator genes, including ER itself, the "artificial neural network" could adequately predict ER-status.

Adjuvant cancer therapy is well-established in the treatment of breast cancer. Chemotherapy or hormonal therapy is able to reduce the risk of disease dissemination in one-third of the patients, but a large number of patients will have already been cured without the application of adjuvant therapy. Taking into account that the application of adjuvant chemotherapy carries a well-defined morbidity and mortality risk, the proper selection of patients with a clear and established benefit from adjuvant chemotherapy would be extremely helpful. Based on the hypothesis that patients with a poor prognosis following surgery would mostly benefit from adjuvant therapy, a Dutch group performed a translational study testing the predictive impact of DNA Microarray data on overall survival prognosis in young females with primary-lymph-node-negative breast cancer [van 't Veer 2002]. In this study, tumor tissue from 117 young patients with primary lymph-node-negative breast cancer was analyzed by DNA microarray technique. A 70-

gene prognosis "predictor" ("poor prognosis signature") for patients with a short interval to the development of distant metastasis was established by supervised classification of the gene expression profiling. This "poor prognosis signature" included genes regulating cell cycle, invasion, metastasis and angiogenesis. A second correlative study was performed by the same group to confirm the predictive power of this 70-gene prognostic marker in a larger and less homogeneous group of patients [van de Vijver 2002]. A series of 295 consecutive patients with stage I and II breast cancer who underwent surgery, were included into this study. All patients had to be younger than 53 years. In this investigation, patients with lymph-node-negative (151 pts) and lymph-node-positive (144 pts) disease were analyzed. A group of 180 patients with "poor prognosis signature" could be separated from 115 patients with "good-prognosis-signature" (Table 4). The overall 10-year survival rate was found to be 54.6 percent within the "poor" and 94.5 percent in the "good prognosis group". The probability of remaining free from distant metastases within 10 years was found to be 50.6 percent in the group with "poor-prognosis-signature" and 85.2 percent in the group with "good-prognosis-signature". In comparison to the "good prognosis group", the estimated hazard ratio for developing distant metastasis in the group with "poor-prognosis-signature" was 5.1 (95% confidence interval, 2.9 to 9.0; P <0.001). This microarray based prognosis profile was identified as a strong inde-

Table 4. Overall Survival and Distant Metastasis Free Survival Probability According to Prognosis Signature (Van't Vijver 2002)

Group	No. of patients	Overall survival (%)		free of distant metastasis (%)	
		5YR	10YR	5YR	10YR
Poor prognosis signature	180	74.1	54.6	60.5	50.6
Good prognosis signature	115	97.4	94.5	94.7	85.2

A 70 gene prognostic marker ("predictor") was tested by van't Vijver in a series of 295 consecutive patients with stage I and II breast cancer who underwent surgery. They could distinguish 180 patients with poor prognosis (Poor prognosis signature) from 115 patients with good prognosis (Good prognosis signature) regarding to overall survival and distant metastasis-free survival.

² **Artificial neural network:** A network of many very simple processors "units" or "neurons", each possibly having a (small amount of) local memory. The units are connected by unidirectional communication channels ("connections", which carry numeric (as opposed to symbolic) data. The units operate only on their local data and on the inputs they receive via the connections.

pendent factor in predicting disease outcome. Interestingly, the prognostic profile did not depend on lymph-node status, well-known to be one of the classical prognostic factors. On

the other hand, the positive-estrogen-receptor status, the other classical predictor, was strongly correlated with a "good prognosis signature" profile. In spite of these impressive results, there has been important criticism towards these findings: An insufficient analysis of intratumoral representativeness of the investigated tumor samples and the measurement of tumor size is critically discussed [Kunkler 2003, Kopans 2003]. However, this pioneer investigation could prove, that a prediction of clinical outcome based on microarray data may in principle be possible.

LUNG CANCER

Lung cancer is still the leading cause of cancer-related deaths in the industrialized world [Jemal 2002]. Physicians treating patients with this disease often face difficult decisions to be made within all clinical disease stages. For example, the issue of adjuvant chemotherapy following complete resection is not conclusively solved with conflicting results published [Scagliotti 2003; International Adjuvant Lung Cancer Trial Cooperative Group 2003, LeChevalier 2003]. A reliable method to predict patient prognosis following surgical treatment of early stage lung cancer could be most helpful to estimate the benefit of adjuvant chemotherapy within the individual patient. Classical histopathology examination is definitely insufficient for this decision making. Beer published in 2002, a translational research study correlating gene expression profile data with overall survival in patients with early stages lung adenocarcinoma after surgery [Beer 2002]. Tumor tissue of 86 primary adenocarcinomas of the lung, including 67 stage I and 19 stage III tumors, was investigated. As an internal control, 10 non-neoplastic lung tissue samples were analyzed. Using "hierarchical clustering" methodology, three different patient

groups with association of cluster and stage ($P=0,030$) or tumor differentiation ($P=0,01$) could be differentiated. All ten non-neoplastic tissue samples could be clustered within the same patient group. In addition, the authors could derive a 50-gene-risk-index by identifying survival related genes using univariate Cox analysis (Table 5). When calculating the 50-gene-risk-index and grouping the patients based on the results, significant differences in overall survival between the individual groups could be identified. Grouped "high-risk" and "low-risk" stage I adenocarcinomas differed significantly between each other ($p=0.003$), whereas low and high risk stage III tumors did not. The robustness of the 50-gene-risk-index in predicting overall survival in early stage lung adenocarcinoma was tested in an independent data set of 84 tumor samples and related to overall survival. A high and a low risk group could be separated ($P=0.003$). Interestingly, among the 62 stage I tumors in this analyzed population, high and low risk groups could be observed differing significantly ($P=0.006$) in their overall survival duration. In conclusion, the authors postulated, that the identification of a high risk group within stage I lung cancer patients would lead to the consideration of a postoperative adjuvant intervention for this group. In 2003, a Japanese group published another important cDNA microarray based study regarding lung cancer [Kikuchi 2003]. A set of 37 tumor tissue samples of non-small cell lung cancer patients were analyzed. To avoid investigation on irrelevant tissue, they only analyzed cancer cells selected by laser capture microdissection. The most frequent NSCLC-subtypes adenocarcinoma and squamous cell carcinoma could be easily distinguished by applying a clustering algorithm to the expression data results. To explore gene expression in post-chemotherapeutic lung cancer tissue, a small pilot-study

Table 5. Selected Examples of the 50 Gene Risk Index of Beer (2002)

Gene name	P (normal versus tumor-t-test)	Coefficient β	Comment
Caspase 4	0,56	0,0022	apoptosis-related cysteine protease
LAMB 1	0,14	0,0027	Laminin β 1
BMP 2	0,54	0,0044	Bone morphogenetic protein 2
CDC 6	1,31E-05	0,0124	cell division cycle 6
Serpine 1	2,89E-03	0,0008	Serine (or cysteine) proteinase inhibitor (clade E)
ERBB2	0,04	0,0013	v-erb-b2 (Receptor)
PDE7A	0,12	- 0,0187	Phosphodiesterase 7 ^a
PLGL	0,04	- 0,0011	Plasminogen like

The 50-gene-risk index was validated in an independent set of 84 tumor samples and corresponding A positive coefficient β is associated with poorer outcome. A 50 gene risk index ("predictor") for lung adenocarcinomas was established in a microarray based correlation study (Beer 2002). Selected examples for interesting genes of this risk index were shown in this table. The coefficient β shows the relation of gene expression and outcome. A positive coefficient β is associated with poorer outcome. This 50 survival data. Among the 62 stage I tumors including this set they could identify a high and a low risk group which differ significant in survival.

^a Le Chevalier, T. for the IALT Investigators (2003): Results of the Randomized International Adjuvant Lung Cancer Trial (IALT): cisplatin-based chemotherapy (CT) vs no CT in 1867 patients (pts) with resected non-small cell lung cancer (NSCLC). *Proc. Am. Soc. Clin. Oncol.* 22, page 2, (abstr 6).

using RNA filter-array was performed in our institution [Ohira 2002]. Lung and normal tissue from three patients who underwent neoadjuvant therapy prior to surgery were collected, following the end of chemotherapy. Gene expression data obtained by a 588 gene filter arrays were

analyzed by “hierarchical clustering” method. Remarkably, normal tissue and tumor tissue from the same patient showed more similarities and clustered nearer than normal and normal tissue or tumor and tumor samples from different patients (Fig. (1)). On the other hand, groups of genes significantly differed in expression profiles between normal and malignant tissue. Especially, angiogenesis and invasion related genes were upregulated in the tumor samples (Fig. (2)). These results suggest that molecules involved in angio-

genesis are suitable targets for novel drugs administered following chemotherapy. This early study is one example of how genomic techniques could help to discover new candidates for target based therapeutics in the future.

GASTROINTESTINAL CANCER

Gastric cancer is still the fourth leading cause of cancer in the world [Parkin 2001]. Due to the lack of sufficient systemic control induced by current anti-neoplastic agents,

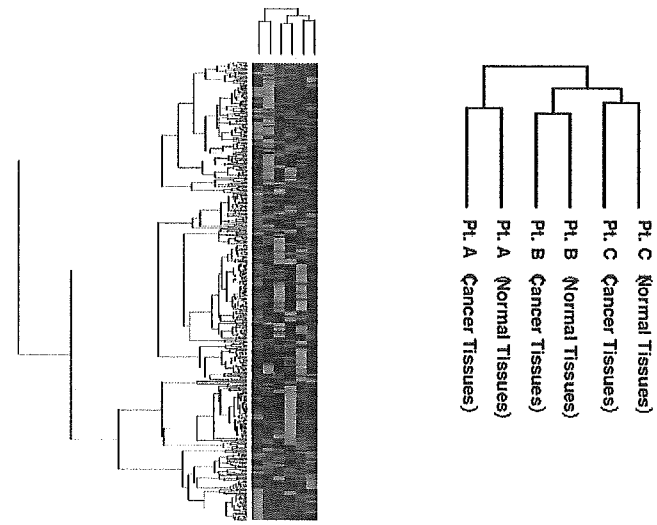
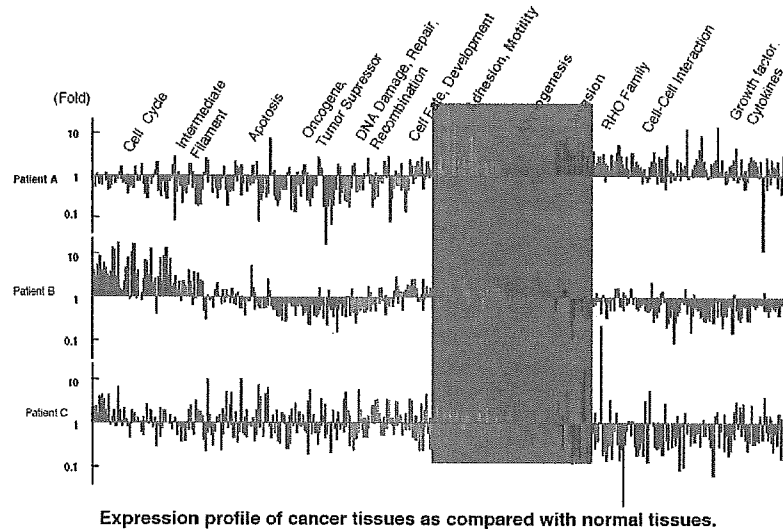


Fig (1). Clustering of gene expressions of tissues 3 from lung cancer patients (Ohira 2002). Tumor tissue and normal lung tissue was collected while surgery after neoadjuvant chemotherapy. Tumor tissue and normal tissue from the same patient show more similarities and clustered nearer than normal.



Expression profile of cancer tissues as compared with normal tissues.

Fig. (2). Histogram of gene expression profile of lung cancer tissue. Expression profile of cancer tissues as compared with normal tissues. Case B; increased expression of the genes related to cell cycle regulator, intermediate filaments, adhesion motility and angiogenesis in the tumor tissues. Expression of the other gene group were decreased in tumor tissue. Case C; increased expression of genes related with cell cycle adhesion were observed in the tumor tissue. Decreased expression of growth factor and cytokine related genes were also observed in tumor of Case C. Taken together, the expression profile of lung carcinoma could be characterized by the increased expression of the genes related with adhesion motility and angiogenesis.

surgery remains the cornerstone treatment approach to this disease. The development of lymph node metastasis is a well-established independent risk factor for recurrence of gastric cancer. In recent years, two independent research groups have established microarray based risk factor scoring systems for the development of lymph-node metastasis in gastric cancer. In 2002, a Japanese group published a study in patients with Intestinal Type Gastric Cancer [Hasegawa 2002]. Primary gastric cancer and corresponding non-cancerous gastric mucosa from 20 patients who underwent surgery were comparatively analyzed. A set of 61 genes that were commonly upregulated and 63 genes downregulated in Intestinal-Type Gastric Cancer in more than 75% of the cases could be identified. In a second step, the expression profiles of nine cases with and cases without lymph-node metastasis were compared. In this approach, 12 genes that were differentially expressed ($P < 0,01$) could be identified by employing a random permutation test. Nine of these 12 genes were overexpressed and three were downregulated in node positive tumors. By use of a "stepwise discriminant analysis", five independent "predictors" were identified among these 12 genes (Table 6). The predictive scoring system was confirmed in nine independent additional tumor tissue samples. All nine cases (four node positive and nine node negative) were correctly assigned to each class by means of the scoring system.

One year later, a Dutch group performed a comparable investigation. The molecular data of 35 gastric carcinomas were analyzed with their clinical data sets [Weiss 2003]. Microarray Comparative Genomic Hybridization (GCH), which allows to analyze accumulation of genetic changes that to a large extent occur on a chromosomal level, was applied to their approach. Three different groups could be distinguished by "hierarchical clustering" of the microarray CGH results. For each cluster, they could define a signature of 204 genes by using a "leave one out" cross validation. Each cluster was analyzed for correlation with clinicopathological data. The lymph-node status and the overall survival were criteria with significant differences between the individual groups. In one group, significantly less lymph-node positive cases (40%) were found than in the other one (83% in comparison to 88%). Patients who belong to the former group were found to have a significantly longer survival duration ($P=0,019$).

Both cited studies focussed on a clinically most important issue. The discussion about the inclusion of

extended lymph node dissection into surgery for gastric cancer remains rather controversial [Bonenkamp 1999, Cuschieri 1999]. The increase of overall morbidity and mortality associated with this treatment strategy has to be well-balanced against the benefit for lymph-node positive patients following this intervention. Predicting the overall risk of lymph-node metastasis by microarray techniques in an individual patient could be a reasonable strategy to select patients for this kind of therapy in the future and would have major implications for the clinical practice in this entity.

In 2003, Suganuma published a study focussed on possible chemoresistance-related genes in gastric cancer [Suganuma 2003]. Tumor samples and corresponding normal mucosa from 35 patients with advanced gastric cancer were differentially examined. The *in vitro* sensitivity of cells from each dissociated tumor sample against cisplatin, 5-fluorouracil, mitomycin C and doxorubicin was measured by MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The results obtained from *in-vitro* cytotoxicity testing assays were correlated with the results of cDNA microarray analysis of corresponding tissue samples. In the case of cisplatin, "hierarchical clustering" could successfully distinguish "sensitive" and "resistant" tumors from each other. A set of 23 potential "cisplatin-resistance-related-genes" could be selected by this method. The latter group included vascular permeability factor, two membrane-transporting subunits and retinoblastoma-binding protein. In a further selection based on strong criteria, metallothionein IG and heparin-binding-epidermal-growth-factor-like-growth factor were also identified as candidates for cisplatin-resistance-related genes. Within this approach, dihydropyrimidine dehydrogenase and HB-EGF-like growth factor were suggested to be 5-FU resistance-related genes. In this innovative approach, the authors could demonstrate that DNA-microarray could be useful to investigate drug-resistance and would be a means to understand some of the complex mechanisms behind it. Although this study is only based on a limited data set, this method could in principal be an important step for an individualized and customized cancer therapy in the future.

Moreover, in 2003, a Japanese group published a correlative study based on gene expression profiling in patients with colorectal cancer [Tsunoda 2003]. To clarify the regulatory factors in this malignant disease, differential gene expression profiles were analyzed by filter-array in

Table 6. Five Genes for Predicting Risk of Lymphnode Metastasis in Intestinal Gastric Cancer (Hasegawa 2002)

Title		Discriminant coefficient
DDOST	dolichyl-diphosphooligosaccharide-protein glycosyltransferase	1.87
GNS	glucosamine (N-acetyl)-6-sulfatase (Sanfilippo disease IIID)	1.26
NEDD8	neural precursor cell expressed, developmentally down-regulated 8	1.29
LOC51096	CGI-48 protein	1.36
AIM2	absent in melanoma 2	-1.54

Five genes were selected based on microarray data for predicting risk of lymph-node metastasis in intestinal gastric cancer (Hasegawa 2002). This "predictor" was validated in 9 additional independent cases. All cases were (four node positive and five node negative) were assigned to each classes.

surgically resected specimen (tumor and normal mucosa) obtained from ten patients with colorectal cancer. The correlation between several clinico-pathological factors and cancer-related genes were investigated by using complex statistical analyses including “average linkage hierarchical clustering and principal component analysis (PCA)”³. As an example, the c-myc-binding-protein and the c-jun-proto-oncogene were both identified as possible correlative markers for histological differentiation and overall clinical prognosis (Fig. (3)). The early-growth-response-protein 1 was selected to play an important role in the progression of clinical stage. The authors concluded that PCA was identified as an appropriate method to select candidate genes relevant to predict clinico-pathological factors in a small population of clinical samples from colon cancer patients.

LYMPHOMA

Diffuse large B-cell lymphoma (DLBCL) represents the most common subtype among the lymphoid neoplasm in adults (International Lymphoma Study Group 1997). Less than 50% of the patients are currently cured with standard combination chemotherapy [Popat 1998]. In 2000, the first large gene expression profiling study was performed in this disease [Alizedah 2000]. Within this study, different types of diffuse large B-cell lymphoma patients could be identified by gene expression profiles. A specific microarray assay for lymphoma was designed. This “Lymphoma-chip” included genes preferentially expressed in lymphoid cells and genes with known or suspected roles in cancer development and immunology. Using this array, 96 normal and different malignant lymphocyte samples were comparatively

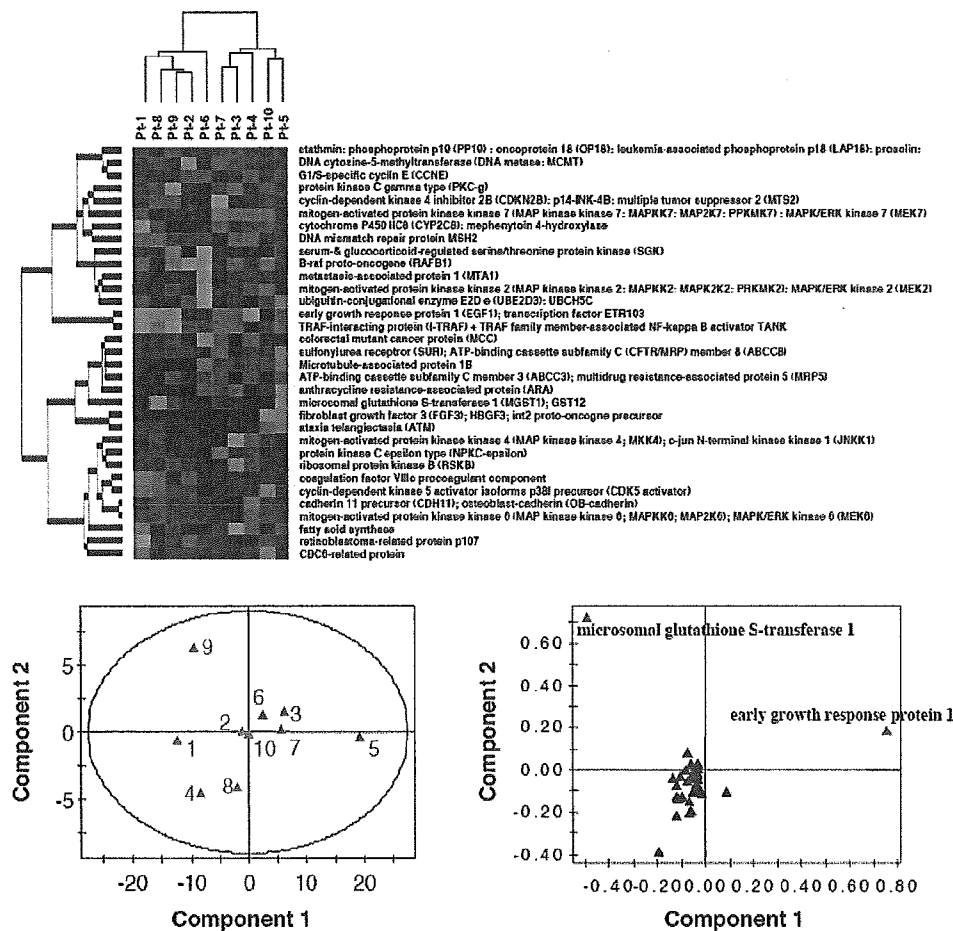


Fig. (3). (A). Average-linkage hierarchical clustering analysis of ten colorectal tumor samples on histological diagnosis. Right cluster shows the group of the well-differentiated and left shows the group of the other differentiations. (B) Principal component analysis on histological diagnosis. The numbers in blue indicate the patients with well-differentiated adenocarcinoma and the numbers in red indicate the patients with the other differentiations. The c-myc binding protein gene and the c-jun proto oncogene were identified as possible markers for histological differentiation.

³ **Principal component analysis:** A method of analyzing multivariate data in order to express their variation in a minimum number of principal components or linear combination of the original, partially correlated variables.

analyzed. All DLBCL samples could clearly be separated from normal lymphocyte samples and other lymphomas. Focused on genes related to a separate stage of B-Cell differentiation and activation, a germinal-centre-B-like

DLBCL and an *in vitro*-activated-B-like DLBCL could be distinguished among the group with diffuse large B-cell lymphoma by “hierarchical clustering” analysis. This clustering was based on the hypothesis that DLBCL derives from normal B-cells within the germinal centres (GC). As a consequence to this, overall survival and clustering to one of these two groups were correlated. Patients belonging to the GC-B-like-group had a significantly higher five-year survival rate than patients belonging to the activated-B-cell-DLBCL group ($P < 0,01$). The average five-year survival rate for all patients was 52%, for patients of the GC-B-like DLBCL was 76% and for patients of the activated B-like DLBCL only was found to be 16%. Two years later, Shipp and co-workers published another prognostic score for DLBCL based on gene expression profiling [Shipp 2002]. Contrary to the latter work, an alternative strategy that was independent of an *a priori* hypothesis was employed. A novel “supervised learning method” was applied. Tumor samples from 77 patients were analyzed. 58 patients with DLBCL and 19 patients with Follicular Lymphoma were clearly distinguishable by the use of this method. Clinical outcome prediction in DLBCL patients based on gene expression profiling data was the further purpose of this study. The long-term follow up was available for all DLBCL patients. While 32 patients were eventually cured, 26 patients turned out to have fatal or refractory lymphoma disease. An “outcome predictor” was designed using a “supervised learning classification approach” (weighted voting algorithm and cross validation test). The highest accuracy was obtained using a “predictor” set of 13 genes (Table 7). Using this “predictor” set, the DLBCL collective could be divided into a “cured” and on the other hand, a “fatal and refractor” group. The Kaplan Maier survival analysis revealed a significantly better 5-year-overall-survival rate for the “cured” group with 70% in comparison to the “fatal and refractory” group with 12% ($P=0,00004$). Based on these results, their model was validated in the dataset from the previously mentioned study. A set of 90genes was represented in their cDNA microarray as well

Table 7. Model of 13 Genes Predicting Outcome in DLBCL Patients (Shipp 2002)

Genes associated with good outcome	Genes associated with poor outcome
-Dystrophin related protein 2	-H731
-3UTR of unknown protein	-Transduction like enhancer protein 1
-uncharacterised	-PDE 4 B
-Protein Kinase C gamma	-uncharacterised
-Minor / NOR 1	-Protein kinase C beta 1
-Hydroxytryptamine 2B Receptor	-Oviductal glycoprotein
-Zinc finger protein C2H2-150	

A 13-gene based “predictor” for outcome in DLBCL patients was developed based on microarray data by a supervised learning method (Shipp 2002). The expression of seven genes were associated with good and the expression of six genes was associated with poor outcome. This “predictor” was superior to “hierarchical clustering” based classification of Alizedah in predicting outcome of DLBCL patients.

as in the oligonucleotide microarray (*lymphoma-chip*) employed by Alizedah. Both patient groups could be divided in the two “cell of origin” groups classified by Alizedah using this 90 gene set. This “cell of origin” differential analysis distinction was strongly associated with clinical outcome in the dataset of Alizedah but not in the actual patient group of DLBCL. Furthermore, the predictive power of their own 13 gene based “predictor” was tested in the dataset of Alizedah. Three of these genes were represented in the “*lymphoma-chip*” of Alizedah: NOR1, PDB4B and PKC β . Significant correlation with outcome was found for NOR1 ($p=0,05$) and PDB4B ($p=0,07$). Results for PKC β were discordant by representing multiple cDNAs on the “*lymphoma-chip*”. These results suggest a significant advantage for a “supervised learning method for the prediction of clinical outcome of disease in comparison to the “unsupervised hierarchical clustering”. The same dataset of Alizedah was re-analyzed by a Japanese group [Ando 2002]. “Fuzzy Neural Network”⁴ as a new statistical method to analyze prediction power of gene expression was applied in their investigation. Their model identified four genes (CD 10, AA807551, AA805611 and IRF-4) that could be used to predict prognosis with 93% accuracy.

In 2003, a Japanese cooperative research group published a study in Primary Central Nervous System Lymphomas (PCNL) using Filter-Array Assays [Yamanaka 2003]. Among 21 brain tumor and normal brain tissue samples, six PCNLs could be clearly distinguished by “hierarchical clustering” (Fig. (4)). The genes encoding for Laminin-receptor-2, thioredoxin-peroxidase and elongation-factor-1 were selected by Principal Component Analysis (PCA) as genes specific for PCNSL. The gene expression profiles of the six PCNL samples were correlated with the clinical outcome of the corresponding patients. These six patients could be distinguished into groups on the basis of post-treatment survival, a parameter likely related to response to therapy: group I >24 month (3 cases); group II < 23month (3 cases). All six patients were treated uniformly with the same chemo-radiotherapy regimen. A set of 76 genes capable of distinguishing the treatment sensitive group from the non-sensitive to treatment group was selected by the Whitney-Mann test. Among these 76 genes, 37 genes were found upregulated and 39 genes were found downregulated in responders. Interestingly, 10 of the 37 upregulated genes were involved in angiogenesis, while 6 of the 39 downregulated genes were involved in apoptosis. Using the selected genes related to response to chemo-radiotherapy, re-clustering was performed. The responders and non-responders could successfully be separated on the basis of subtle differences in distribution of gene expression (Fig. (5)). This study represents another example of how genomic techniques may improve evaluation of complex treatment and prognosis evaluation of neoplastic disease.

RELIABILITY AND REPRODUCIBILITY OF ARRAY DATA

These examples of investigations with microarray technique surely represent landmark studies in their

⁴ Fuzzy neural network: A special kind of artificial neural network.

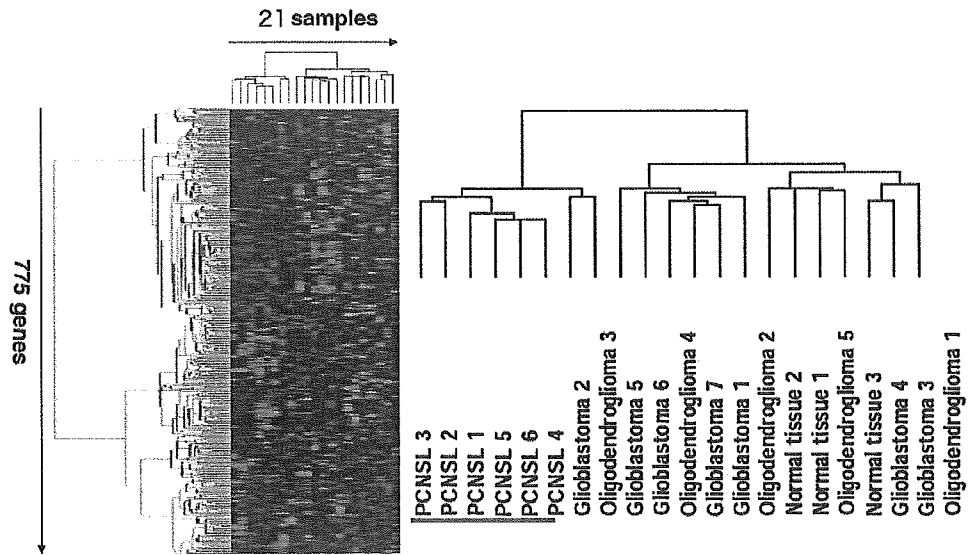


Fig. (4). Macroarray analysis of the 21 samples including PCNSCL, Glioblastoma, Oligodendroglioma and normal tissue. The phylogenetic tree obtained by application of the “clustering algorithm” shows separation of the PCNSL.

individual fields. They have brought a realistic hope to the scientific community that long time open issues will be solved within the near future. But besides all well-founded hope and enthusiasm, it cannot be overlooked that analyzing and interpreting array data remains a rather complex technology. Applying these novel genomic techniques uncritically to clinical data sets and clinical trials could lead to potentially problematic results and conclusions. It has to be critically taken into account that these novel techniques are still experimental. The question of validation and reproducibility remains the major issue. A number of studies regarding these technical and statistical problems have been

reported within recent years. We will try to summarize the most important ones of these in the following paragraph.

A fundamental problem for comparing gene expression profiles from different populations or groups (e.g. normal/disease) are large variations between individuals within the same population. It is difficult to distinguish differences in gene expression that appear associated with a specific disease from random genetic variations. Oleksiak has performed a landmark study regarding this important issue for all gene expression based studies [Oleksiak 2003]. Gene expression profiles within and among populations of the teleost fish of the genus *Fundulus* were analyzed in

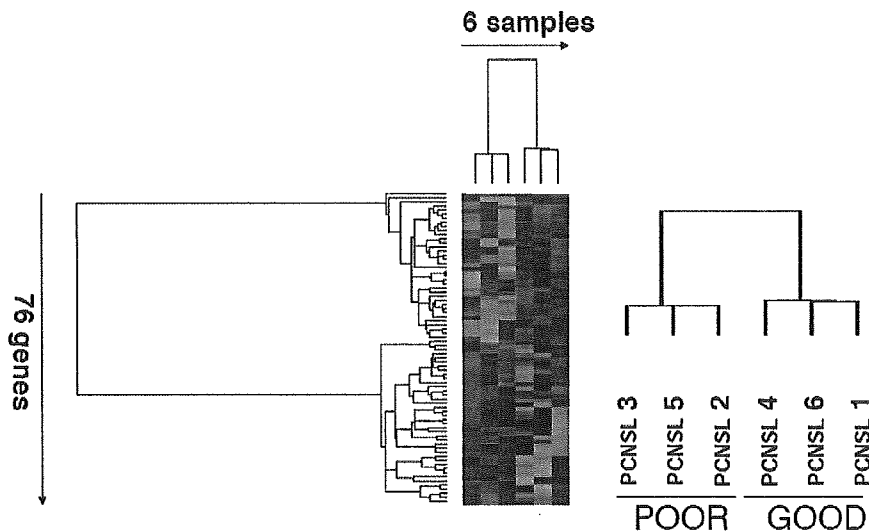


Fig. (5). Re-clustering was performed using selected genes related with response to chemo-radiotherapy. The responders (described as GOOD) and non-responders (described as POOR) were clearly separated clearly by the re-clustering method.

this investigation. Statistically, significant differences in expression profiles between individuals within the same population for approximately 18% of 907 genes were observed. Typically, expressions differed by a factor of 1.5 and often even more than a factor of 2.0. In addition to that, Enard *et al.* found in global comparisons of mRNA-levels of chimpanzee and human brain tissue, greater variations within the human population than between the human and the chimpanzee population [Enard 2002]. Both studies point out the importance to recognize the large variations between individuals within a population in study design as well as in the appropriately selected statistical analysis.

Our group has focussed largely on relevant questions of gene-profiling practice within the recent years. From small tumor samples, often only a small amount of RNA can be obtained. In some cases, this amount is not enough to perform gene profiling assays. Amplification of RNA to a.cRNA (amplified RNA) is one common way to enlarge the given RNA amount. Possible artificial changes of gene expression influenced by amplification have not yet been examined in detail. A two step amplification method was used in our validation experiment (Fig. (6)). By this approach, 10-100 μg amplified cRNA from a small amount of total RNA (1 μg or less) could be obtained. Then we have focussed on the question whether differential gene expression is conserved after amplification. The differential gene expression profiles of the PC 14 cell line and a sample of peripheral blood lymphocytes (PBL) were compared using mRNA and after amplification, a.cRNA. Although the R-Ratio was lower, we could conserve significant differences in the gene expression profiles after amplification (Fig. (7)). These preliminary results suggest that a gene-profiling study could be based on only small samples with a small amount of RNA if an appropriate amplification would be performed.

Another promising application of genomic techniques is to observe time or dose dependent changes of gene expression profiles in tumor tissue under the influence of a given drug application. However, repeated tumor sampling is necessary. This remains a very encumbering approach for the patient and often not possible in clinical every day practice. Therefore, we have examined, if a more easily performed method to obtain peripheral blood lymphocytes might be useful as a method to identify surrogate tissue for observing drug related changes in gene expression profiles. Within a clinical phase-I study with a novel Farnesyl-transferase Inhibitor, we have collected tumor samples and peripheral blood lymphocytes predose and on days two and eight following drug application (Fig. (8)). A cDNA filter-array assay including 775 genes chosen for predicting chemosensitivity was used for analyzing gene expression profiles (Fig. (9)). Interestingly, changes in gene expression were not only observed in tumor sample but also in the PBL. In still ongoing clinical research, we are currently trying to determine the role of PBL as surrogate tissue in pharmacogenomic cancer research.

In 2002, Churchill presented a basic review article about the fundamentals of experimental design for cDNA microarrays [Churchill 2002]. The appropriate design of a microarray experiment is essential for the scientifically based interpretation of the results. He pointed out the importance to analyze an adequately high number of biological samples to achieve representative, predictive and validated results. A higher number of technical replication with the same biological sample could not lead to validation of the results in most cases. Although the optimal design of an experiment or a study is the basis for successful results, the appropriate statistical analysis of the obtained data turns out to be of further importance too. An inadequate data analysis can lead

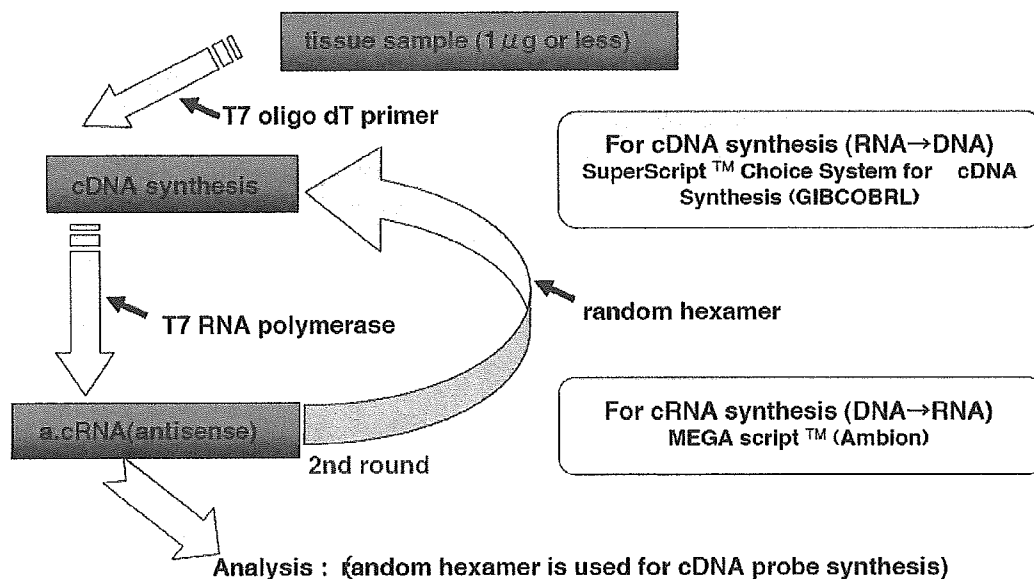


Fig. (6). Gene amplification by T7-based RNA amplification method. In a 2 step approach, first cDNA was synthesized (RNA \rightarrow DNA) followed by c.a.cRNA synthesis (DNA \rightarrow RNA), we could purify 10-100 μg RNA of amplified cRNA from small amount of total RNA (1 μg or less).

Is differential expression conserved even after amplification?

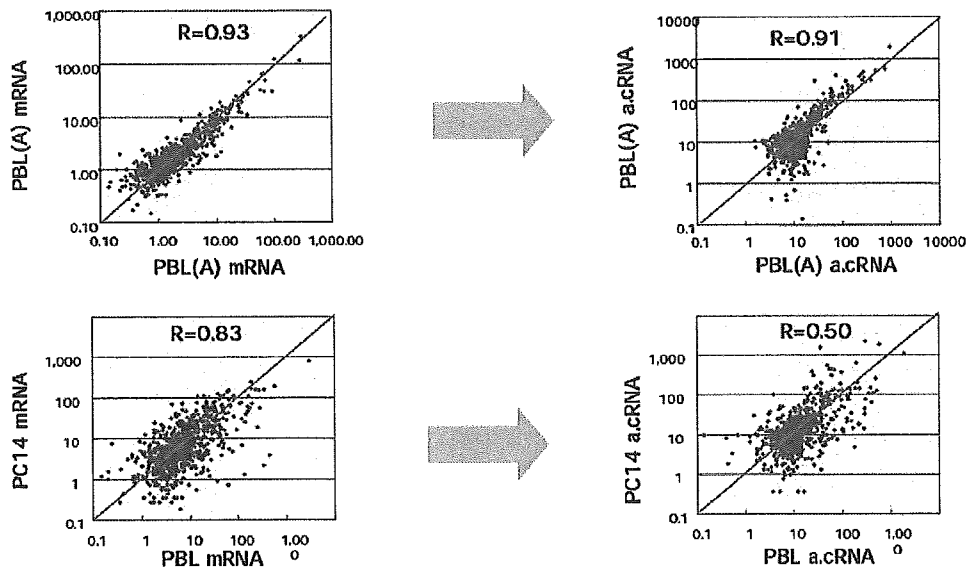


Fig. (7). Is differential expression conserved even after amplification? In order to analyze the reproducibility of the clinical samples, the gene expression profile of non-amplified and amplified samples were compared in scattered plot. Upper: gene expression data of duplicate samples of peripheral blood mononuclear cells were compared in scattered blot. High reproducibility ($R=0.93$) was obtained. These reproducible profiling was also observed in the amplified samples ($R= 0.91$). Lower: In a second experiment, we compared the differential gene expression of the PC 14 cell line and of peripheral blood mononuclear cells using mRNA and after amplification a.cRNA (amplified cRNA). Also the reproducible profiling was lower after amplification ($R=0.50$) than in non-amplified samples ($R=0.83$); we could conserve significant differences in gene expression after amplification.

Experimental design and drawing PBL samples (tissue sample)

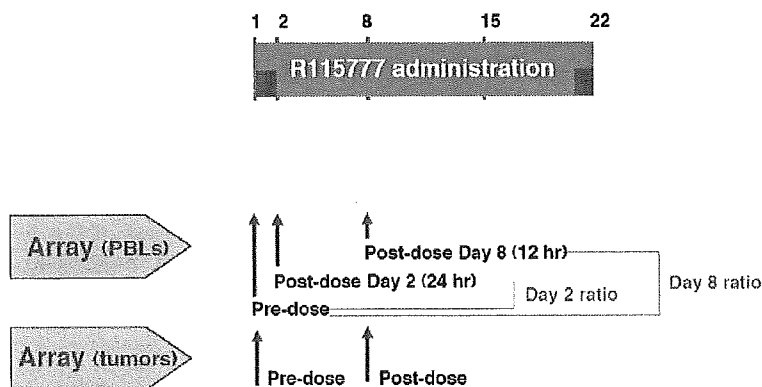
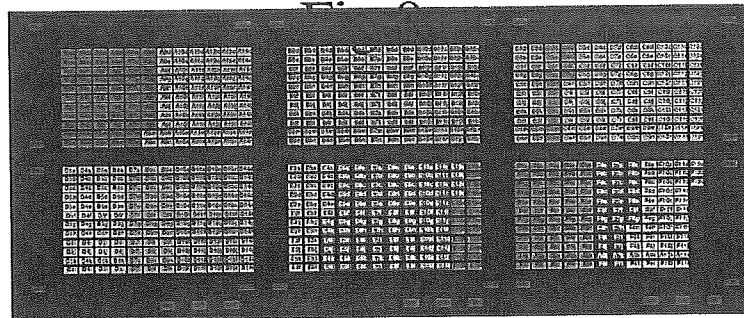


Fig. (8). Experimental design: sampling of PBL and tissue samples in correlative study in clinical phase I study of a farnesyltransferase inhibitor (FTI). Peripheral Blood Lymphocytes and tumor samples were collected predose, postdose day 2 and post dose day 8. Gene alteration after administration of FTI was analyzed for proof the pharmacodynamic effect of FTI.

Custom Atlas™ Array (cDNA filter-array) A set of 775 genes was chosen for predicting chemosensitivity analysis



- | | |
|--|--|
| 1: Cell Cycle Regulation | 7: Cytokines |
| 2: Signal / Oncogenes | 8: Apoptosis - Related Proteins |
| 3: Rho Family - Related Proteins | 9: DNA Transcription Factors / Damage Response, Repair & Recombination |
| 4: FTI - Related Proteins | 10: Metabolism |
| 5: Angiogenesis / Adhesion / Cell - Cell Interaction | 11: Translation / Protein Turnover / Detoxification Enzymes |
| 6: Growth Factors | 12: Transporters / Nucleocytoplasmic Transporters / Symporters & Antiporiers / Cytoskeletal Proteins |

Produced by Pharmacology Division, National Cancer Center Research Institute, Tokyo, Japan

Fig. (9a). The cDNA filter-array with a set of 775 genes chosen for predicting chemosensitivity analysis.

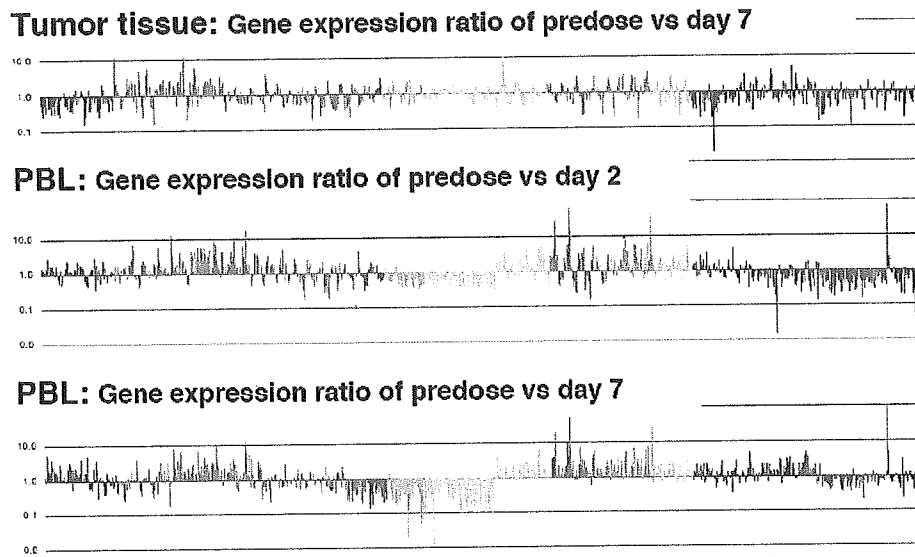


Fig. (9b). Gene expression changes of tumor tissue and PBL in the melanoma patient after administration of FTI. Specific gene groups were modulated by FTI. Changes in gene expression influenced by FTI were not only observed in the tumor samples but also in the peripheral blood lymphocytes. This findings suggest that drug modulated changes of gene expression in peripheral blood lymphocytes could be useful as surrogate markers in pharmacogenomic studies.

to potential pitfalls. As previously shown, the lymphoma data set of Alizedah is analyzed by different groups with different statistical methods, thus leading to partially different or even conflicting results. It is most important to recognize that different purposes of studies require different methods of statistical analyses. For example, the commonly

used “unsupervised hierarchical clustering” although useful for discovery subsets in a number of tumor samples within the same histological group, is not appropriate to compare these amongst each other or establish a meaningful “predictor” [Simon 2003]. In 2001, Tusher *et al.* published a new method for analyzing microarray data [Tusher 2001]. In

their investigation, they focussed on the problem to identify significant changes in gene expression profiles between different functional biological states. Cluster analysis provides only little information about statistical significance, and conventional *t* test is not appropriate for the thousands of data obtained within these microarray experiments. This problem led them to develop a statistical method adapted specifically for microarray analysis. This "Significance Analysis of Microarrays (SAM)" assign a score to each gene on the basis of gene expression relative to the standard deviation of repeated measurements. This method was used in the breast cancer study of Sorlie [Sorlie 2001]. Another important challenge is the integration of microarray data generated by different research groups on different array

platforms. Moreau has currently summarized three major problems: (1) the efficient access and exchange of microarray data; (2) the validation and comparison of data from different platforms (cDNA and short long oligonucleotides); and (3) the integrated statistical analysis of data sets [Moreau 2003]. Tan has reported a considerable divergence of results from three different commercial available microarray platforms analyzing the same RNA sample [Tan 2003]. The most common application of microarray technology is the prediction of clinical outcome in cancer. The most important reports have been referred to within in the first part of our review. Nitzani and Ioannidis systematically analyzed studies correlating outcome with genetic profiles based on microarray data published from

Table 8. Analysis of Microarray Based Correlation Studies (1999-2003) by Nitzani and Ioannidis (Nitzani 2003)

Characteristic	Studies of major clinical outcomes (n=30)	Other studies (n=54)	Total (n=84)
Year of publication			
1999	1(3%)	2(4%)	3(4%)
2000	2(7%)	1(2%)	3(4%)
2001	6(20%)	18(33%)	24(29%)
2002	18(60%)	28(52%)	46(55%)
2003	3(10%)	5(9%)	8(10%)
Malignant disorder			
Haematological	9(30%)	9(17%)	18(21%)
Solid tumor	21(70%)	45(83%)	66(79%)
Median (IQR) number of samples			
Total	62(29-96)	30(18-44)	37(20-57)
Specific cancer	43(24-69)	20(13-36)	25(15-45)
Microarray type			
cDNA	19(63%)	31(57%)	50(60%)
Oligonucleotide	11(37%)	23(43%)	34(40%)
Median (IQR) number of probes	8683 (6817-18 624)	6936 (4569-12 600)	7014 (5534-12 600)
Training			
Independent	9(30%)	17(32%)	26(31%)
Dependent	8(27%)	20(37%)	28(33%)
Both	13(43%)	17(32%)	30(36%)
Validation			
Independent	3(10%)	1(2%)	4(5%)
Cross-validation	6(20%)	4(7%)	10(12%)
Both	3(10%)	5(9%)	8(10%)
None	18(60%)	44(82%)	62(74%)
Outcomes/correlates assessed			
One	9(30%)	35(65%)	44(52%)
Two to four	12(40%)	11(20%)	23(27%)
Five or more	9(30%)	8(15%)	17(20%)
Significant associations reported			
Yes	21(70%)	20(37%)	41(49%)
No	9(30%)	34(63%)	43(51%)

Microarray correlation studies focused on prediction outcome or other important clinico-pathological features were systematically analyzed by Nitzani and Ioannidis in 2003. This table shows the results of their investigations. In 70% of the studies correlating major clinical outcome with gene expression significant associations were reported. However, in only 30 percent of the major outcome focused studies cross-validation or independent validation was performed. These findings underline the need for consequent quality control and validation in microarray based clinical studies.

1995-2003 [Nitzani 2003] (Table 8). They concluded that the predictive performance of this new technique was variable and in many cases, molecular classifications were not subject to an appropriate validation. Of note is, that they found out that only in 30% of the studies with major clinical implication an appropriate cross-validation or independent validation check was performed.

Another substantial open issue in the proceedings of DNA-microarray techniques in translational cancer research is the lack of information of the concrete biological function of encoding proteins. Most investigators have validated their DNA-microarray results by Real-Time-PCR [Chaqui 2002]. Although we can measure the level of expression of the genes of interest in a reliable way, we will miss information of the post-translational protein modifications, time course of protein expression, conditions of protein synthesis, cellular location of the protein, activation of the protein and interaction with other molecules. Therefore, more and more authors combine in their investigations DNA-techniques as DNA microarray and Real-Time-PCR with non-DNA techniques such as tissue-array, immunohistochemistry and western blotting. White and co-workers published a remarkable study focused on the correlation between mRNA and protein expression [White 2004]. This British group performed a microarray analysis to compare transcription in response to the ErbB-2 receptor tyrosine kinase activity in a model of a mammary luminal epithelial cell system. They compared the differences of mRNA expression with changes at protein level using a parallel proteomic strategy employing two-dimensional difference gel electrophoresis (2-D-DIGE) and quantification of multiple immunoblotting experiments. Interestingly, they found a high correlation between transcription and translation for the subset of genes studied. Moustafa and colleagues include immunoblotting in the validation of their DNA microarray experiment [Moustafa 2002]. To identify genes involved in head and neck cancer, they compared the gene expression profile in matched primary normal epithelial cells and primary head and neck cancer cells from the same patients employing a cDNA microarray consisting of 12530 genes human genes. They found significant changes in the expression of 213 genes. 91 genes were found upregulated and 122 downregulated in the cancer-cells. In general, most of the genes that are overexpressed in the head and neck cancer cells encode for growth factors and cell structure. The underexpressed genes are involved in cell-cell adhesion and motility, apoptosis and metabolism. To validate their results at protein level, they investigated the expression of nine selected genes from the cell-cell adhesion and motility group by immunoblotting and Reverse Transcriptase-PCR. They found in three of the four cell line pairs consisting results of DNA-microarray, Reverse Transcriptase-PCR and immunoblotting. However, in one sample, they found conflicting results between the protein and the mRNA expression of E-cadherin and γ -catenin. These differences may be explained by differing rates of translation or protein stability in the cancer cell versus their normal counterparts. Tissue microarrays (TMA) are promising approaches for validation of DNA-microarray results [Chaqui 2002, Hao 2004, Mousses 2002]. A TMA is a slide with dozens to hundred predefined microscopic sections of tissue. This makes it feasible for an

investigator to measure DNA, mRNA and protein expression in a large number of samples, providing enough statistical power for meaningful analysis. Immunohistochemistry is the most common method applied to TMAs, but *in situ hybridization* is increasingly used. In spite of many clear advantages for TMAs in the validation of microarray results, this technique is not without any limitations. The critical issues involve sensitivity and lack of quantification.

SUMMARY

In summary, these new techniques will play an important role in future of translational cancer research. However, a consequent and critical evaluation is definitely needed. An internationally commonly accepted standardization must be established. Public microarray databases should allow critical comparisons of independent experiences within the same malignant tumor entity. [Stoeckert 2002]. Published investigations should in detail report all key features of the experimental design, the samples used, the extract preparation and labeling performed, hybridization procedures and variables employed, measurement data and specifications generated. Future studies should generally be performed on the basis of the recommendations proposed by the Microarray Gene Expression Database Group (MEGD) [Brazma 2001].

In spite of many unsolved issues, genomic techniques have taken translational cancer research a significant step forward. In some cancer types such as lymphomas and solid tumors, more detailed and biologically relevant risk classifications could be developed using these novel techniques. For several anticancer agents, significant knowledge about mechanisms of action and resistance could be gained. As a consequence to this, genomic techniques will eventually become the backbone of translational cancer research in the upcoming future.

ACKNOWLEDGEMENTS

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Element Array by Scanning X-ray Fluorescence Microscopy after *Cis*-Diamminedichloro-Platinum(II) Treatment

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Abstract

Minerals are important for cellular functions, such as transcription and enzyme activity, and are also involved in the metabolism of anticancer chemotherapeutic compounds. Profiling of intracellular elements in individual cells could help in understanding the mechanism of drug resistance in tumors and possibly provide a new strategy of anticancer chemotherapy. Using a recently developed technique of scanning X-ray fluorescence microscopy (SXFM), we analyzed intracellular elements after treatment with *cis*-diamminedichloro-platinum(II) (CDDP), a platinum-based anticancer agent. The images obtained by SXFM (element array) revealed that the average Pt content of CDDP-resistant cells was 2.6 times less than that of sensitive cells, and the zinc content was inversely correlated with the intracellular Pt content. Data suggested that Zn-related detoxification is responsible for resistance to CDDP. Of Zn-related excretion factors, glutathione was highly correlated with the amount of Zn. The combined treatment of CDDP and a Zn(II) chelator resulted in the incorporation of thrice more Pt with the concomitant down-regulation of glutathione. We propose that the generation of an element array by SXFM opens up new avenues in cancer biology and treatment. (Cancer Res 2005; 65(12): 4998-5002)

Introduction

Cis-Diamminedichloro-platinum(II) (CDDP) is an effective anticancer agent, but tumor cells can become resistant after CDDP-based therapy (1). Detoxification of CDDP, an increase in DNA repair, and excretion of CDDP have been implicated as major factors contributing to CDDP resistance (1). Incorporated CDDP is excreted by several molecules, such as overexpressed P-glycoprotein (2), a zinc-related defense system that is regulated by increased intracellular glutathione (GSH; ref. 3), and the ATP-dependent glutathione S-conjugate export pump (GS-X pump), which plays a role in the vesicle-mediated excretion of GSH-CDDP conjugates from resistant cells (4). Recent reports suggest

that minerals such as zinc (Zn) and copper (Cu), important for normal cellular functions (5), are involved in CDDP resistance (6, 7). The simultaneous monitoring of multiple numbers of cellular elements would be helpful in identifying the mechanism of drug resistance in a malignant cell. The recently developed technique of scanning X-ray fluorescence microscopy (SXFM; refs. 8, 9) has made it possible to detect elements of interest by a single measurement and give a profile of these elements at the single-cell level (termed an element array). To examine the efficacy of element array analysis, we analyzed elements before and after treatment with CDDP and compared the element profiles of CDDP-sensitive and CDDP-resistant cells. We showed that the Zn content has an inverse correlation with Pt incorporation owing to a positive linkage with glutathione (GSH), a Zn-dependent detoxification factor. The combined treatment with CDDP and *N,N,N,N*-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), a Zn (II)-chelator (10), increased Pt uptake with a concomitant reduction of intracellular GSH. We propose that the element array is a versatile method suitable for obtaining information about metals involved in drug metabolism and could contribute to a novel strategy for anticancer chemotherapy.

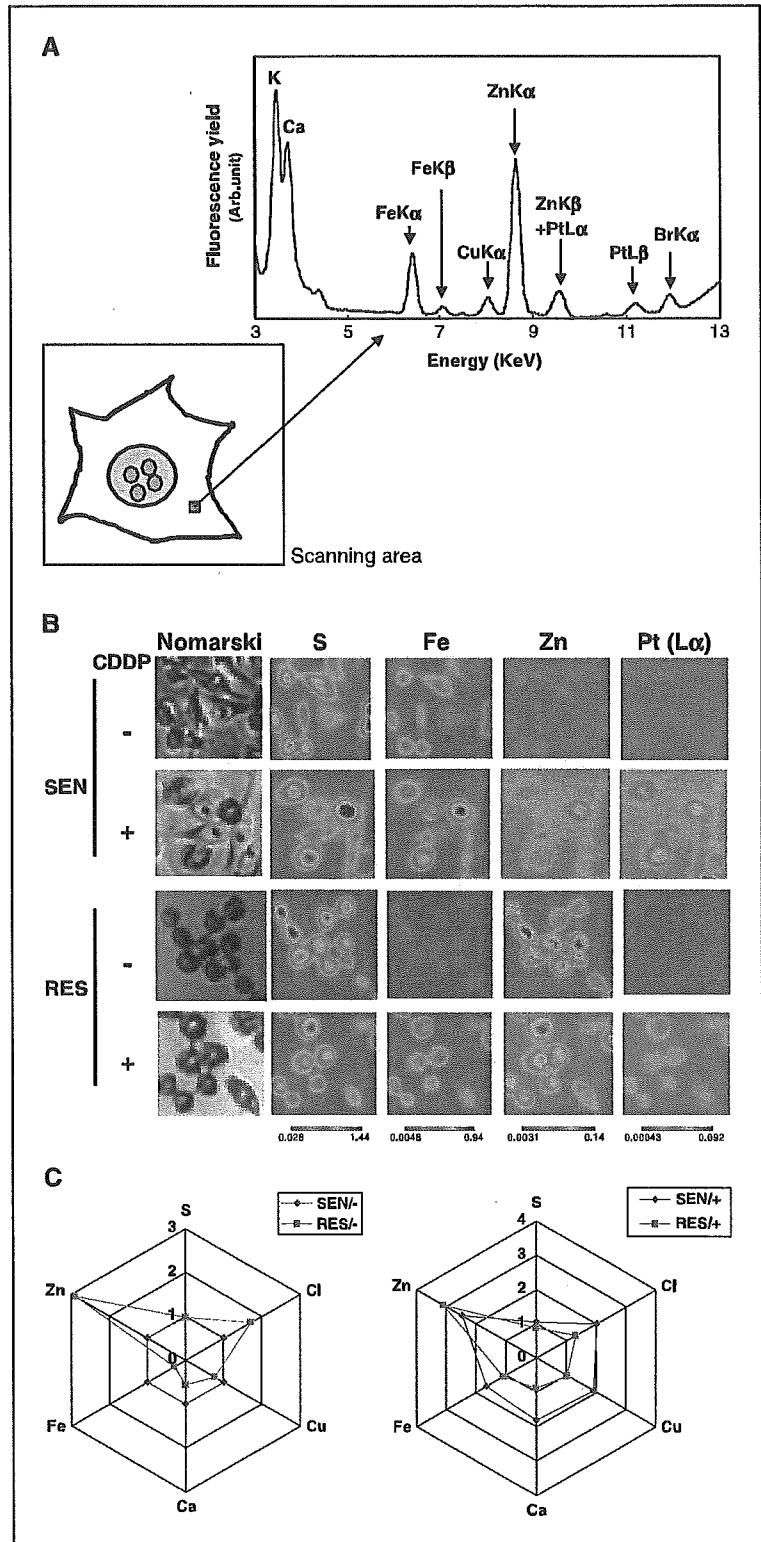
Materials and Methods

Element array analysis by scanning X-ray fluorescence microscopy. SXFM was set up at an undulator beamline, BL29XU, of the Spring-8 synchrotron radiation facility (11) by combining a Kirkpatrick-Baez-type X-ray focusing system (12, 13), an XY-scanning stage for sample mounting, and an energy-dispersive X-ray detector (SDD, Röntec, Co., Ltd.). Monochromatic X-rays at 15 keV for Pt *L*-line excitation were focused into a 1.5 μm (*H*) \times 0.75 μm (*W*) spot with a measured flux of $\sim 1 \times 10^{11}$ photons/s. The focused X-rays simultaneously yielded the fluorescence of various chemical species in a small volume of sample cells, as shown in Fig. 1A. The fluorescence from each element was taken independently and did not overlap except for the Pt *L* α signal, which was contaminated by Zn *K* β (Fig. 1A). This was corrected by subtraction, as described previously (8). In this study, we could also measure Pt *L* β as a unique signal of Pt (Fig. 1A). After counts were collected for 4.0 to 8.5 seconds at each pixel of scanning, the detected counts were normalized by incident beam intensity. In addition to the mapping images, an elemental concentration per single cell was calculated from the integrated elemental intensity over the whole mapping image.¹⁰

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Figure 1. Element array by SXFM. *A*, scheme of imaging cellular elements by SXFM. Coherent X-rays are focused on each area (*pixel*), and the X-ray fluorescence from each element is detected. Each pixel gives an elemental spectrum, as shown in the right panel, and an integrated intensity of the individual element was mapped to the corresponding area of analyzed cells. *B*, SXFM analysis after CDDP treatment. Cell morphologies obtained by Nomarski are shown at $\times 100$ magnification (*left*). Each field of view is equivalent to an area of $70 \times 70 \mu\text{m}$. Representative results are shown. Brighter colors indicate a higher signal intensity of each element. Results are shown for PC/SEN (*top*) and PC/RES cells (*bottom*). Note the high intensity of PtL α in PC/SEN cells after CDDP treatment (*second panel of the Pt column*) and the higher signal intensity of Zn in PC/RES cells compared with that of PC/SEN cells. *C*, element array based on SXFM analysis. The mean signal intensity of each element obtained by SXFM analysis was calculated, and the fold increase of elements in PC/RES cells (*red*) was depicted by using the intensity in PC/SEN cells (*blue*) as a standard (*left*). A part of analyzed elements is shown. The fold increase of elements in PC/SEN (*blue*) and PC/RES cells (*red*) after CDDP treatment was also shown by using the intensity in PC/SEN before CDDP treatment as a standard (*right*).



Chemicals and biochemical assays. TPEN (Sigma, St. Louis, MO; ref. 10), GSH (Calbiochem, La Jolla, CA), and CDDP (Daiichi Kagaku, Tokyo, Japan) were purchased. A GSH colorimetric assay kit (Calbiochem) and a BCA protein assay kit (Bio-Rad, Hercules, CA) were used for measuring

intracellular GSH. About 3×10^5 to 4×10^5 cells were subjected to GSH measurement, and the data were normalized by cell number.

Cell lines. PC-9 cells (PC/SEN) and PC-9 cells resistant to CDDP (PC/RES), originally derived from a lung carcinoma cell line (14), were

maintained in DMEM (Nissui, Co., Tokyo, Japan) supplemented with 10% FCS (Sigma). The viability of PC/SEN cultured for 72 hours in the presence of 1 $\mu\text{mol/L}$ CDDP was 40%, whereas that of PC/RES was ~90%. In this study, each cell line when treated with 1 $\mu\text{mol/L}$ CDDP for 24 hours showed >85% viability.

Colony formation. After treatment, aliquots of PC/SEN and PC/RES were plated into culture dishes or soft agar, and the numbers of cell aggregates consisting of >50 cells were counted. Each number was normalized by plating efficiency, and the mean and SD of the number of formed colonies were calculated.

Sample preparation. Cells were plated on a silicon nitride base (NTT Advanced Technology, Tokyo, Japan) 1 day before the experiment. After incubation for 24 hours in the presence of 1 $\mu\text{mol/L}$ CDDP, the cells were washed with PBS, fixed in 2% paraformaldehyde in PBS for 10 minutes at room temperature, and incubated in cold 70% ethanol for 30 minutes. The cells were then placed in a 1:3 solution of glacial acetic acid and methanol for 10 minutes, washed with 70% alcohol, and dried overnight at room temperature.

Measurement of cellular platinum and zinc. To measure Pt and Zn, $\sim 5 \times 10^6$ cells were subjected to inductively coupled plasma mass spectroscopy (ICP-MS; Toray Research Center, Shiga, Japan; ref. 15).

Statistical analysis. The Pearson product-moment correlation coefficient and Student's *t* test were used to evaluate statistical significance (16).

Results and Discussion

Incorporation of platinum and element array after *cis*-diamminedichloro-platinum(II) treatment. We analyzed intracellular elements by SXFM after CDDP treatment (Fig. 1A). At 12 hours after treatment with 1 $\mu\text{mol/L}$ CDDP, the level of Pt was increased in PC/SEN cells, whereas little increase in the Pt level was seen in PC/RES cells (Fig. 1B). The intensity of Pt in PC/RES cells was 2.6-fold less than that in PC/SEN cells, as confirmed by the results of ICP-MS, which indicated that the amount of Pt in PC/RES cells (5.5 fg/cell) was 3.6-fold less than that in PC/SEN cells (19.7 fg/cell). Therefore, the decreased accumulation of CDDP is likely to be responsible for resistance in PC/RES cells.

Based on the mean signal intensity obtained by SXFM, element array analysis was carried out (Fig. 1C). The element profile

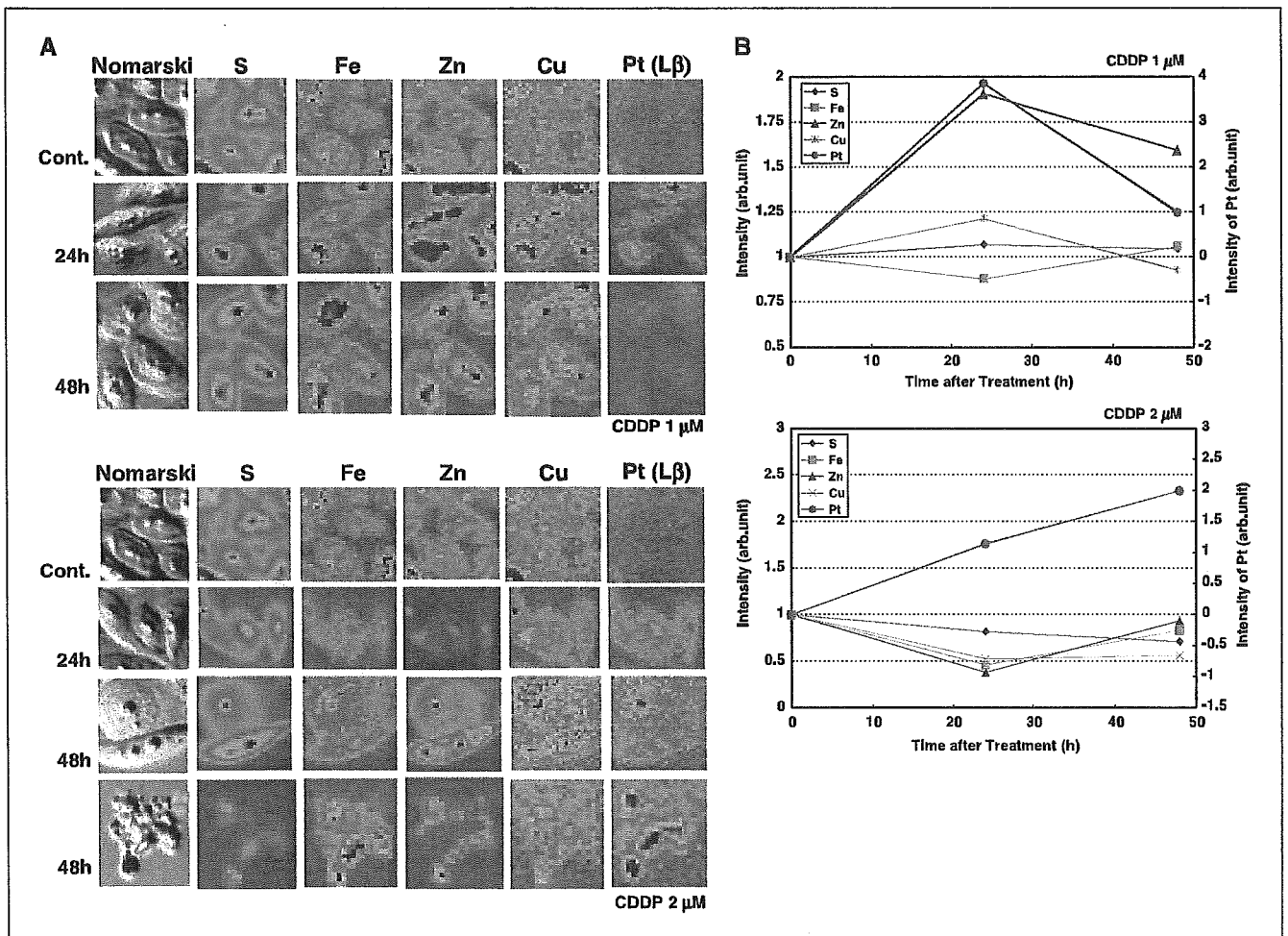


Figure 2. Chronological changes in elements after CDDP treatment. A, detection of elements in CDDP-treated PC/SEN cells. From the left, Nomarski images, signals of S, Fe, Zn, Cu, and Pt are shown. Top and bottom sets of panels show cells treated with 1 and 2 $\mu\text{mol/L}$ CDDP, respectively. In each set of panels, control cells (top) and cells treated with CDDP for 24 hours (middle) and 48 hours (bottom) are shown. In this experiment, the signals of PtL β were measured instead of PtL α (see Materials and Methods). The lowest panels show an apoptotic cell after 48 hours. B, summarized results of chronological changes of elements. The results after treatment with 1 $\mu\text{mol/L}$ (top) and 2 $\mu\text{mol/L}$ CDDP (bottom) are shown. The mean signal intensity was calculated from the results partly shown in (A). Among the cellular elements, Zn was most influenced by both 1 and 2 $\mu\text{mol/L}$ CDDP treatment and had an inverse correlation with Pt content.

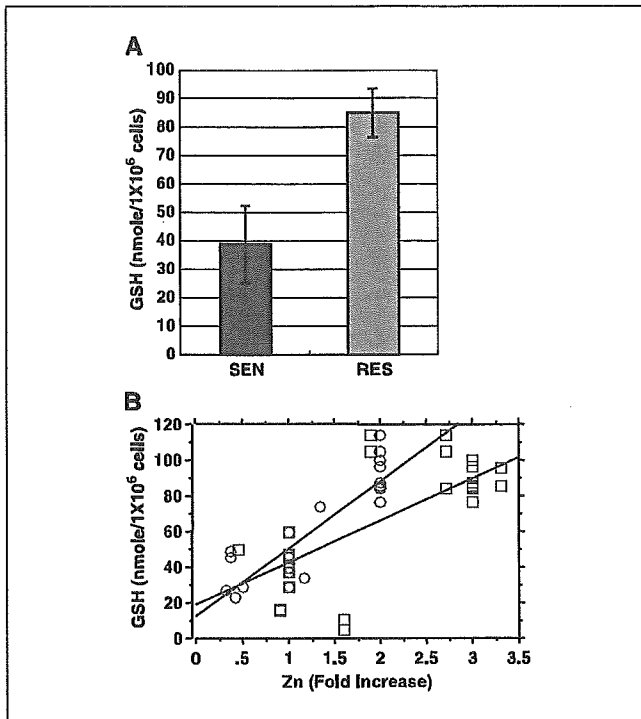


Figure 3. Cellular Zn content and GSH. **A**, basal level of intracellular GSH. The intracellular GSH levels in PC/SEN (black) and PC/RES cells (gray) were measured. GSH was significantly higher in PC/RES than in PC/SEN cells (t test, $P < 0.05$). **B**, correlation between Zn and intracellular GSH. A scatter diagram for Pearson product-moment correlation coefficient is depicted. Zn, measured by SXFM (red squares, $n = 27$) and by ICP-MS (green circles, $n = 29$), was plotted against intracellular GSH. Scattered values were based on data from both PC/SEN and PC/RES cells. The correlation coefficient r was calculated, and the statistical significance was determined ($P < 0.05$).

facilitates the identification of the elements related to the mechanism of drug resistance to CDDP. First, we noticed that the Zn content of untreated PC/RES cells was ~3-fold of that in PC/SEN cells (Fig. 1C, left). The difference in the Zn contents of these cells was confirmed by ICP-MS analysis (105 fg/cell for PC/SEN cells and 189 fg/cell for PC/RES cells, respectively). When 1 $\mu\text{mol/L}$ CDDP was used for treatment, constitutive high Zn was observed in PC/RES (Fig. 1C, right). In PC/SEN cells, the amounts of all the elements were slightly increased, but the amount of Zn was increased most markedly.

We then analyzed the chronological changes in the levels of elements in PC/SEN cells following CDDP treatment. Representative results for S, Fe, Zn, Cu, and Pt are shown in Fig. 2A. Pt was clearly observed at 24 hours after treatment with 1 or 2 $\mu\text{mol/L}$ CDDP (Fig. 2A). It was, however, barely detectable at 48 hours after the cells were treated with 1 $\mu\text{mol/L}$ CDDP (Fig. 2A, top), suggesting that the cells excreted CDDP. In contrast, the cellular content of Pt gradually increased after treatment with 2 $\mu\text{mol/L}$ CDDP (Fig. 2A, bottom), and apoptotic cells with high levels of incorporated CDDP were observed after 48 hours (Fig. 2A, bottom).

The element profile was plotted against the time after treatment with CDDP (Fig. 2B). When the cells were treated with 1 $\mu\text{mol/L}$ CDDP, the Zn content increased remarkably and reached a peak at 24 hours (Fig. 2B, top, red line). In these cells, the Pt content was reduced after 48 hours. When the cells were treated with 2 $\mu\text{mol/L}$ CDDP, the Zn content decreased within 24 hours (Fig. 2B, bottom),

and the Pt content increased within 48 hours. In this analysis, Cu did not show significant changes. The results imply that the intracellular Zn content has an inverse correlation with the incorporated Pt content.

Cellular zinc and zinc-related detoxification. We studied Zn-related detoxification factors, such as metallothioneins (17), GSH (18), and the GSH-coupled excretory pump GS-X (4), and we observed that intracellular GSH was high in PC/RES cells (Fig. 3A). We then examined the possible correlation between the intracellular Zn content and GSH. As shown in Fig. 3B, the GSH levels showed a significant correlation with the levels of Zn detected by both ICP-MS and SXFM (Pearson product-moment correlation coefficient $r = 0.794$, $P < 0.05$ and $r = 0.533$, $P < 0.05$, respectively). The levels of Zn detected by SXFM may have less correlation with GSH than do the levels detected by ICP-MS because SXFM analyzed Zn in a small number of cells, whereas the analyses of GSH using ICP-MS were carried out on $>10^5$ cells.

Effects of zinc depletion and cis-diamminedichloroplatinum(II) uptake. To examine ways of increasing the sensitivity of PC/RES cells to CDDP, we used the Zn(II) chelator TPEN, as it was thought that CDDP uptake would increase when the GSH level was down-regulated by decreased Zn. Consistent with this hypothesis, treatment with 7.5 $\mu\text{mol/L}$ of TPEN decreased cellular Zn to ~40 fg/cell at 30 hours after treatment in PC/SEN cells (Fig. 4A, left, solid line). The decrease seen in PC/RES cells owing to TPEN treatment was more rapid, with the Zn concentration being reduced to ~40 fg/cell within 7 hours (Fig. 4A, left, dashed line). The intracellular GSH also decreased with the reduction in intracellular Zn (Fig. 4A, right, dashed line).

To determine the effects of TPEN on the growth of PC/RES cells, the cells were pulse-treated for 2 hours with TPEN for 5 consecutive days and the growth was examined. Although treatment with 1 $\mu\text{mol/L}$ CDDP did not induce apparent morphologic changes (Fig. 4B, bottom, left), the combined treatment with TPEN and CDDP caused prominent changes (Fig. 4B, bottom, right). A colony formation assay clearly showed that the combination of CDDP and TPEN, as well as single TPEN treatment, significantly impaired the growth of PC/RES cells (Fig. 4C). Consistent with these changes, ICP-MS indicated that the intracellular Pt content increased 3.5-fold after the combined treatment (from 0.38 to 1.35 fg/cell with TPEN treatment). It is important to note that the same dose of TPEN did not attenuate the growth of PC/SEN cells (Fig. 4C). These data indicate that the GSH level seems to be critical for resistance in PC/RES cells, consistent with previous reports that CDDP-resistant cells have high levels of GSH and that a decrease in GSH results in loss of resistance (3, 19). Our data also suggest that the high GSH content was maintained by the effects of Zn in PC/RES cells. Overall, our trial treatment with combined TPEN and CDDP suggests that this combination would be effective in eliminating tumors even if they include a CDDP-resistant population of cells with high Zn content.

We showed the use of element array analysis by SXFM to examine a mechanism of CDDP resistance. Based on element profiles, we successfully overcame CDDP resistance in PC/RES cells by using a Zn chelator that down-regulated the GSH level. Although it has been reported that Cu is a necessary factor for CDDP incorporation (7), the present work revealed that Cu was not involved in PC/RES cells. It is tempting to speculate that drug resistance is generated by various elements, and we propose that an element array can contribute to better understanding of cancer biology as well as other fields of medical science.

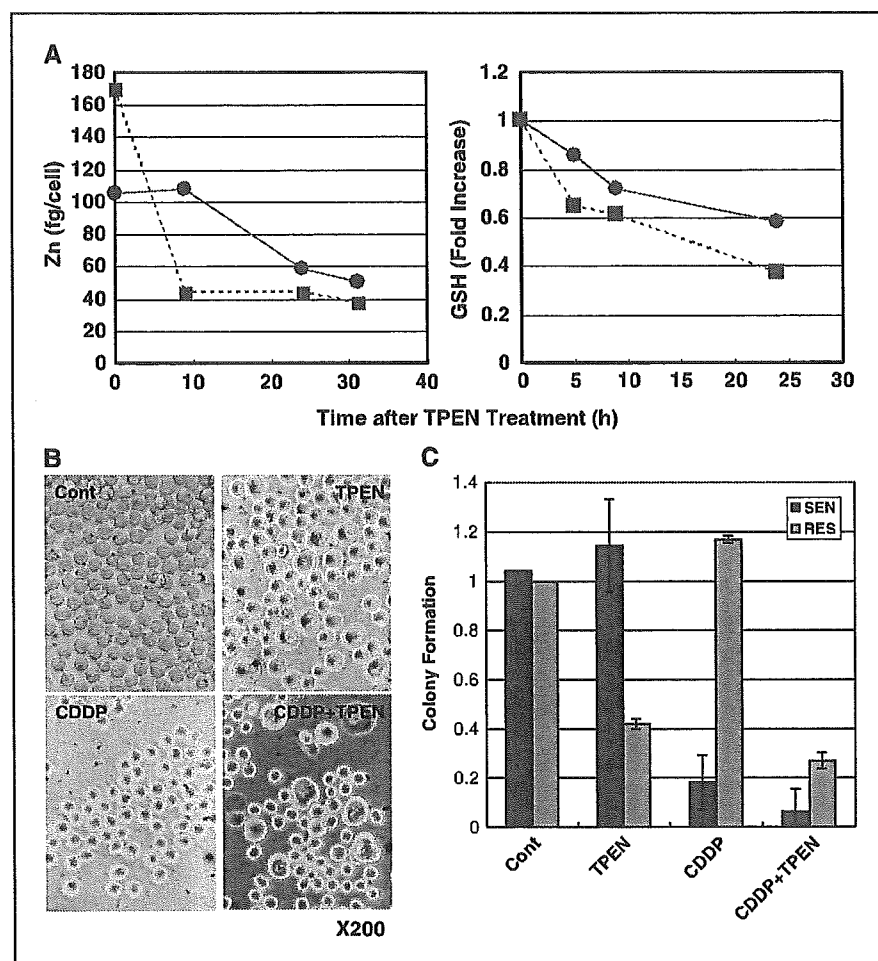


Figure 4. Cellular Zn content and Pt uptake with TPEN. **A**, TPEN-induced depletion of cellular Zn and down-regulation of GSH. TPEN (7.5 $\mu\text{mol/L}$) was added to the culture medium for the indicated time periods, and cellular Zn was measured by ICP-MS (*left*). Intracellular GSH content was also monitored (*right*). The Zn contents in PC/SEN (solid lines) and PC/RES cells (dashed lines) are shown. **B**, morphologic changes after pulse treatment with TPEN and CDDP. The morphologies of untreated PC/RES cells (*top, left*) and of cells treated with TPEN (*top, right*), CDDP (*bottom, left*), and CDDP plus TPEN (*bottom, right*) are shown. The cells were exposed to 1.0 $\mu\text{mol/L}$ CDDP with or without 7.5 $\mu\text{mol/L}$ TPEN for 2 hours, and then the medium was replaced with fresh medium. Pulse treatment was carried out for 5 consecutive days. Magnification, $\times 200$. Note that large cells are observed after treatment with TPEN alone, and larger cells with irregular shape are observed following the combination treatment. The data showed that TPEN caused cellular accumulation at G₂-M phase with mitotic failure (data not shown). **C**, colony formation after pulse treatment with CDDP with or without TPEN. After pulse treatment for 5 consecutive days, as described in (**B**), the cells were plated in soft agar and the colony formation assay was done. The means and SDs of colony numbers of PC/SEN (black columns) and PC/RES cells (gray columns) are shown. The experiments were carried out in triplicate.

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