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Protein Expression Profiles Indicative for Drug Resistance of Kidney Carcinoma

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Abstract. *The purpose of this investigation was to evaluate whether different protein patterns exist between drug-sensitive and drug-resistant kidney carcinomas. As a first step, expressions of drug resistance proteins (P-glycoprotein (P-gp), glutathione S-transferase-π (GST-π), DNA topoisomerase IIα (Topo IIα), alkaline phosphatase (AP), catalase, thymidylate synthetase, metallothionein), signal transducers (protein kinase Cα/β (PKCα/β)), proliferation-associated proteins (Ki-67) and proteins of proto-oncogenes and tumor suppressors (ErbB1, ErbB2, Fos, Jun, Myc, Ras and p53) of primary cell cultures of human kidney carcinomas of 18 patients were determined. The expression levels of the proteins were compared with the response to doxorubicin, vincristine or mafosfamide measured by growth inhibition and nucleotide incorporation assays. As a second step, those proteins showing a relationship to doxorubicin resistance (P-gp, GST-π, Topo IIα, PKCα/β, AP, ErbB1, ErbB2, Fos, K-Ras, p53, and Ki-67) were analyzed by hierarchical cluster analysis and clustered image mapping. The resulting clusters were correlated with the drug resistance data. The data shows that different protein expression profiles exist between drug-resistant and –sensitive kidney carcinoma cell cultures. Finally, the clustered image map (CIM) demonstrates a sensitive area that is characterized by a lower expression of proteins and a resistant area with a higher expression of proteins. These results may have important implications for the diagnosis and therapy of kidney cancer. The resistance proteins and the resistance-related factors found in the present analysis may be suitable parameters to predict treatment outcome of kidney cancer.*

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Kidney carcinomas are mainly treated by surgery and radiotherapy, because they are frequently resistant to chemotherapeutical agents. Kidney tumors represent, therefore, a suitable model to study drug resistance phenomena. Drug resistance is frequently multi-factorial and depends on drug transport, drug metabolism, drug detoxification, DNA repair, cell proliferation and many other factors (1-4). It is not sufficient to investigate single proteins to understand clinical drug resistance.

Therefore, different cellular parameters including drug resistance proteins (P-glycoprotein (P-gp), glutathione S-transferase-π (GST-π), DNA topoisomerase IIα (Topo IIα), alkaline phosphatase (AP), catalase, thymidylate synthetase, metallothionein), signal transducers (protein kinase Cα/β (PKCα/β)), proliferation-associated proteins (Ki-67) and proteins of proto-oncogenes and tumor suppressors (ErbB1, ErbB2, Fos, Jun, Myc, Ras, and p53) were investigated in primary cell cultures of 18 human kidney carcinomas. The protein resistance profile of kidney cancer was determined by hierarchical cluster analysis.

Materials and Methods

Cell lines. Primary cell cultures of human renal cell carcinomas of 18 patients were obtained from the Tumorbank of the German Cancer Research Center (Heidelberg, Germany). Their generation has been described earlier (5). These cell lines were numbered from RCC1 to RCC18 according to the 18 patients. The cell cultures were maintained in DMEM medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (Seromed) and 5% L-glutamine at 37˚C in a cell culture incubator. All investigations were performed using sub-confluent cultures of the third to fifth passage.

Growth inhibition assay. For evaluation of drug sensitivity, cell growth was measured after application of doxorubicin (Pharmacia, Freiburg, i. Br., Germany), vincristine (Eli Lilly, Giessen, Germany), or mafosfamide (an *in vitro* active derivative of cyclophosphamide) (Baxter Oncology, Frankfurt a. M., Germany) (6).

Nucleotide incorporation test. Measurement of incorporation of nucleic acid precursors for predicting drug sensitivity has been described earlier (7, 8).

Table I. *Protein expression in 18 primary cell cultures of kidney carcinomas (semiquantitative scores).*

RCC	DOX	DOX	VCR	MAFO	P-gp	GST-π	Topo IIα	PKC α/β	AP	CAT	MT	TS	Ki-67	ErbB1	ErbB2	Fos	Jun	Myc	K-Ras	N-Ras	H-Ras	p53
1	94[a]							3[c,d]	3[c,e]	3[c]	1[c]	1[c]	65[b]	2[c]	3[c]	3[c]	2[c]	3[c]	0[c]	1[c]	0[c]	0[c]
2	77				60[a,b]	3[a,c]		2	3	3	1	2	19	1	2	3	3	3	1	2	1	2
3	77	165[a]	160[a]	10000[a]	67		29[a,b]	3	2	3	1	1		3	2	2	1	2	0	2	0	3
4	75	400	82	4	52	3	39	2	2	2	2	2	15	3	3	2	2	3	0	2	1	3
5	71	175	150	115	55	3	38	2	2		2	1	61	3	2		0		0	1	0	
6	70	310	48	4	49	3	34	2	2	1	1	1	23	2	2	1	1	2	0	1	0	3
7	69				52	3	53	1	2				15	2	2							
8	69	100	190	9	47	3	52	3	1	3	1	3	12	2	2	2	2	2	0	3	0	3
9	48	65	100	180	35	3	66	3	2	2	2	2	16	2	1		2	3	1	1	0	3
10	45	31	66	3300	37	2	73	0	2	1	2	1	31	2	3	2	2	3	0	2	0	3
11	44	12	22	1000	42	1		0					33	2								
12	36	14	15	8	10	1	93	0	3	2	0	0	41		3		0	2	0	0	0	3
13	36	8	48	1	5	3	77	2	0	2	1	2		0	1	3	1	3	0	1	0	3
14	30	7	33	1000	9	1		2	1	3	2	2	45	1	0	1	1	0	1	3	1	2
15	30		8	1		0	86	0	1		0	0		0	2	0	0		0	0	0	
16	22	6	44	2600	9	2	90	1	0	2	1	2	46	0	2	1	1	3	1	2	1	3
17	20	5	25	235	8	1	72	3	1	2	2	1	39	2	1	1	3	2	1	2	0	0
18	12				1			1	1	3	2	1	39	2	1	2	1	3	0	2	0	3

[a] according to (15)
[b] % positive cells
[c] semiquantitative scores (0, negative; 1, weak ; 2, moderate ; 3, strong reaction)
[d] according to (16)
[e] according to (17)
P-gp, P-glycoprotein; GST-π, glutathione S-transferase-π, Topo IIα, DNA topoisomerase IIa, PKC α/β, protein kinase C α/β, AP, alkaline phosphatase, CAT, catalase; MT, metallothionein, TS, thymidylate synthetase.

Immunocytochemistry. Protein expression was detected by means of a streptavidin-biotin peroxidase method as described earlier (9, 10). Cells were suspended in Hank's balanced salt solution and centrifuged by Cytospin 2 resulting in a monolayer cell smear on glass slides. Air-dried cells were fixed in acetone (10 min , -20˚C). After preincubation with non-immune sheep serum (dilution 1:10, 10 min; Dianova, Hamburg, Germany), the cell smears were incubated with primary antibody in a moist chamber for 20 h at 4˚C. Some of the antibodies were gifts from several laboratories, others were commercially available. The polyclonal anti-glutathione S-transferase-π (GST-π) antibody was kindly donated by Dr. K. Satoh (University School of Medicine, Hirosaki, Japan; working dilution 1:2,000), the polyclonal anti-DNA topoisomerase IIα (Topo IIα) antibody by Dr. L. Liu (John Hopkins Oncology Center, Baltimore, MD, USA; working dilution 1:500), and the polyclonal anti-thymidylate synthetase (TS) antibody by Dr. B. Yates (Burroughs Welcome, Research Triangle Park, Cornwallis USA; working dilution 1:500). The anti-P-glycoprotein (P-gp) monoclonal antibody C219 was obtained from Centocor (Malvern, PA, U.S.A.; dilution: 10 μg/ml), the polyclonal anti-catalase (CAT) antibody from Calbiochem (La Jolla, CA, USA; working dilution 1:100), the monoclonal anti-alkaline phosphatase (AP) antibody AP-59 from BioMakor (Israel; working dilution 1:50), and the monoclonal anti-metallothionein (MT) antibody E9 from DAKO Diagnostika (Hamburg, Germany; working dilution 1:100). The monoclonal anti-protein kinase C α/β (PKC α/β) antibody MC5 was purchased from Amersham (Braunschweig, Germany) and used in a concentration of 10 μg/ml. Proliferative activity was detected by the monoclonal antibody Ki-67 (Dianova, Hamburg, Germany; working dilution 1:10). For detection of the proteins of proto-oncogenes and suppressors, we used the following antibodies: ErbB1 (EGF-R Ab-4), ErbB2 (c-neu Ab-1), Fos (Ab-2), Jun (c-jun/AP-1), Myc (33), K-Ras (234-4.2), H-Ras (235-1.7.1.), N-Ras(F155-277), and p53 (Pab1801). These antibodies were from Dianova and were used at a concentration of 10 μg/ml. As secondary antibodies, biotinylated goat-anti-rabbit or sheep-anti-mouse immunoglobulins (dilution 1:50; Dianova) were applied to the cell smears for 45 min at room temperature. Subsequently, the streptavidin-biotinylated-peroxidase complex (dilution 1:100; Amersham) was added for 15 min at room temperature. The peroxidase activity was visualized with 3-amino-9-ethylcarbazole. Negative controls were done omitting the primary antibody. Endogenous peroxidase activity was quenched using 0.5% hydrogen peroxidase (H_2O_2). The cell smears were preincubated with unlabeled streptavidin (dilution 1:50, 10 min; Amersham) to suppress endogenous biotin activity.

Statistical analysis. Most of the immunohistochemical parameters were evaluated on an ordinary scale: "no reaction" (0), "weak" (1), "moderate" (2), and "strong reaction" (3). The remaining parameters (P-gp, Topo IIα, Ki-67) were counting variables. The investigation of protein expression profiles by hierarchical cluster analysis has been described (4, 11-14). It is an explorative statistical method which groups heterogeneous objects into clusters of homogeneous

Table II. *Correlation between drug resistance and protein expression (according to Kendall's τ-test).*

	Doxorubicin NIT	Doxorubicin GIA	Vincristine GIA	Mafosfamide GIA
P-gp	< 0.0001	0.0006	0.0178	n.s.
GST-p	0.0004	0.0036	0.0001	n.s.
Topo IIa	0.0003	0.0051	0.0053	n.s.
PKC a/b	0.0320	n.s.	0.0026	n.s.
AP	0.0007	0.0308	n.s.	n.s.
CAT	n.s.	n.s.	n.s.	n.s.
MT	n.s.	n.s.	n.s.	n.s.
TS	n.s.	n.s.	0.0309	n.s.
Ki-67	n.s.	0.0258	n.s.	n.s.
ErbB1	0.009	0.0022	0.0108	n.s.
ErbB2	0.009	0.0415	n.s.	n.s.
Fos	0.0197	n.s.	0.0056	n.s.
Jun	n.s.	n.s.	n.s.	n.s.
Myc	n.s.	n.s.	n.s.	n.s.
H-Ras	n.s.	n.s.	n.s.	n.s.
K-Ras	n.s.	0.0052	n.s.	0.0344
N-Ras	n.s.	n.s.	n.s.	0.0173
p53	n.s.	0.0472	n.s.	n.s.

P-values < 0.05 were considered as statistically significant
Abbreviations: GIA, growth inhibition assay; NIT, nucleotide incorporation test
n.s., not significant

objects. Cluster analyses, applying the complete-linkage method, were done by means of the WinSTAT program (Kalmia, Cambridge, MA, USA). Missing values were automatically omitted by the program, and the closeness of two joined objects was calculated by the number of data points they contained. In order to calculate distances of all variables included in the analysis, the program automatically standardizes the variables by transforming the data with mean = 0 and variance = 1.

Kendall's τ-test was used to calculate significance values and rank correlation coefficients as a relative measure for the linear dependency of two variables. This test was implemented into the WinSTAT Program (Kalmia). Kendall's τ-test determines the correlation of rank positions of values. Ordinal or metric scaling of data is suited for the test and are transformed into rank positions. There is no condition regarding normal distribution of the data set for the performance of Kendall's τ-test.

Results

The purpose of the present investigation was to evaluate, whether different protein patterns exist between drug-sensitive and drug-resistant kidney carcinomas. As a first step, cell cultures of primary human kidney carcinoma of 18 patients (RCC1 to RCC18) were analyzed by immunohistochemistry. The semiquantitative scores of the protein expressions are shown in Table I.

A growth inhibition assay was applied for the determination of resistance to doxorubicin, vincristine and mafosfamide (an *in vitro* active derivative of cyclophosphamide) (Table I). In addition, a nucleotide incorporation assay was used as a second test method. A significant correlation was found between the results of the growth inhibition assay and the nucleotide incorporation assay after exposure of the renal carcinoma cells to doxorubicin, indicating that the sensitivity of the cell cultures is independent of the assay used ($p<0.001$; $R=0.86$; Kendall's τ-test).

Furthermore, the IC_{50} values for doxorubicin (measured by the growth inhibition assay) correlated with the IC_{50} values for vincristine ($p=0.02$) but not with the IC_{50} values for mafosfamide ($p=0.13$). That means that a cross-resistance exists between doxorubicin and vincristine but not between doxorubicin and mafosfamide.

The expression levels of the proteins were correlated to the response to doxorubicin, vincristine, or mafosfamide. We found that the expression of P-glycoprotein (P-gp), glutathione S-transferase-π (GST-π), DNA topoisomerase IIα (Topo IIα), protein kinase Cα/β (PKC α/β), alkaline phosphatase (AP), ErbB1, ErbB2 and Fos correlates to doxorubicin resistance as determined by the nucleotide incorporation assay (Table II). If doxorubicin resistance was measured by the growth inhibition assay, significant correlations were observed with P-gp, GST-π, Topo IIα, AP, ErbB1, ErbB2, K-Ras, p53 and Ki-67. Furthermore, the expression of P-gp, GST-π, Topo IIα, PKC α/β, TS, ErbB1 and Fos were correlated with vincristine resistance. Only the expression of K-Ras and N-Ras was associated with mafosfamide resistance (Table II).

As a second step, proteins with a relationship to doxorubicin resistance ($p<0.05$) were analyzed by hierarchical cluster analysis. The resulting dendrogram can be separated into two main clusters (Figure 1, right side).

Then, the two clusters were correlated with the drug resistance data of doxorubicxin, vincristine and mafosfamide (Table III). Cluster 2 was enriched with doxorubicin-resistant cell cultures ($p<0.001$; Fisher's exact test). The same is true for vincristine ($p=0.016$). In contrast, this difference is not visible for mafosfamide ($p=0.53$).

Finally, a clustered image map (CIM) was generated (Figure 1, left side). In fact, the sensitive area was characterized by a less frequent expression of drug resistance proteins than resistant tumors.

Discussion

There is increasing evidence that a wide variety of drug resistance mechanisms operate simultaneously in the development of clinically relevant drug resistance. In the present investigation, we used immunocytochemical assays

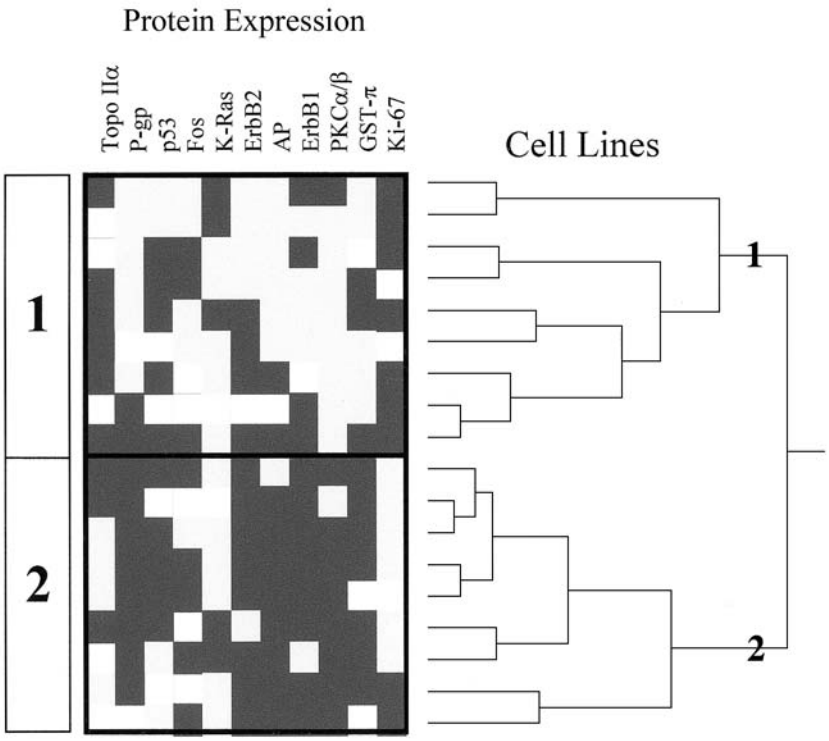

Figure 1. *Dendrogram of hierarchical cluster analysis (complete linkage method) and clustered image map (CIM) obtained from protein expression of 11 parameters in 18 primary renal cell carcinoma cell cultures. The dendrogram and CIM can be separated into two main clusters. Light fields, low or absent protein expression; dark fields, high protein expression; white fields, missing values.*

Table III. *Separation of two clusters obtained by hierarchical cluster analysis shown in Fig. 1 and comparison to drug resistance.*

		Cut-off	Cluster 1	Cluster 2	*P*-Value
Doxorubicin (NIT[a])	sensitive	< 45	9	0	
	resistant	≥ 45	0	9	< 0.001
Doxorubicin (GIA[b])	sensitive	< 50	7	0	
	resistant	> 50	0	6	< 0.001
Vincristine (GIA)	sensitive	< 50	7	1	
	resistant	> 50	1	5	0.016
Mafosfamide (GIA)	sensitive	< 100	3	3	
	resistant	> 100	5	3	0.53

[a] NIT, nucleotide incorporation assay (cut-off in % of control)
[b] GIA, growth inhibition assay (cut-off: IC_{50}, ng/ml)

to analyze expression profiles of proteins of kidney cancer. By means of cluster analysis, we could define sensitive and resistant cell lines of renal cell carcinomas.

The up-regulation of P-gp and GST-π and the down-regulation of Topo IIα for multidrug resistance (MDR) has been well characterized in the past for many tumor types. This is also valid for kidney carcinomas.

Protein kinase Cα/β and alkaline phosphatase (AP) correlated with drug resistance in kidney carcinoma cells as well. This is in accordance with an earlier observation that overexpression and increased enzyme activity is linked to resistance phenotypes (18, 19). Ki-67, a cellular marker for cell proliferation, shows only a weak correlation with resistance.

The role of oncoproteins and tumor suppressor proteins for carcinogenesis and tumor progression is well-known. In addition, they are also involved in drug resistance. In the present investigation, the expression of ErbB1, ErbB2, Fos, K-Ras and p53 correlated significantly with drug resistance except for p53. Growth factor receptors (ErbB1, ErbB2), signal transducers (Ras), transcription factors (Fos, Jun, Myc) and tumor suppressors affect cellular response to

cytostatic drugs by regulation of apoptosis, DNA repair or the induction of resistance gene expression (20).

The determination of protein expression profiles by hierarchical cluster analysis may facilitate the development of novel diagnostic options for an individualized tumor treatment. In the present investigation, we have shown the general feasibility of this concept for a limited number of proteins and cell lines. Future investigations should clarify, whether the set of proteins analyzed in the present investigation are sufficient or whether other proteins should also be included for the prediction of drug resistance. The recently thriving microarray technology may allow simultaneous analyses of hundreds or thousands of genes or proteins of a single patient in a single experiment. On the other hand, it has been suggested that a minimal set of about 10 to 50 proteins or genes may provide more robust results than sets of thousands of them (21). In the present investigation, it was shown that a limited number of proteins is indeed sufficient to predict resistance. We found that resistant cell cultures can be separated from sensitive ones according to the expression profiles in the cluster analysis.

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