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Gene Signatures Developed from Patient Tumor Explants Grown in Nude Mice to Predict Tumor Response to 11 Cytotoxic Drugs

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Abstract. *Patient tumor explants established subcutaneously in serial passage in nude mice were characterized for their sensitivity towards 11 standard cytotoxic anti-cancer agents. The latter include the alkylating agents cyclophosphamide, ifosfamide, mitomycin C, cisplatin and CCNU, the antimetabolites 5-FU and methotrexate; the topoisomerase II inhibitors adriamycin and etoposide as, well as the tubulin binders paclitaxel and vindesine. The mean number of tumors treated with any of the various drugs was 54 (range 31-78). The tumor xenografts' gene expression profiles were determined using the Affymetrix HG-U133 plus 2.0 mRNA expression array representing ~38.500 human genes. The hypothesis was that the correlation of drug response to gene expression would identify gene signatures that can predict the drug response of individual tumors to these agents. Predictive gene signatures were found and subsequently verified using the leave-one-out cross-validation (LOOCV) technique. Tumors were considered as responsive if the drugs effected a tumor volume inhibition to less than 11-41% of the volume of vehicle control tumors (T/C%). The median cut-off over all drugs was a T/C of 25%. Using these criteria, on average one third of the test tumors were sensitive (responders) and two thirds were resistant (non-responders). The bio-informatic analysis yielded predictive gene signatures consisting of 42-129 genes (mean for the 11 drugs: 87 genes). On average, the response rate for predicted responders (83%) was 2.45 fold higher than that for all test tumors (random testing, 34%). This increase of response rates, following signature-guided testing, was consistent for all 11 agents. Conversely, 94% of the predicted non-responders (range: 84-100%) proved to be non-responders in nude mouse studies while the proportion of non-responders among all test tumors was approximately 66%. The majority of genes (59%) making up the predictive gene signatures had an unknown function. Known genes were implicated in cell proliferation, apoptosis, DNA repair, cell cycle, metabolism and transcription. The predictive gene signatures presented here for 11 cytotoxic agents have the potential, if employed in the clinic, to substantially increase tumor response rates compared to empirical drug treatment. However they need to be further validated, both preclinically and clinically.*

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Cancer chemotherapy is still largely empirical. The response rates of the various tumor types to given combination or single agent therapies are well established and are the basis of standard clinical treatment regimens that aim at maximizing the chances of therapeutic success for the whole cancer patient population. Because of the diversity of tumors however, this approach is not necessarily most effective for the individual patient.

In contrast to the standard approach, the concept of individualized cancer therapy aims at accurately predicting the most effective therapy for a given patient. If successful, this approach would spare the patient adverse effects and save costs associated with ineffective therapies. Numerous attempts were made to predict the drug sensitivity of a given tumor by analysing tumor samples obtained from patients *in vitro*. For example, the capacity of drugs to inhibit colony formation by patient-derived tumor cells in agar was used to identify effective drugs. These approaches, however, were not generally accepted and are therefore not widely used (1). An exception is the highly reliable prediction of drug resistance using an *in vitro* thymidine incorporation assay (2).

The recent advent of DNA microarrays representing the complete human transcriptome made it possible to derive global mRNA expression profiles of human tumors. Correlation of tumor gene expression profiles with the drug sensitivities of tumors allows us to derive gene signatures that are indicative of tumor sensitivity to a given drug. Matching such predictive gene signatures for all registered drugs with the mRNA profile of a given tumor has the potential to predict which treatment would be most effective for the tumor in question.

Table I. *Comparison of tumor responses in nude mice and in patients.*

Nude mouse	Patient	Response
remission	remission	19
no remission	remission	2
no remission	no remission	57
remission	no remission	2

80 comparisons were performed in 55 tumors. Xenografts were predicted correctly for resistance in 97% (57/59) and for remission in 90% (19/21) of cases (from Fiebig *et al.* (4)).

Over the years we have established a large collection of patient tumor explants passaged as subcutaneous tumor xenografts in nude mice. We have shown earlier (3) that tumor response in the mouse faithfully recapitulates the response of the same tumor to the same treatment in the patient (Table I). Thus, of 21 tumors that underwent remission in the patient, 19 tumors also underwent remission as a tumor xenograft in the mouse if the mouse received the same treatment as the patient. Only 2 tumors that were sensitive in the patient, did not undergo remission in the mouse. Conversely, of 59 tumors that were resistant in the patient, 57 tumors were also resistant in the mouse. Overall, the correct prediction for resistance was 97% and for sensitivity 90%.

Using the Affymetrix chip HG-U-133 plus 2.0 representing approximately 38.500 human genes (4) we have now determined the gene expression profiles of 200 patient tumor xenografts passaged subcutaneously in nude mice. By correlating tumor sensitivity to 11 registered cytotoxic drugs with gene expression data, we have derived gene signatures that can predict tumor sensitivity to the 11 drugs. The next goal is to develop, for further clinical validation, a custom-made chip to assess tumor gene expression and thereby predict tumor drug sensitivities.

Materials and Methods

Animals. Athymic nude mice of NMRI genetic background were either supplied by our own breeding facility or purchased from Bomholtgard, now Taconic, Ry, DK. In the first passage, tumors from male patients were implanted into male mice, tumors from female patients into female mice. All efficacy tests were carried out in female mice with the exception of testicular and prostate cancers. All animal experiments were performed according to German Animal License regulations and were approved by the local authorities.

Tumors. From more than 1,600 resected solid human malignancies, we were able to establish more than 400 tumor models growing subcutaneously in serial passage in nude mice (5-7). For efficacy tests, two fragments of 1.5-2 mm in diameter were implanted subcutaneously into both flanks of each mouse, for tumors inducing cachexia only one tumor per mouse was implanted. Testings were performed using tumor xenografts in serial passage after their growth had become regular, for most tumors this was between passage 2 and 10. Tumors were not used for more than 10-15 passages after thawing and implanting tumor pieces from frozen master stocks. Four tumor xenografts used in this study were established from human tumor cell lines (MCF7, T24, SKOV3 and CCRF-CEM).

Table II. *Dose levels, schedules and routes of administration for 11 anti-cancer agents in tumor bearing nude mice.*

Drug	Dose level [mg/kg/day]	Schedule [Days]	Route
Cyclophosphamide	200	1,15	*i.p.*
Ifosfamide	130	1-3,15-17	*i.p.*
Mitomycin C	2	1,15	*i.v.*
Cisplatin	6,4 or 8	1,15	*s.c.*
CCNU	20	1	*i.p.*
5-FU	40 or	1-4,15-18	*i.p.*
	100	1,8,15	*i.p.*
Methotrexate	4	1,4,8,11,15,18	*i.p.*
Adriamycin	6 or 8	1,15	*i.v.*
Etoposide	24	1-3, 15-17	*s.c.*
Paclitaxel	15 or 20	1,8,15	*i.v.*
Vindesine	1,5	1,8,15	*i.v.*

Tumor measurements. Tumors were measured either weekly or, for fast growing tumors, twice weekly and volumes were calculated according to the formula $axb^2/2$ where a is the longest diameter and b the perpendicular axis. Relative tumor volumes were calculated for each individual tumor according to tumor size on Day x divided by tumor size on Day 0, *i.e.* at randomization, multiplied by 100. Group median relative tumor volumes were used for evaluation.

Design of efficacy tests. For efficacy tests, tumor-bearing mice were selected randomly for the untreated or vehicle treated control group and the test groups approximately 10-30 days after implantation when tumors had grown to mean tumor diameters of 6 mm (range: 5-7 mm) equal to a tumor volume of approximately 100 mm^3. Each test group consisted of 5 or 6 animals collectively bearing between 6 and 10 evaluable tumors. The day of randomization was designated as Day 0. Day 0 was also the first day of dosing. The experiments were evaluated after 4 - 6 weeks or after 2-3 weeks for very fast growing tumors.

Chemotherapy. Eleven established anti-cancer agents were administered in monotherapy to tumor-bearing nude mice at dose levels, schedules and routes as indicated in Table II (4). The treatment regimens corresponded to clinical schedules with the exception that treatment in mice was usually repeated after 2 weeks instead of the 3 or 4 week intervals used in the clinic. Two treatment cycles were given. Growth curves of 2 sensitive models with long term remissions are shown in Figure 1. Drug dose levels causing up to 20% lethalities after 3 to 4 weeks were considered as the maximum tolerable doses in tumor bearing nude mice and were used as the maximum test dose levels.

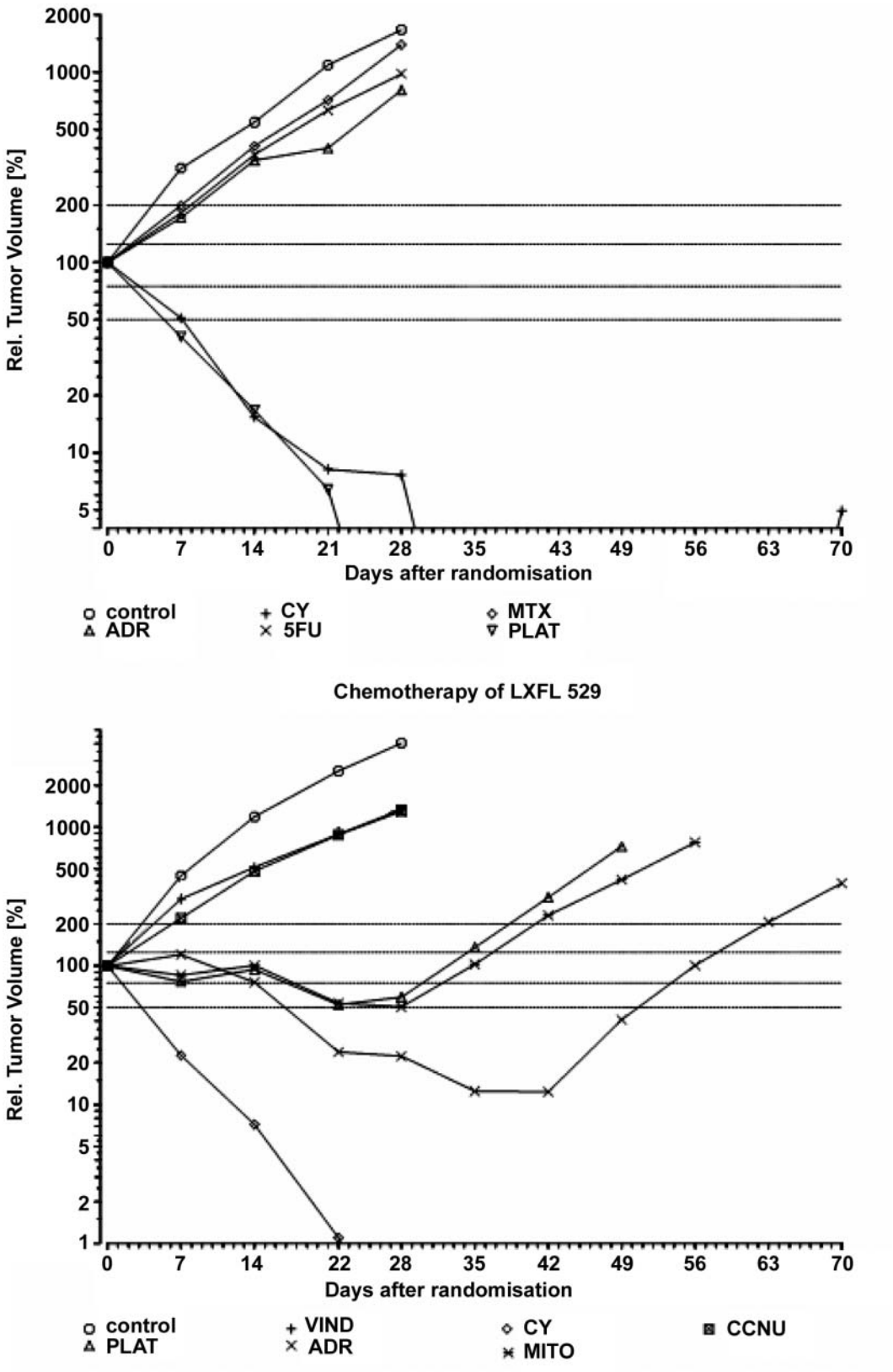

Figure 1. *Effect of chemotherapy in the mammary cancer model MAXF 401 and in the large cell lung cancer model LXFL 529. ADR, adriamycin; CY, cyclophosphamide; MITO, mitomycin C; MTX, methotrexate; PLAT, cisplatin; VIND, vindesine.*

Evaluation parameters for tumor response. Test/control (T/C%) values, *i.e.* the ratio of median relative tumor volumes for test and control groups were calculated for all measurement time points, and the minimum (optimal) T/C value recorded during an experiment was taken as the endpoint for evaluation of tumor sensitivity. Mice had to be sacrificed when the volume of their tumors exceeded 1600 mm^3. For all drugs, the tested tumors were classified either as responders (R) or as non-responders (NR), depending on the optimal T/C value. The cut-off points differed for the various drugs (Table IV) and were chosen such that the ratio of R to NR was approximately 1:2, thus roughly matching the clinical situation and allowing statistically sound correlations of gene expression and efficacy data.

Tumor excision and RNA extraction. For mRNA preparation, tumors were grown in untreated mice. Following sacrifice of mice by cervical dislocation, tumors were excised without delay and tumor pieces free of necrosis were flash frozen in liquid nitrogen. Following mechanical tissue disruption, total tumor RNA was extracted using the RNeasy Mini kit (QIAGEN, Hilden, Germany). Prior to array analysis, one round of T7 promotor-based RNA amplification was performed.

Microarrays. Affymetrix® HG-U133 Plus 2.0 mRNA expression arrays were used to determine the expression of 47,400 transcripts, corresponding to human 38,500 genes. The HG-U133 Plus 2.0 mRNA expression arrays have proven high reproducibility for mRNA expression analysis (8).

Data preprocessing and normalization. CEL result files were pre-processed using the gc-RMA (9) algorithm independently for training and validation sets. Chip normalization to the 50th percentile was performed afterwards.

Data analysis. Predictive transcripts were identified by an iterative leave-one-out/intersection process utilizing Genespring BioScript SG2c-2 (Agilent, Santa Clara, US). Support vector machines were used as the class prediction algorithm (10-12).

Leave-one-out cross-validation. Predictive gene signatures were generated using the expression profiles and sensitivity data of all n test tumors as a training set. Leave-one-out cross-validation (LOOCV) involved removing a single tumor from the original training set of n tumors and using the remaining n-1 tumors as the training set and the removed tumor for validation. This procedure was repeated such that each tumor in the original training set was used once for validation.

Results

Efficacy of 11 cytotoxic agents in tumors with known gene expression profiles. A prerequisite for the derivation of gene signatures predicting tumor sensitivity towards each of the 11 cytotoxic drugs was the availability for the same tumors of gene expression profiles and efficacy data for monotherapy with the 11 drugs. Global mRNA expression profiles were determined for approximately 200 different patient tumor explants passaged as subcutaneous xenografts in nude mice. The tumors were of various types (Table III), including mainly non-small cell lung (n=44), colon (38), kidney (17), breast (12), and head and neck (12) cancers as well as melanomas (22) and sarcomas (10). Efficacy tests for the various drugs were predominantly carried out in the subset of tumor types for which the respective drugs are registered or for which clinical activity has been demonstrated. The cut-off points for efficacy differed for the various drugs and were chosen such that the ratio of responders (R) to non-responsders (NR) was approximately 1:2, thus roughly matching the clinical situation and allowing statistically sound correlations of gene expression and efficacy data. An overview over the numbers of tumors tested, the classification borders and the proportion of sensitive and resistant test tumors (referred to as R for responder and NR for non-responder, respectively) for the various agents is given in Table IV.

Table III. *Identity of human tumor models used for gene expression profiling.*

Tumor	Gene expression HG-U133 Plus 2.0
Lung non-small cell	44
Colon	38
Melanoma	22
Breast	12
Kidney	17
Head and Neck	12
Sarcomas	10
Gastric	7
Bladder	8
Pancreas	6
Lung small cell	4
Miscellaneous	20
Total	200

The *alkylating agents* cyclophosphamide, ifosfamide, mitomycin C, cisplatin, and CCNU were tested in a total of (depending on the drug) 31-78 tumors. The main test tumor types were non-small cell lung cancer (all 5 compounds), mammary cancer (all compounds except CCNU) as well as small cell lung cancer, gastric cancer, cancer of the testis and melanoma (all compounds except cyclophosphamide and ifosfamide). The cut-off point for activity of the various drugs was a tumor volume inhibition (T/C%) to smaller than (depending on the drug) 11-34% of the volume of vehicle control tumors. The proportion of sensitive tumors ranged from approximately 32 to 42%. About one half of these responders underwent remission while the volume of most of the remaining responders was stable (no change). "No change" still translates into a clinical benefit, especially since drugs are administered in combination.

Table IV. *Derivation of predictive gene signatures for 11 anti-cancer agents: number of predictive genes, classification borders and results of efficacy tests.*

Drugs	Predictive genes n	T/C, cut-off	Number of tumors in training set				
			R	R in %	NR	NR in %	Total
Alkylating agents							
Cyclophosphamide	129	<11%	26	35.1%	48	64.9%	74
Ifosfamide	81	<24%	15	38.5%	24	61.5%	39
Mitomycin C	115	<30%	23	36.5%	40	63.5%	63
Cisplatin	84	<25%	25	32.1%	53	67.9%	78
CCNU	85	<11%	12	38.7%	19	61.3%	31
Antimetabolites							
5-FU	86	<40%	18	31.6%	39	68.4%	57
Methotrexate	42	<41%	6	21.4%	22	78.6%	28
Topoisomerase II inh.							
Adriamycin	79	<35%	23	34.8%	43	65.2%	66
Etoposide	66	<30%	10	23.8%	32	76.2%	42
Tubulin binder							
Paclitaxel	96	<12%	21	41.2%	30	58.8%	51
Vindesine	95	<13%	23	38.3%	37	61.7%	60
Total	958		202		387		589
Mean	87.1%		34.3%		65.7%		54

The *antimetabolites* 5-FU and methotrexate were tested in 57 and 28 tumors, respectively. The main test tumor types for 5-FU were colon, mammary, gastric and pancreatic cancer. For methotrexate test tumors were evenly distributed among the various tumor types. The threshold for activity was set at relatively high optimal T/C values of 40 and 41%, respectively, taking into account that for both drugs patients tolerate more than three times the maximum tolerated dose of mice, based on body surface. Using these criteria, 32 and 21% of the tumors were classified as responsive.

The *topoisomerase-II-inhibitors* adriamycin and etoposide were tested in 66 and 42 tumors, respectively. The main test tumors for both compounds were non-small cell lung cancer, mammary cancer, for adriamycin in addition small cell lung, pancreatic, bladder and renal cell cancer and for etoposide ovarian and testis cancer. Tumors showing tumor volume inhibition to <35% of the vehicle control group were considered as sensitive which was the case for 35 and 31% of the tested tumors, respectively.

The *tubulin binders* paclitaxel and vindesine were tested in 49 and 68 tumors, respectively, among them predominantly non-small cell lung and mammary cancers for both compounds and for vindesine in addition small cell lung and gastric cancers. The cut-off points were at minimum T/C values of 12 and 13%, respectively, and the proportion of sensitive (responsive) tumors was 41 % for paclitaxel and 38% for vindesine. As for alkylating agents, about one half of the responders underwent remission while the volume of most of the remaining responders was stable.

Utility of predictive gene signatures. The efficacies of mitomycin-C and adriamycin in the respective 63 and 66 test tumors are given in Tables V and VI. The derived gene signatures predicting tumor sensitivities towards the 2 drugs, as assessed based on leave-one-out cross-validation, comprise 79 and 115 genes, respectively. Out of 26 tumors predicted to be sensitive to mitomycin C, 20 (77%) were sensitive in animal testings. For adriamycin, out of 28 tumors predicted to be sensitive, 19 (=68%) actually were. In summary, if only the tumors predicted to be sensitive had been tested with mitomycin C and adriamycin, response rates of 77% (20 out of 26) for mitomycin C and of 68% (19 out of 28) for adriamycin would have been obtained as compared to response rates of 37% and 35% for random testing, *i.e.* using the gene signatures for test tumor selection, the response rates increased by factors of 2.08 and 2.06 compared with random testings. The correct predictions for non-responders were 34 out of 37 (92%) for mitomycin C and 34 out of 38 (89%) for adriamycin. The proportion of non-responders among all test tumors was 40 out of 63 (63.5%) and 43 out of 66 (65.2%) for mitomycin C and adriamycin, respectively.

The distribution of the real T/C values among the predicted mitomycin C and adriamycin responders and non-responders is presented in Figure 2. Among the predicted responders, the median T/C achieved by mitomycin C in real testings was 10% as compared to 56% for the predicted non-responders. For adriamycin the median T/C values among predicted responders and non-responders were 31% and 56%,

Table V. *Real and predicted efficacy and cross-validation for mitomycin C in human tumor xenografts.*

Tumor	T/C[%]	real	predicted
CXF_280	1	R	R
GXF_281	1	R	R
GXF_97	1	R	R
LXFL_529	1	R	R
LXFS_605	1	R	R
MAXF_401	1	R	R
MAXF_449	2	R	R
LXFA_289	3	R	R
LXFE_211	3	R	R
MAXF_MX1	5	R	R
LXFE_409	6	R	R
OVXF_1023	8	R	R
CXF_260	9	R	R
MAXF_583	11	R	R
MAXF_508	13	R	R
LXFA_629	16	R	R
GXF_251	17	R	R
LXFA_400	18	R	R
MAXF_1384	18	R	R
GXF_602	24	R	R
CXF_1103	45	NR	R
LXFE_397	56	NR	R
MAXF_1162	58	NR	R
MAXF_857	69	NR	R
MAXF_MCF7	73	NR	R
OVXF_899	76	NR	R

Tumor	T/C[%]	real	predicted
PXF_541	32	NR	NR
CXF_264	34	NR	NR
PAXF_546	34	NR	NR
CXF_269	35	NR	NR
CXF_676	39	NR	NR
MAXF_574	39	NR	NR
RXF_944LX	42	NR	NR
CXF_975	44	NR	NR
MEXF_274	45	NR	NR
OVXF_1353	48	NR	NR
LEXF_1189	52	NR	NR
MEXF_1341	53	NR	NR
CNXF_498	54	NR	NR
MEXF_276	54	NR	NR
MEXF_1732	55	NR	NR
CXF_158	56	NR	NR
CXF_609	57	NR	NR
LXFL_1072	59	NR	NR
CXF_647	64	NR	NR
RXF_423	64	NR	NR
LXFA_526	67	NR	NR
MEXF_514	67	NR	NR
RXF_631	68	NR	NR
CXF_1729	69	NR	NR
PAXF_736	70	NR	NR
RXF_1393	72	NR	NR
CXF_886	74	NR	NR
CXF_243	79	NR	NR
MEXF_535	79	NR	NR
RXF_486	80	NR	NR
BXF_T24	81	NR	NR
RXF_1220	81	NR	NR
RXF_393	89	NR	NR
SXF_1410	89	NR	NR
GXF_209	2	R	NR
MEXF_989	10	R	NR
PAXF_1657	27	R	NR

***In vivo* study**

63 Tumors tested

23 (37%) Responsive(R) T/C<30%
40 (63%) Not responsive (NR) T/C>=30%

Predicted	*in vivo* real
R 26	**R 20 (77%)**
	NR 6 (23%)

Predicted	*in vivo* real
NR 37	R 3 (8%)
	NR 34 (92%)

respectively. For both mitomycin C and adriamycin, rank sum tests demonstrated significance of the difference in drug efficacy among predicted responders and non-responders ($p<0.001$). In summary, the utility of the predictive mitomycin C and adriamycin gene signatures was demonstrated based on the overall response rates and the median T/C values for predicted responders and non-responders on the one hand and randomly tested tumors on the other.

With the same methodology, predictive gene signatures for 9 additional compounds (cyclophosphamide, ifosfamide, cisplatin CCNU, 5-FU methotrexate, etoposide, paclitaxel and vindesine) were evaluated. For all compounds, the actual response rate among predicted responders was on average 2.45-fold higher than the response rate for all tumors, *i.e.* for random testing. The increase of the response rate ranged from 2.06- to 4.17-fold for the individual compounds.

Table VI. *Real and predicted efficacy and cross-validation for adriamycin in human tumor xenografts.*

Tumor	T/C[%]	real	pred
LXFL_529	2	R	R
LXFS_573	3	R	R
LXFS_650	3	R	R
MEXF_276	4	R	R
LXFE_409	5	R	R
RXF_393	8	R	R
LXFS_605	9	R	R
SXF_627	22	R	R
PXF_537	23	R	R
LEXF_1189	24	R	R
LXFE_937	25	R	R
MEXF_989	25	R	R
LXFL_1072	29	R	R
RXF_944LX	29	R	R
GXF_281	32	R	R
BXF_1218	33	R	R
MAXF_MX1	33	R	R
CXF_280	34	R	R
GXF_97	34	R	R
MAXF_574	42	NR	R
SXF_1410	49	NR	R
LXFE_397	52	NR	R
MEXF_514	55	NR	R
LXFS_615	62	NR	R
PXF_541	64	NR	R
MEXF_535	81	NR	R
LEXF_CCRFCEM	86	NR	R
LXFA_289	95	NR	R

Tumor	T/C[%]	real	pred
BXF_1228	35	NR	NR
BXF_T24	35	NR	NR
RXF_1393	35	NR	NR
OVXF_1353	36	NR	NR
CXF_1788	37	NR	NR
RXF_486	37	NR	NR
GXF_602	39	NR	NR
MAXF_401	39	NR	NR
LXFL_430	41	NR	NR
LXFA_629	42	NR	NR
OVXF_899	43	NR	NR
PAXF_1657	47	NR	NR
OVXF_SKOV3	50	NR	NR
PAXF_546	53	NR	NR
RXF_631	54	NR	NR
RXF_1220	56	NR	NR
LXFE_211	57	NR	NR
MAXF_1322	57	NR	NR
MAXF_1384	58	NR	NR
MAXF_857	59	NR	NR
CXF_158	60	NR	NR
CXF_975	62	NR	NR
CXF_1103	66	NR	NR
GXF_251	67	NR	NR
MAXF_583	67	NR	NR
MAXF_MCF7	71	NR	NR
MEXF_274	72	NR	NR
LXFA_677	75	NR	NR
LXFS_538	75	NR	NR
CXF_609	80	NR	NR
MAXF_1162	82	NR	NR
CXF_243	84	NR	NR
BXF_1258	96	NR	NR
RXF_423	98	NR	NR
GXF_209	5	R	NR
PAXF_736	10	R	NR
MAXF_449	12	R	NR
LXFA_526	15	R	NR

***In vivo* study**
66 Tumors tested
23 (35%) Responsive(R) T/C<35%
43 (65%) Not responsive (NR) T/C>=35%

Predicted	***in vivo* real**
R 28	**R 19 (68%)**
	NR 9 (32%)

Predicted	***in vivo* real**
NR 38	R 4 (11%)
	NR 34 (89%)

For all 11 compounds, random testings of all tumors resulted in an average tumor response rate of 34% which increased to 82% if only the subgroup of gene signature-predicted responders were tested. Gene signature-guided prediction of resistance (no response) was 94% correct, *i.e.* 94% (range: 84-100% for the various compounds) of the predicted non-responders actually did not respond. The proportion of non-responders following random testing was on average only 66%. Details are shown in Table VII.

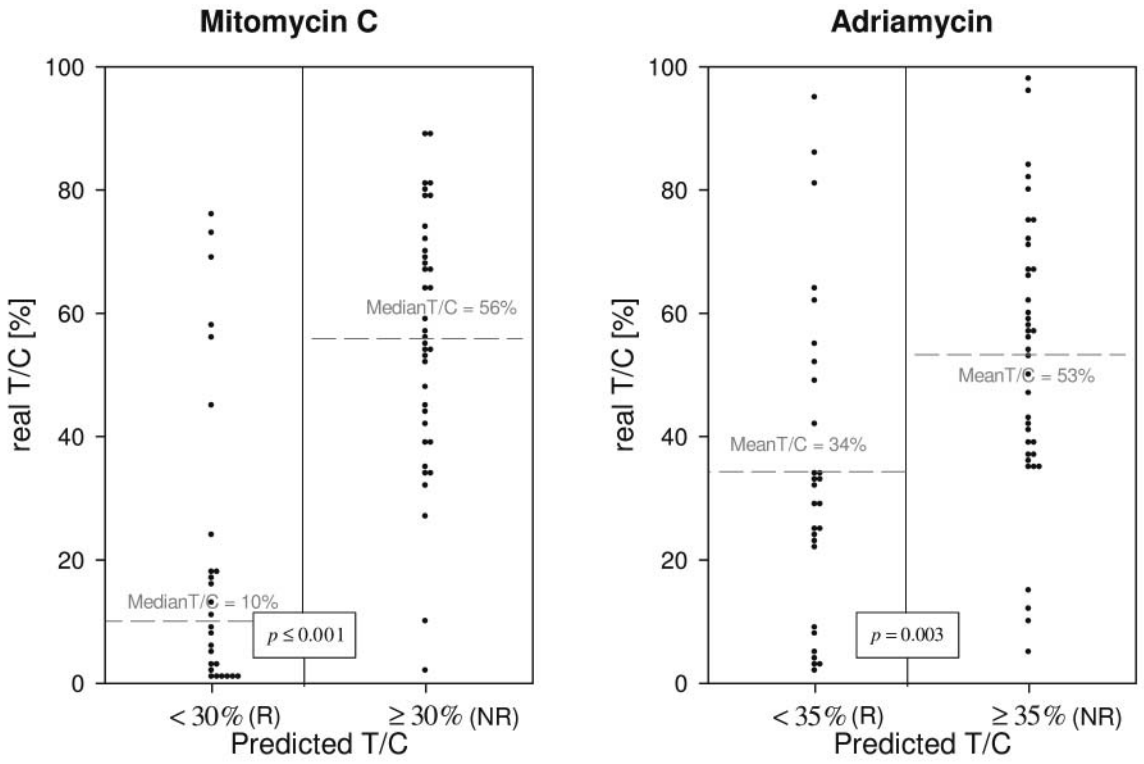

Figure 2. *Mitomycin C and adriamycin activity (expressed as T/C values) in tumors predicted as responsive vs. non-responsive. P-values according to Mann-Whitney Rank Sum Test.*

Prediction of the mitomycin C and adriamycin responses for an independent set of 106 additional tumors. In addition to the 63 and 66 tumors, respectively, whose gene expression profiles and mitomycin C and adriamycin sensitivities were used to derive predictive gene signatures, 106 untested tumors with known gene expression profiles were available. The predictive gene signatures were used to predict mitomycin C and adriamycin responses of those tumors. Table VIII (for mitomycin C) and IX (for adriamycin) show the response predictions separately for tumor types known to be sensitive in the clinic (A) and for tumor types that are either predominantly resistant or have not yet been tested clinically (B).

For tumor types considered as mitomycin C-sensitive, 32 out of 70 individual untested tumors (46%) were predicted to be mitomycin C-sensitive, including 3 out of 4 small cell lung cancers, 3 out of 4 cervix uteri cancers, 2 out of 4 bladder cancers, 13 out of 28 non-small cell lung cancers, 5 out of 14 colorectal cancers, 1 each of gastric, mammary and pancreatic cancers and 1 sarcoma. Among the 63 tested tumors used to derive the mitomycin C gene signature (see above), 47 belonged to mitomycin C-sensitive tumor types. Among those 47 tumors, sensitivity was demonstrated for 23 (49%). In summary, in the case of the mitomycin C-sensitive tumor types, 55 out of a total of 117 (38%) were either demonstrated or predicted to be sensitive. Conversely, for tumor types clinically considered as mitomycin C-resistant (or that had never been tested in the clinic), only 3 out of 36 untested tumors (8%) were predicted to be sensitive, including 2 out of 5 head and neck cancers and 1 leukemia but no responses were predicted for any of the liver cancers, renal cell cancers, melanomas and a myeloma (Table VIII B). Among the 63 tested tumors used to derive the mitomycin C gene signature (see above), 16 belonged to mitomycin C-resistant tumor types. Among those 16 tumors, sensitivity was demonstrated for one melanoma only (6%). Summing up for the mitomycin C-resistant tumor types, 4 out of a total of 52 (8%) were either demonstrated or predicted to be sensitive.

For tumor types expected to be adriamycin-sensitive, 20 out of 54 untested tumors (37%) were predicted as responsive including 5 out of 5 leukemias, 2 out of 3 sarcomas, 6 out of 20 non-small cell lung cancers, 1 out of 3 mammary cancers, 1 out of 3 small cell lung cancers, 1 out of 3 bladder cancers, 1 out of 3 pleuramesotheliomas, the only myeloma and the only testicular cancer (Table IX A). Among the 66 previouisly tested tumors used to derive the adriamycin-specific predictive gene signature (see above), 44 belonged to adriamycin-sensitive tumor types. Among those 44 tumors, sensitivity was demonstrated for 17 (39%).

Table VII. *Leave-one-out cross-validation of predictive gene profiles for 11 anticancer agents.*

Substance	# Genes	# Tumors tested		Class. Border		Responsive		Response Rate	Correct Pred.
			R		NR	Random	Signature	Increase	NR
Alkylating Agents									
Cyclophosphamide	129	74	<	11%	>	35%	79%	x2.26	97%
Ifosfamide	81	39	<	25%	>	38%	93%	x2.45	92%
Mitomycin C	115	63	<	30%	>	37%	77%	x2.08	92%
Cisplatin	84	78	<	25%	>	32%	73%	x2.28	84%
CCNU	85	31	<	11%	>	39%	83%	x2.13	89%
Antimetabolites									
5-FU	86	57	<	40%	>	32%	84%	x2.63	95%
Methotrexate	42	28	<	41%	>	21%	75%	x3.57	100%
Topoisomerase II Inh.									
Adriamycin	79	66	<	35%	>	33%	68%	x2.06	89%
Etoposide	66	42	<	30%	>	24%	100%	x4.17	94%
Tubulin Binder									
Paclitaxel	96	51	<	12%	>	41%	86%	x2.1	93%
Vindesine	95	60	<	13%	>	38%	87%	x2.29	92%
Ø	87	54				34%	82%	x2.45	93%

Table VIII. *A) Mitomycin C efficacy in clinically sensitive tumor types: results for 47 tested tumors and predictions for 70 untested tumors.*

Cancer type	Mitomycin C real				Mitomycin C predicted				Mitomycin C real + predicted			
	R	NR	Total	%	R	NR	Total	%	R	NR	Total	%
Bladder		1	1	0%	2	4	6	33%	2	5	7	29%
Colon	2	11	13	15%	5	9	14	36%	7	20	27	26%
Gastric	6		6	100%	1	1	2	50%	7	1	8	88%
Lung NSCLC	6	3	9	67%	13	15	28	46%	19	18	37	51%
Lung small cell	1		1	100%	3	1	4	75%	4	1	5	80%
Mammary	6	4	10	60%	1	1	2	50%	7	5	12	58%
Ovary	1	2	3	33%	1	2	3	33%	2	4	6	33%
Pancreas	1	2	3	33%	1	1	2	50%	2	3	5	40%
Sarcoma		1	1	0%	1	3	4	25%	1	4	5	20%
Testicle					1		1	100%	1		1	100%
Uterus cervix					3	1	4	75%	3	1	4	75%
Total	23	24	47	49%	32	38	70	46%	55	62	117	47%

Table VIII. *B) Mitomycin C efficacy in clinically resistant tumor types: results for 16 tested tumors and predictions for 36 untested tumors.*

Cancer type	Mitomycin C real				Mitomycin C predicted				Mitomycin C real + predicted			
	R	NR	Total	%	R	NR	Total	%	R	NR	Total	%
Head + Neck					2	3	5	40%	2	3	5	40%
Leukemias+ Lymphomas		1	1	0%	1	5	6	17%	1	6	7	14%
Liver						2	2	0%		2	2	0%
Melanoma	1	6	7	14%		9	9	0%	1	15	16	6%
Myeloma						1	1	0%		1	1	0%
Pleuramesothelioma		1	1	0%		4	4	0%		5	5	0%
Renal		7	7	0%		9	9	0%		16	16	0%
Total	1	15	16	6%	3	33	36	8%	4	48	52	8%

Table IX. *A) Adriamycin efficacy in clinically sensitive tumor types: results for 44 tested tumors and predictions for 54 untested tumors.*

Cancer type	Adriamycin real				Adriamycin predicted				Adriamycin real + predicted			
	R	NR	Total	%	R	NR	Total	%	R	NR	Total	%
Bladder	1	3	4	25%	1	2	3	33%	2	5	7	29%
Gastric	3	2	5	60%		2	2	0%	3	4	7	43%
Leukemias+ Lymphomas	1	1	2	50%	5		5	100%	6	1	7	86%
Lung NSCLC	5	6	11	45%	6	20	26	23%	11	26	37	30%
Lung small cell	3	2	5	60%	1	2	3	33%	4	4	8	50%
Mammary	2	8	10	20%	1	2	3	33%	3	10	13	23%
Myeloma					1		1	100%	1		1	100%
Ovary		3	3	0%	1	3	4	25%	1	6	7	14%
Pleuramesothelioma	1	1	2	50%	1	2	3	33%	2	3	5	40%
Sarcoma	1	1	2	50%	2	1	3	67%	3	2	5	60%
Testicle					1		1		1		1	100%
Total	17	27	44	39%	20	34	54	37%	37	61	98	38%

Table IX. *B) Adriamycin efficacy in clinically resistant tumor types: results for 22 tested tumors and predictions for 52 untested tumors.*

Cancer type	Adriamycin real				Adriamycin predicted				Adriamycin real + predicted			
	R	NR	Total	%	R	NR	Total	%	R	NR	Total	%
CNS						1	1			1	1	0%
Colon	1	6	7	14%	1	19	20	5%	2	25	27	7%
Head+Neck					1	4	5	20%	1	4	5	20%
Melanoma	2	3	5	40%	3	8	11	27%	5	11	16	31%
Pancreas	1	2	3	33%		2	2	0%	1	4	5	20%
Renal	2	5	7	29%		9	9	0%	2	14	16	13%
Uterus cervix						4	4			4	4	0%
Total	6	16	22	27%	5	47	52	10%	11	63	74	15%

Summing up for the adriamycin-sensitive tumor types, 37 out of a total of 98 tumors (38%) were either demonstrated or predicted to be sensitive. For tumor types being considered as resistant in the clinic, only 5 out of 47 untested tumors (10%) were predicted to be adriamycin-sensitive, among them 3 out of 11 melanomas (27%), 1 out of 19 colorectal cancers (5%), and 0/9 renal cancers (Table IX B). Among the 66 tested tumors used to derive the adriamycin gene signature, 22 belonged to adriamycin-resistant tumor types. Among those 22 tumors, sensitivity was demonstrated for 6 tumors (27%). Summing up for the adriamycin-resistant tumor types, 11 out of a total of 74 (15%) were either demonstrated or predicted to be sensitive.

In summary, when combining the results of real efficacy tests with the predicted tumor responses, the proportion of sensitive tumors among clinically sensitive and clinically resistant tumors was in agreement with clinical data.

Function of genes contained in predictive gene signatures. The genes making up the predictive gene signatures were identified using an algorithm comprising a combination of different statistical tests (12). Collectively, the genes allow predictions of tumor drug sensitivities. For the gene signatures presented here, the mean number of genes per signature was 87 (range: 42-129). On average 58.9% of those genes have an unknown function, as apparent from the fact that they were not annotated to any of the different functional categories of genes by the gene ontology consortium (Table X). Looking at the six functional categories considered as particularly relevant to oncology, a mean of 1.5% of the genes per signature were implicated in cell proliferation, 1.8% in apoptosis, 1.3% in DNA repair, 3.4% in cell cycle, 26.1% in metabolism and 10.3% in transcription. Please note that genes can be assigned to more than one functional class.

Table X. *Representation of selected gene classes* the 11 predictive gene signatures.*

Substance	Gene number	Proliferation	Apoptosis	DNA repair	Cell cycle	Metabolism	Transcription	Not-annotated
Alkylating agents								
Cyclophosphamide	129	1	5	1	7	29	15	59
Ifosfamide	81	0	1	0	3	18	10	49
Mitomycin C	115	4	3	6	5	38	8	63
Cisplatin	84	0	1	2	5	11	3	61
CCNU	85	2	2	2	3	24	12	54
Antimetabolites								
5-FU	86	4	2	0	3	17	6	53
Methotrexate	42	0	1	0	1	9	4	27
Topoisomerase II inh.								
Adriamycin	79	0	1	0	2	22	11	50
Etoposide	66	1	1	1	1	28	10	28
Tubulin binder								
Paclitaxel	96	2	0	0	1	30	11	54
Vindesine	95	0	0	0	2	24	9	66
mean	87.1	1.3	1.5	1.1	3.0	22.7	9.0	51.3
		1.5%	1.8%	1.3%	3.4%	26.1%	10.3%	58.9%

*as defined by the gene ontology consortium. Some genes were annotated to more than one class.

Discussion

Predictive gene signatures for 11 cytotoxic agents. We have used gene expression profiles and drug sensitivities of patient-derived tumor xenografts grown in nude mice to identify gene signatures that can predict tumor sensitivities to 11 cytotoxic drugs. Compared with random testing, the predictive gene signatures significantly improved tumor response rates in leave-one-out cross-validation. Conversely, the proportion of resistant tumors was clearly higher among predicted non-responders as compared to all test tumors. Actual and predicted response rates were substantially higher for clinically sensitive tumor types as compared to clinically resistant tumor types. The 11 predictive gene signatures reported here consist on average of 87 genes (range: 42-129). For all 11 signatures, the proportion of genes implicated in metabolism clearly exceeds the proportion of genes involved in other relevant cellular processes such as cell proliferation and apoptosis, indicating that not only genes directly involved in the mechanism of drug action impact on tumor sensitivity.

A prerequisite for the validity of our approach to deriving predictive gene signatures is that gene expression patterns and drug sensitivities of tumor xenografts remain stable during tumor passages in mice. While we have shown in numerous experiments over many years (5) that drug sensitivities do not change significantly over at least 15 passages, preliminary data indicate that gene expression profiles also remain stable. Thus, correlation coefficients for mRNA profiles of the same tumor at different passages starting at passage 0 (original patient tumor) up to passage 12 were generally above 0.8 for all transcripts and above 0.9 for the transcripts of the predictive genes (unpublished data).

We estimate that the tumor training sets required to derive predictive gene signatures should ideally comprise 50 tumors or more. To obtain the necessary tumor numbers, in this study we included tumors of, depending on the drug, as many as 6-10 different types. Consequently, our signatures are applicable to all tumor types represented in the training sets. Experiments are underway to generate tumor type-specific gene signatures by admitting only tumors of one type, *e.g.* colorectal cancer, to the training set. We expect that the resulting signatures will be more accurate and possibly comprise fewer genes.

Clinical perspectives of predictive gene signatures derived from patient tumor explants passaged in nude mice. Gene expression profiles mirror the complexity and diversity of human tumors. Since they are easily accessible, they could form the basis of individualized cancer therapy. Indeed, by correlating gene expression profiles determined for patient tumor samples with clinical outcome, gene signatures have been developed that predict the prognosis and drug response of human tumors. Gene signatures for the prediction of patient prognosis were published for breast and non-small cell lung cancers, lymphomas and mesotheliomas (13-18). Diagnostic tests have been developed by Genomic Health (Oncotype DX, 21 genes assessed by PCR) and Agendia (mamma-print, 70 genes assessed by array analysis) to identify primary breast

cancer patients with a high risk of relapse (19, 20) and to then decide on adjuvant chemotherapy accordingly. Both tests have now been approved by the FDA. They will need further validation in prospective clinical studies which are underway. Examples of gene signatures for the prediction of drug response include a 92 gene signature for Docetaxel in the neoadjuvant setting in advanced breast cancer patients (21) and 512 and 30 gene signatures for the prediction of pathological complete remission, following neoadjuvant chemotherapy of breast cancer patients with either gemcitabine, epirubicine and docetaxel (22) or with paclitaxel/ fluorouracil, doxorubicin and cyclophosphamide (23).

A disadvantage inevitably associated with the use of clinical data for the generation of predictive gene signatures is that most patients receive combination therapies, making it impossible in most settings to derive gene signatures for monotherapies and to prove how much a single agent actually contributed to a therapeutic outcome. In the absence of controls, it is strictly speaking not even possible to attribute a therapeutic outcome for a given patient (*e.g.*, stable disease) unequivocally to the treatment of that patient. These disadvantages can be avoided using experimental systems such as human tumor cell lines cultured *in vitro*. Combining the gene expression profiles of tumor cell lines with their drug sensitivities, gene signatures were derived that successfully predict tumor cell sensitivity to monotherapy with investigational and registered anti-cancer agents (24-26). Depending on the choice of tumor cells for the training set, gene signatures can be independent of a test cells' tissue of origin. Interestingly, at least in one instance predictive gene signatures derived from cultured tumor cell lines were used successfully to predict the drug sensitivity of patient tumors (25). On the other hand, tumor cell line-based experimental systems have been criticised for lack of clinical relevance and are, therefore, not generally accepted. For example, as a consequence of prolonged *in vitro* culture, variants of the cell lines were selected that are well adapted to rapid *in vitro* growth but differ from the original patient tumor. Indeed, nude mouse tumor xenografts derived from most cultured tumor cell lines are histologically homogeneous and undifferentiated and differ in this regard from patient tumors. Second, the *in vitro* culture conditions of tumor cell lines and the assay parameters (induction of apoptosis and/or inhibition of cell proliferation on a plastic surface) are not representative for the clinical situation (inhibition of 3D tumor growth in the context of tissue).

Patient tumor explants passaged in nude mice combine most of the advantages of the clinic and tumor cell line-based experimental systems and avoid most of the restrictions. As demonstrated by extensive comparisons of drug sensitivities and histology, the patient tumor xenografts retain most of the characteristics of the original patient tumor (5) and the principal assay parameter, *i.e.* inhibition of a real tumor, is similar in patients and mice. On the other hand, in contrast to the clinic, experiments are rapid and well controlled and efficacy in a given tumor can be unequivocally attributed to individual drugs. Using this system, predictive gene signatures can be generated for experimental compounds, prior to any clinical tests. Restrictions for drugs directed toward tumor stroma components may arise from the fact that after 2 or 3 passages in the mouse the tumor stroma is of mouse origin, while the tumor cells themselves are of human origin. Restrictions may also exist for hormone-dependent tumors which exhibit relatively low take rates in mice and whose clinical diversity may not be adequately represented among nude mouse tumor xenografts.

In summary, we believe that predictive gene signatures derived from patient tumor explants passaged in nude mice have great potential to predict drug sensitivities of patient tumors and could complement the signatures generated with clinical data. Accordingly, we have initiated the clinical validation of our signatures, using the gene expression profiles of patient tumor samples to predict tumor sensitivities.

Conclusion

Gene expression profiles represent the diversity and individuality of human tumors. Predictive gene signatures link tumor gene expression profiles with therapeutic outcome and therefore have the potential to form the basis of individulalized cancer therapy.

Human tumor explants passaged subcutaneously in nude mice are a promising system to derive predictive gene signatures. On the one hand, they faithfully recapitulate the sensitivity of the original patient tumors to cytotoxic drugs, a fact indicating their clinical relevance. On the other hand, they have the advantages of experimental systems, *i.e.* there is the capacity to rapidly obtain clean and well controlled data under defined conditions.

Predictive gene signatures for 11 cytotoxic drugs have been derived using the tumor xenograft system. Initial data generated with these gene signatures are consistent and plausible. However, validation of the signatures, in particular for clinical application, is still required.

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Conflicts of Interest

All authors are employees of Oncotest GmbH, HHF is the owner.

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