Gene Signature-based Prediction of Tumor Response to Cyclophosphamide

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Abstract. *Cyclophosphamide (CY) is a clinically used cytotoxic agent that is effective in a wide range of tumor types including breast and small cell lung cancers. However, by far not all patients benefit from CY therapy. We used patient tumor explants grown in nude mice as an experimental model system to identify a gene signature that, based on a tumor's gene expression profile, predicts its CY response. Forty-nine human tumor xenografts of different histologies were defined as the training set. Correlation of the gene expression profiles of untreated tumors to the sensitivity of the same tumors to CY led to the identification of 129 transcripts as predictive biomarkers for CY response. Interestingly, the products of 12 of these genes were known to interact at least indirectly with CY. A leave-one-out cross-validation approach led to a correct prediction of the CY response of the training set tumors in 15 out of 18 cases (83%) as compared to a response rate of 18 out of 49 (32%), following random testing. For an independent set of 25 previously untested tumors with known gene expression profiles (validation set) CY sensitivity was predicted correctly for 6 out of 8 tumors (75%), and CY resistance for 15 out of 17 tumors (88%). In comparison, random testing of the same tumors resulted in a response rate of 8 out of 25 (32%). For the same 25 tumors, the median minimum T/C value for predicted responders was 1% as compared to 49% for predicted non-responders. Finally, for tumor types considered as CY sensitive such as small cell lung and breast cancers as well as melanoma, the combined real and predicted response rates for 37 tested and 26 untested tumors was 49%. In contrast, for tumor types considered as CY resistant, including colon and renal cancer, the combined real and predicted response rate for 37 tested and 75 untested tumors was only 13%. Taken together, we identified a gene signature that can predict tumor response to CY and warrants clinical validation.*

In the clinic, the alkylating agent CY is active in a broad spectrum of tumor types. It is used in combination chemotherapy regimens for the treatment of breast, small cell lung and endometrial cancers as well as in Hodgkin's and non-Hodgkin's lymphoma, sarcomas and multiple myelomas (1-7). However, only a subset of patients benefit from combination chemotherapy. Most patients relapse and many suffer from side effects. It would, therefore, be of interest to identify in advance patients that do not benefit from treatment with CY and select an alternative therapy that is likely to be more effective.

The advent of DNA array technology has made it possible to derive global tumor gene expression profiles. In principle, it should be possible to correlate such profiles with tumor response and thereby identify gene signatures that can predict drug sensitivity. However, since CY is mainly administered in combination therapy, even for patients that do benefit, it is virtually impossible to assess the contribution of CY to the therapeutic success. Even worse, there is no information on how the tumor would have grown in the absence of any treatment.

Patient tumor explants subcutaneously transplanted and passaged in nude mice, termed human tumor xenografts, allow it to study the efficacy of drugs as single agents and in combination by using tumor-bearing vehicle control mice as an internal control and thus, avoiding the described limitations in the clinic. On the other hand, the patient-derived tumor xenografts typically retain the histology and drug sensitivity of the original tumors. Thus, 90% of the tumors, that underwent remission in the patient, also regressed as xenografts in mice if mice received the same treatment as the patient. Conversely, 97% of the tumors that progressed in patients, also progressed as xenografts in mice (8). Taken together, these observations demonstrate the clinical relevance of this experimental system.

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Here, we used the patient tumor xenograft system to obtain relevant CY monotherapy efficacy data for correlation with the tumors' gene expression profiles and subsequent identification of a gene signature predictive for tumor response to CY. By employing this signature we show that the CY response rate of predicted responders is significantly higher than that of randomly tested tumors.

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, Rye, 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 (8). 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).

Tumor measurements. Tumors were measured either weekly or, for fast growing tumors, twice weekly and volumes were calculated according to the formula $a*b^2/2$ 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 (see below), 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 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 mm³. 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. CY was administered in monotherapy at i.p. (intraperitoneal) doses of 200 mg/kg on days 0 and 14. This was the maximum tolerated dose (MTD) for tumor-bearing nude mice, as apparent from a lethality of 20% after 3 weeks. The CY treatment regimen used for mice mimicked the clinical schedules, with the exception that in the clinic treatment is usually repeated after 3 weeks.

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 T/C value recorded during an experiment was taken as the endpoint for evaluation of tumor sensitivity. The observation period was 3 to 4 weeks for progressive tumors and longer for tumors undergoing remission. Mice had to be sacrificed when the volume of their tumors exceeded 1600 mm³. For the bioinformatic analysis, the tested tumors were classified either as responders (R, minimum T/C ≤10%) or as non-responders (NR, minimum T/C >10%). The classification borders were chosen in such a way 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, microarray data processing and normalization. Affymetrix® HG-U133 Plus 2.0 mRNA expression arrays were used to determine the expression of 47,400 transcripts, corresponding to 38,500 human genes (9). These arrays have proven high reproducibility for mRNA expression analysis (10).

CEL result files were pre-processed using the gc-RMA (11) algorithm independently for array data of training and validation set tumors. Afterwards each transcript was normalizined to the 50th percentile of each single array.

Identification of predictive transcripts. To identify predictive transcripts, an iterative leave-one-out/intersection process was used (Figure 1). Briefly, in a first step all transcripts with a standard deviation >0.75 for at least one of the two classification groups (R, NR) were filtered out because they were unlikely to discriminate between the two groups. With 49 tumors in the training set, 49 gene lists consisting of the 300 most specific transcripts were generated by iteratively removing one tumor at a time from the training set. The final list of 129 transcripts consisted of the intersection of these 49 gene lists. The purpose of this iterative process was to reduce the risk of underestimating the prediction error by reducing the number of false positive transcripts.

In each of these iteration steps the most specific transcripts, which have a raw signal intensity of at least 30 in at least (49-1)/4 tumors, were determined by choosing the 300 transcripts with the highest specificity rank defined by 1/(ANOVA *p*-value + 1/Fishers rank). This specificity rank is a measure for the ability of a transcript to discriminate between the two classification groups. ANOVA *p*-value was calculated by Welch ANOVA and Fisher's rank describes the rank of every transcript according to the hypergeometric Fisher's exact test. This calculation was performed by Genespring's BioScript SG2c-2 (Agilent, Santa Clara, USA).

Class prediction algorithm. Support-vector-machines (SVMs) are powerful pattern recognition techniques assigning objects unequivocally to one of two classes. They build robust prediction
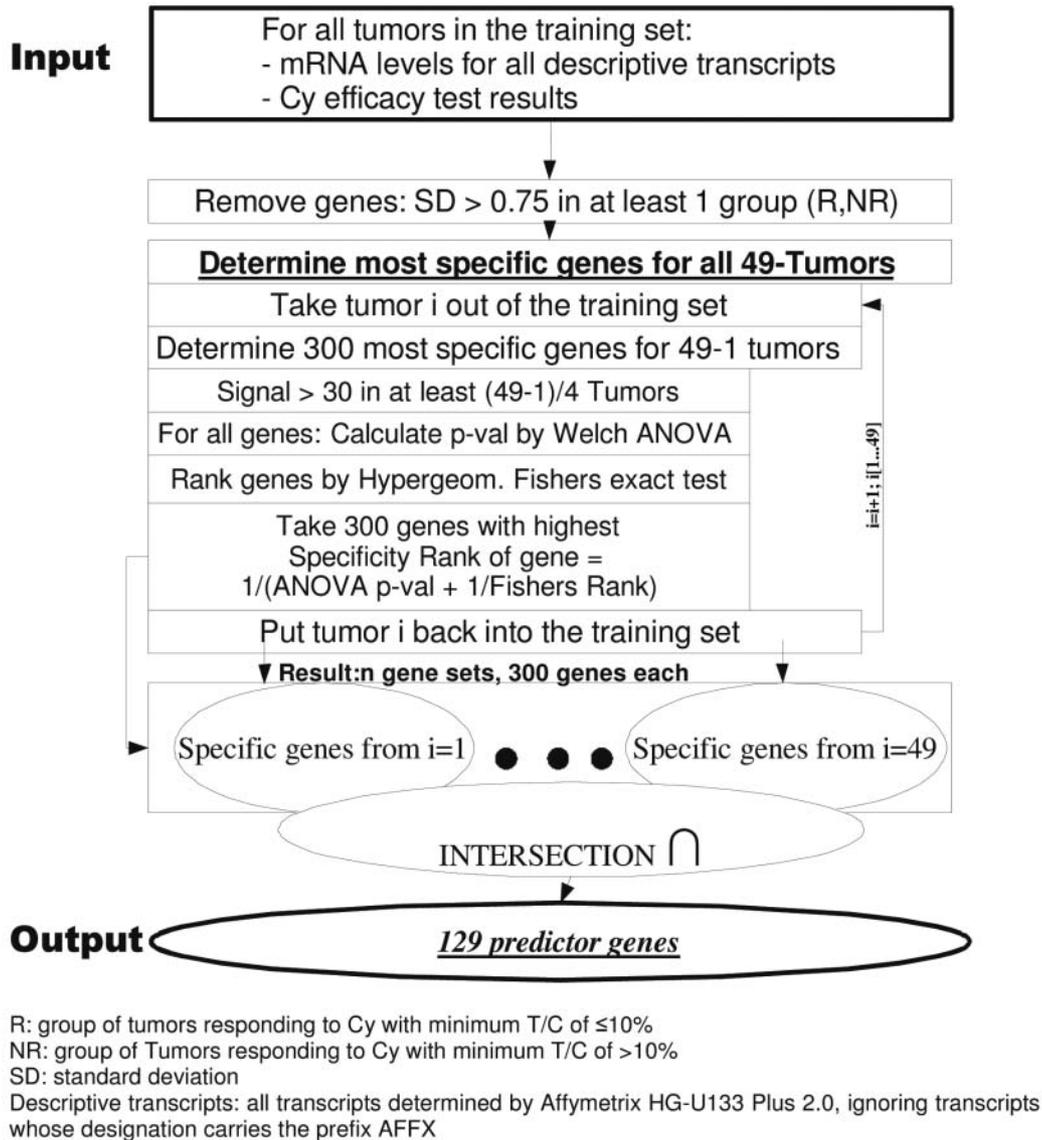
Figure 1. *Algorithm for the derivation of gene sets predicting the cyclophosphamide sensitivity of tumors.*

models even when the dimensionality is high and the number of training samples is small. They have recently been used successfully to classify microarray data (12-14). A Gaussian SVM (radial basis) was used as class prediction algorithm. This type of SVM gave the best prediction results in the leave-one-out cross-validation and, consequently, was used to predict the independent data set as well.

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

Tumor classification based on response to CY treatment. In this study, a total of 74 tumor xenografts of different histologies were subjected to efficacy tests with CY. As many as 49 out of the 74 test tumors formed the tumor training set (Table I A), and their CY responses and gene expression profiles were used to derive a gene signature predictive for CY response. The remaining 25 test tumors were used for independent validation of the gene signature (Table I B and see below).

Based on their response to CY, the 74 tested tumors were grouped into 2 categories, 26 responsive tumors (35%) with a

Table I. *CY sensitivity of tested tumor xenografts.*

A) Tumor Training Set

Tumor	T/C	class	Tumor	T/C	class
LEXF_1189	1%	R	PXF_1118	11%	NR
LXFS_605	1%	R	PXF_537	14%	NR
MAXF_MX1	1%	R	RXF_1220	14%	NR
MEXF_1765	1%	R	BXF_1228	16%	NR
RXF_944LX	1%	R	MAXF_1162	17%	NR
TXF_404	1%	R	LXFA_526	20%	NR
MAXF_1322	2%	R	RXF_1393	20%	NR
LXFE_409	3%	R	MEXF_274	26%	NR
MAXF_1384	3%	R	PRXF_PC3M	27%	NR
MAXF_449	3%	R	PXF_541	27%	NR
CEXF_633	4%	R	LXFS_650	29%	NR
MEXF_276	5%	R	OVXF_1353	31%	NR
MEXF_1341	6%	R	PAXF_736	34%	NR
LXFS_615	7%	R	SXF_1410	36%	NR
MMXF_L363	7%	R	MAXF_583	40%	NR
MEXF_462	7%	R	CXF_1103	41%	NR
CXF_280	9%	R	LXFL_1072	42%	NR
LXFL_430	10%	R	CXF_158	45%	NR
			CXF_886	46%	NR
			MEXF_535	49%	NR
			MAXF_MCF7	52%	NR
			MAXF_857	58%	NR
			RXF_486	58%	NR
			HNXF_536	62%	NR
			LXFE_397	68%	NR
			OVXF_899	68%	NR
			RXF_423	69%	NR
			CXF_1729	73%	NR
			OVXF_SKOV3	74%	NR
			RXF_393	82%	NR
			RXF_631	84%	NR

B) Tumor Validation Set

Tumor	T/C	true	Tumor	T/C	true
BXF_1218	1%	R	OVXF_1023	11%	NR
LEXF_CCRFCEM	1%	R	CNXF_498	17%	NR
LXFS_538	1%	R	LXFA_629	25%	NR
MAXF_401	1%	R	GXF_251	36%	NR
MEXF_989	1%	R	LXFA_289	39%	NR
LXFL_529	1%	R	GXF_281	42%	NR
MAXF_574	1%	R	MEXF_514	44%	NR
LXFS_573	3%	R	OVXF_1544	44%	NR
			PAXF_546	49%	NR
			LXFA_400	53%	NR
			LXFE_211	58%	NR
			OVXF_550	58%	NR
			CXF_609	63%	NR
			BXF_T24	73%	NR
			CXF_243	74%	NR
			CXF_975	78%	NR
			PRXF_DU145LX	80%	NR

The CY response of each tumor is expressed as the minimum T/C value recorded during the observation period; tumors with a minimum T/C ≤10% were classified as responsive, tumors with a minimum T/C >10 as non-responsive.

minimum T/C value during the observation period of ≤10% (referred to as R) and 48 non-responsive tumors (65%) with a minimum T/C value of >10% (referred to as NR). Most of the responders underwent a complete or a partial remission in response to CY treatment, some tumors displayed stasis. With regard to tumor types (see Table I for details), R tumors were mainly found among small cell lung cancers (4 out of 5 tested), breast tumors (6 out of 10) and melanomas (5 out of 8). The responses of R tumors would certainly translate into a clinical benefit for the tumor-bearing patient. The NR tumors comprised 48 out of the 74 tested tumors (65%). Most of the NR tumors progressed despite CY-treatment. The NR tumors included, among others, 7 out of 8 tested colon cancers, 7 out of 10 non-small cell lung cancers, 6 out of 7 renal cell cancers, 6 out of 6 ovarian cancers and 3 out of 3 pleuramesotheliomas (Table I).

Biological function of the genes contained in the predictive gene signature. Bioinformatic analysis of the CY responses and the gene expression profiles of the 49 training set tumors led to the definition of a gene signature predictive for CY response. This gene signature is made up of 129 different genes. The protein products of 67 of these genes have a known biological activity, as apparent from the overlap of the predictive gene signature with the gene ontology biological process categories as defined by the gene ontology consortium (Table II). The fact that overlaps with DNA-related gene ontology categories were most significant was not surprising, given that CY is a DNA alkylating agent. Twelve genes (ANP32A, BTAF1, CBL, DNMT1, DPP4, FEN1, GADD45A, PLSCR1, PRKDC, PSEN1, TIEG, VAMP3) of the predictive gene list are known to interact at least indirectly with CY (Figure 2).

Leave-one-out cross-validation of the predictive gene signature. To demonstrate the utility of the predictive gene signature, in a first step leave-one-out cross-validation (LOOCV) was performed, using the training set of 49 tested tumors. Predictions for CY response were correct in 15 out of 18 cases (83%) and predictions of CY resistance were correct in 28 out of 31 cases (90%) (sensitivity 0.83, specificity 0.90). The corresponding figures for random testing were 36.7 and 63.3%, respectively.

Validation based on a set of independent, previously untested tumors. More convincing than a leave-one-out cross-validation based solely on the training set is a validation based on a set of independent, previously untested tumors. For this, 25 tumors previously not tested for sensitivity to CY were selected. Using the predictive gene signature and the available gene expression profiles of the 25 tumors, their responses to CY monotherapy were predicted and the predictions were compared with the results of real efficacy tests. As shown in
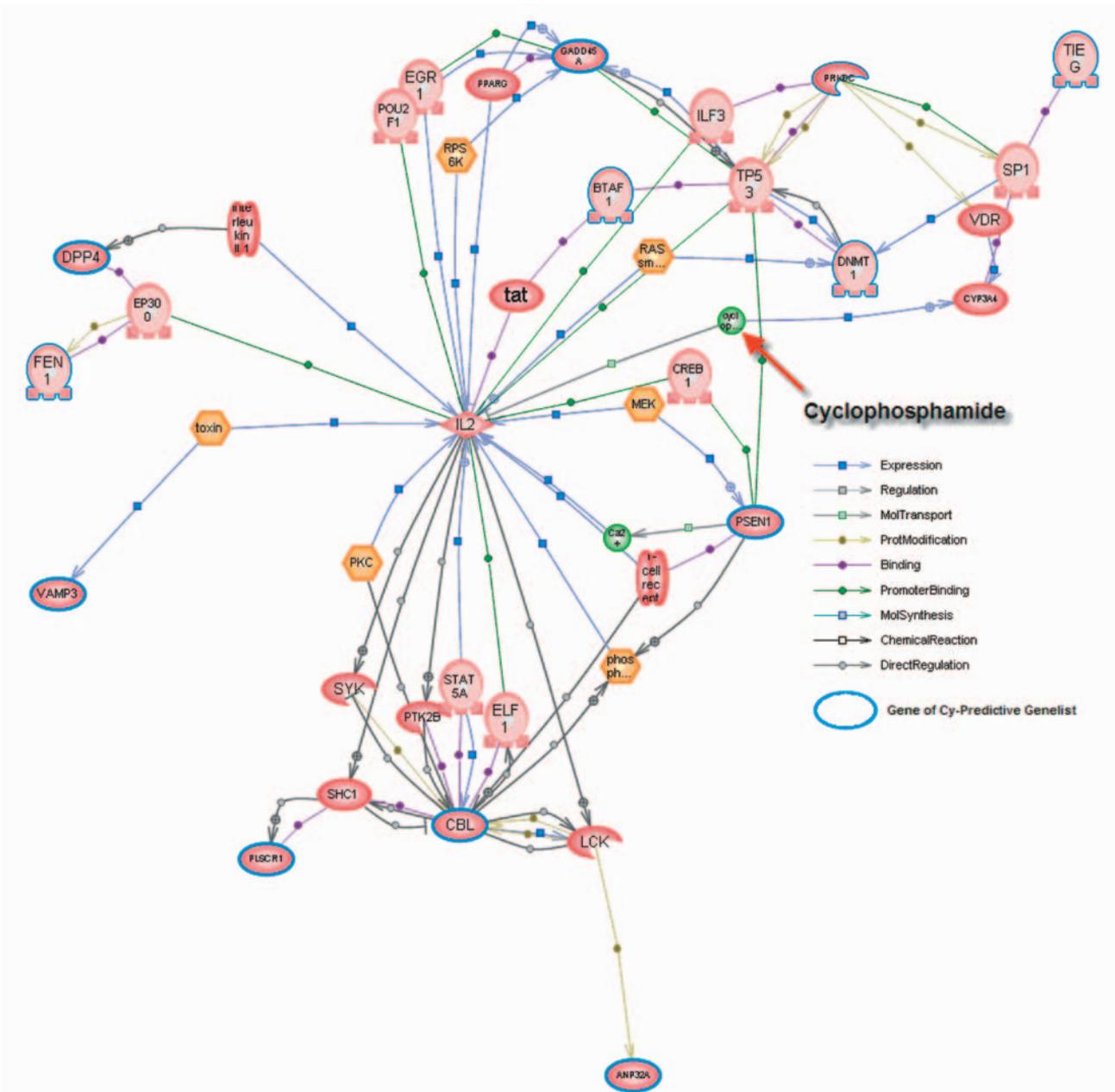
Figure 2. *The products of 12 genes present in the gene signature predictive for tumor response to CY have a known direct or indirect relationship to CY. Data from PathwayStudio 4, Ariadne Genomics.*

Table III, 6 out of 8 tumors (75%) predicted to respond to CY actually did respond. Conversely, out of 17 tumors predicted to be non-responders, 15 (88%) were non-responders in real testings. For comparison, following random testing of the same 25 tumors, 8 tumors (32%) responded to CY treatment while 17 out of the 25 tumors (68%) fell into the class of non-responders. In summary, compared with random testing, biomarker-guided testing increased the

response rate to CY treatment from 32 to 75% and the frequency of non-responders increased from 68% for random testing to 88% among predicted non-responders.

Comparison of real T/C values for predicted responders versus predicted non-responders. If the predictions of tumor CY responses match real CY tumor sensitivities, then the real minimum T/C values of predicted responders should on

Table II. *Overlap of the 129 genes predictive for tumor response to CY and the Biological Process Gene-Ontology.*

Biological process	Genes in category	% of Genes i	Genes in Lis	% of Genes i	p-value
GO:6260: DNA replication	273	1,24	8	11,94	1,75E-06
GO:46080: dUTP metabolism	3	0,0136	2	2,985	2,73E-05
GO:9211: pyrimidine deoxyribonucleoside triphosphate metabolism	8	0,0363	2	2,985	2,53E-04
GO:9200: deoxyribonucleoside triphosphate metabolism	8	0,0363	2	2,985	2,53E-04
GO:6259: DNA metabolism	1049	4,766	11	16,42	3,02E-04
GO:46125: pyrimidine deoxyribonucleoside metabolism	11	0,05	2	2,985	0,000493
GO:9120: deoxyribonucleoside metabolism	11	0,05	2	2,985	0,000493
GO:6213: pyrimidine nucleoside metabolism	13	0,0591	2	2,985	0,000697
GO:6261: DNA-dependent DNA replication	135	0,613	4	5,97	0,000769
GO:9219: pyrimidine deoxyribonucleotide metabolism	14	0,0636	2	2,985	0,000811
GO:9262: deoxyribonucleotide metabolism	17	0,0772	2	2,985	0,00121
GO:46907: intracellular transport	1090	4,952	10	14,93	0,00161
GO:51649: establishment of cellular localization	1095	4,975	10	14,93	0,00167
GO:9147: pyrimidine nucleoside triphosphate metabolism	20	0,0909	2	2,985	0,00167
GO:51641: cellular localization	1096	4,979	10	14,93	0,00168
GO:16043: cell organization and biogenesis	2440	11,08	16	23,88	0,00224
GO:6886: intracellular protein transport	802	3,643	8	11,94	0,00293
GO:16481: negative regulation of transcription	210	0,954	4	5,97	0,00386
GO:15031: protein transport	844	3,834	8	11,94	0,00399
GO:45934: negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	220	0,999	4	5,97	0,00455
GO:45184: establishment of protein localization	867	3,939	8	11,94	0,00469
GO:6281: DNA repair	360	1,635	5	7,463	0,0048
GO:8104: protein localization	880	3,998	8	11,94	0,00512
GO:6302: double-strand break repair	36	0,164	2	2,985	0,00538
GO:6167: AMP biosynthesis	2	0,00909	1	1,493	0,00608
GO:15879: carnitine transport	2	0,00909	1	1,493	0,00608
GO:6606: protein import into nucleus	130	0,591	3	4,478	0,00732
GO:51170: nuclear import	130	0,591	3	4,478	0,00732
GO:31324: negative regulation of cellular metabolism	254	1,154	4	5,97	0,00752
GO:6974: response to DNA damage stimulus	404	1,835	5	7,463	0,00773
GO:9892: negative regulation of metabolism	258	1,172	4	5,97	0,00793
GO:45005: maintenance of fidelity during DNA-dependent DNA replication	44	0,2	2	2,985	0,00795
GO:6298: mismatch repair	44	0,2	2	2,985	0,00795
GO:9719: response to endogenous stimulus	411	1,867	5	7,463	0,00829
GO:17038: protein import	137	0,622	3	4,478	0,00845
GO:305: response to oxygen radical	3	0,0136	1	1,493	0,0091
GO:9650: UV protection	3	0,0136	1	1,493	0,0091
GO:6220: pyrimidine nucleotide metabolism	51	0,232	2	2,985	0,0106
GO:9116: nucleoside metabolism	52	0,236	2	2,985	0,011
GO:19321: pentose metabolism	4	0,0182	1	1,493	0,0121
GO:6014: D-ribose metabolism	4	0,0182	1	1,493	0,0121
GO:46033: AMP metabolism	4	0,0182	1	1,493	0,0121
GO:7175: negative regulation of epidermal growth factor receptor activity	4	0,0182	1	1,493	0,0121
GO:45892: negative regulation of transcription, DNA-dependent	159	0,722	3	4,478	0,0126
GO:6364: rRNA processing	57	0,259	2	2,985	0,0131
GO:17121: phospholipid scrambling	5	0,0227	1	1,493	0,0151
GO:16072: rRNA metabolism	63	0,286	2	2,985	0,0158
GO:7059: chromosome segregation	63	0,286	2	2,985	0,0158
GO:51169: nuclear transport	176	0,8	3	4,478	0,0166
GO:7046: ribosome biogenesis	67	0,304	2	2,985	0,0178
GO:302: response to reactive oxygen species	6	0,0273	1	1,493	0,0181
GO:7176: regulation of epidermal growth factor receptor activity	6	0,0273	1	1,493	0,0181
GO:42254: ribosome biogenesis and assembly	68	0,309	2	2,985	0,0183
GO:6913: nucleocytoplasmic transport	199	0,904	3	4,478	0,0228
GO:7582: physiological process	18808	85,44	63	94,03	0,0252
GO:7009: plasma membrane organization and biogenesis	9	0,0409	1	1,493	0,0271
GO:7028: cytoplasm organization and biogenesis	87	0,395	2	2,985	0,0289
GO:48523: negative regulation of cellular process	982	4,461	7	10,45	0,0294
GO:9141: nucleoside triphosphate metabolism	91	0,413	2	2,985	0,0314
GO:6271: DNA strand elongation	11	0,05	1	1,493	0,033
GO:43170: macromolecule metabolism	6428	29,2	27	40,3	0,0337
GO:7020: microtubule nucleation	12	0,0545	1	1,493	0,0359
GO:43283: biopolymer metabolism	4151	18,86	19	28,36	0,038
GO:6378: mRNA polyadenylylation	13	0,0591	1	1,493	0,0389
GO:48519: negative regulation of biological process	1057	4,802	7	10,45	0,0412
GO:31124: mRNA 3'-end processing	14	0,0636	1	1,493	0,0418
GO:31123: RNA 3'-end processing	14	0,0636	1	1,493	0,0418
GO:6396: RNA processing	626	2,844	5	7,463	0,0418
GO:6605: protein targeting	253	1,149	3	4,478	0,042
GO:51181: cofactor transport	15	0,0681	1	1,493	0,0447
GO:122: negative regulation of transcription from RNA polymerase II promoter	113	0,513	2	2,985	0,0465
GO:7034: vacuolar transport	16	0,0727	1	1,493	0,0476
GO:51180: vitamin transport	16	0,0727	1	1,493	0,0476

Gene ontology groups that have a significant overlap ($p<0.05$) with the gene list are shown according to decreasing statistical significance.

average be smaller than the minimum T/C values of predicted non-responders. Using the validation set tumors as test tumors, this was indeed the case (Figure 3). Thus, the median minimum T/C value for the 8 tumors predicted to display a minimum T/C value ≤10% (predicted responders) was 1% while the minimum median T/C value of the 17

Table III. *Real and gene signature-predicted efficacy of CY in 25 tumor xenografts form an independent validation set.*

Tumor	T/C	true	pred
BXF_1218	1%	R	R
LEXF_CCRFCEM	1%	R	R
LXFS_538	1%	R	R
MAXF_401	1%	R	R
MEXF_989	1%	R	R
LXFS_573	3%	R	R
CNXF_498	17%	NR	R
MEXF_514	44%	NR	R

Predicted	*in vivo* real
R 8	R 6 (75%)
	NR 2 (25%)

In vivo study

25 Tumors tested

8 (32%) Responsive (R) T/C<11%
17 (68%) Not responsive (P)T/C>=11%

Tumor	T/C	true	pred
OVXF_1023	11%	NR	NR
LXFA_629	25%	NR	NR
GXF_251	36%	NR	NR
LXFA_289	39%	NR	NR
GXF_281	42%	NR	NR
OVXF_1544	44%	NR	NR
PAXF_546	49%	NR	NR
LXFA_400	53%	NR	NR
LXFE_211	58%	NR	NR
OVXF_550	58%	NR	NR
CXF_609	63%	NR	NR
BXF_T24	73%	NR	NR
CXF_243	74%	NR	NR
CXF_975	78%	NR	NR
PRXF_DU145LX	80%	NR	NR
LXFL_529	1%	R	NR
MAXF_574	1%	R	NR

Predicted	*in vivo* real
NR 17	R 2 (12%)
	NR 15 (88%)

Figure 3. *Real cyclophosphamide sensitivities for validation set tumors expressed as T/C values: predicted responders (predicted T/C ≤10%) vs. predicted non-responders (predicted T/C >10%). The p-value was determined using the Mann-Whitney Rank Sum Test.*

Frequency of real and predicted responders among CY sensitive and CY resistant tumor types. In addition to the 74 combined training and validation set tumors whose CY sensitivities and gene expression profiles had been used to derive and validate a gene signature predictive for CY response, 101 additional untested tumors with known gene expression profiles were available. We used the gene signature predictive for CY tumor responses to predict the CY response of those 101 tumors and subsequently compared the experimentally determined CY responses of the 74 training and validation set tumors with the predicted CY responses of 101 untested tumors. The results are presented separately for tumor types known to be CY-sensitive in the clinic on the one hand (Table IV A) and for tumor types that are largely resistant to CY or whose CY sensitivity has not yet been tested in the clinic (Table IV B).

Among the 74 training and validation set tumors, 37 (50%) belonged to CY-sensitive tumor types. Twenty of them (54%), including 6 breast cancers, 5 melanomas and 4 small cell lung cancers, proved to be sensitive to CY in real testings while the remaining 17 tumors, among them 6 ovarian cancers, 4 breast cancers and 3 melanomas, proved to be resistant. For the 101 untested tumors, 26 (25.7%) belonged to CY sensitive tumor types and 11 out of the 26 (42%) were predicted to be CY

predicted non-responders (predicted minimum T/C value >10%) was 49%. Statistical analysis of minimum T/C values based on the Mann-Whitney rank sum test demonstrated the significance of the difference ($p=0.004$). In conclusion, in real testings predicted responders exhibited a clearly better CY response than predicted non-responders.
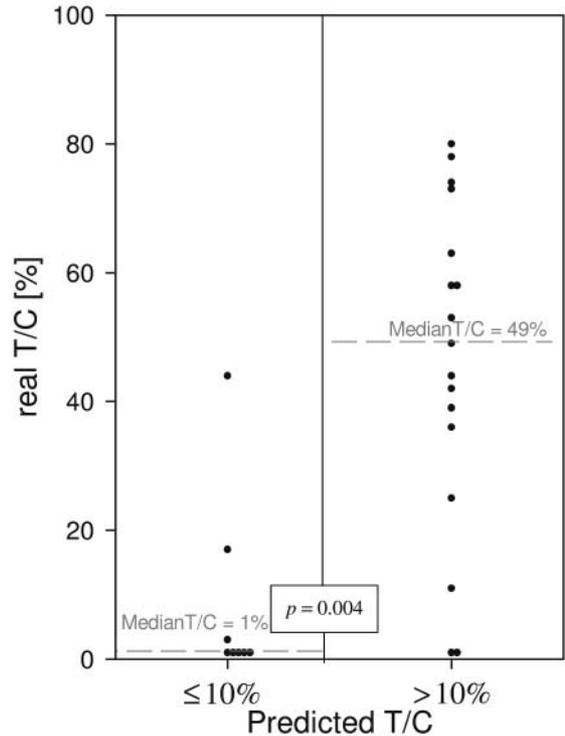
Table IV. *A. Cyclophosphamide efficacy in clinically sensitive tumor types: results for 37 tested tumors and predictions for 26 untested tumors.*

Cancer type	CY real				CY predicted				Cy real + predicted			
	R	NR	Total	%	R	NR	Total	%	R	NR	Total	%
Bladder	1	2	3	33%	1	3	4	25%	2	5	7	29%
Leukemias+Ly	2		2	100%	5		5	100%	7		7	100%
Lung small cell	4	1	5	80%	2	1	3	67%	6	2	8	75%
Mammary	6	4	10	60%	0	2	2	0%	6	6	12	50%
Melanoma	5	3	8	63%	2	6	8	25%	7	9	16	44%
Myeloma	1		1	100%			0				0	1
Ovary		6	6	0%			0			6	6	0%
Sarcoma		1	1	0%	1	3	4	25%	1	4	5	20%
Testicle	1		1									
Total	20	17	37	54%	11	15	26	42%	31	32	63	49%

Table IV. *B. Cyclophosphamide efficacy in clinically resistant or untested tumor types: results for 37 tested tumors and predictions for 75 untested tumors.*

Cancer type	CY real				CY predicted				Cy real + predicted			
	R	NR	Total	%	R	NR	Total	%	R	NR	Total	%
CNS		1	1	0%								
Colon	1	7	8	13%	3	16	19	16%	4	23	27	15%
Gastric		2	2	0%		6	6	0%		8	8	0%
Head and Neck		1	1	0%	1	3	4	25%	1	4	5	20%
Liver						2	2	0%		2	2	0%
Lung NSCLC	3	7	10	30%	4	24	28	14%	7	31	38	18%
Pancreas		2	2	0%		3	3	0%		5	5	0%
Pleuramesothelioma		3	3	0%	1	1	2	50%		4	5	0%
Prostate		2	2	0%								
Renal	1	6	7	14%		9	9	0%		15	16	0%
Uterus cervix	1					2	2	0c		2	2	0%
Total	6	31	37	16%	9	66	75	12%	15	97	112	13%

sensitive, including 5 leukemias and lymphomas, 2 small cell lung cancers and 2 melanomas while the remaining 15 tumors (58%) were predicted to be non-responders, including 6 melanomas, 3 bladder cancers, 3 sarcomas and 2 breast cancers. Summing up for the CY sensitive tumor types, 31 out of a total of 63 tumors (49%) were either demonstrated or predicted to be sensitive, while the remaining 32 tumors (51%) were either demonstrated or predicted to be resistant.

The second half of the training and validation set tumors belonged to tumor types considered as CY resistant. Six of them (16%), among them 3 non-small cell lung cancers, were sensitive in real testings while the remaining 31 (84%), among them 10 non-small cell lung cancers, 8 colorectal carcinomas, 7 renal cell cancers and 3 pleuramesotheliomas, were resistant. For the 101 untested tumors, 75 (74.3%) belonged to CY-resistant tumor types and 9 of the 75 (12%) were predicted to be sensitive, including 4 non-small cell lung cancers and 3 colon cancers, while the remaining 66 tumors were predicted

to be non-responders, including 24 non-small cell lung cancers, 16 colorectal carcinomas, 9 renal cell cancers, 6 gastric cancers, 4 head and neck cancers and 3 pancreatic cancers. Summing up for the CY-resistant tumor types, 15 out of a total of 112 tumors (13%) were either demonstrated or predicted to be sensitive, while the remaining 97 tumors (87%) were either demonstrated or predicted to be resistant.

Taken together and in agreement with clinical data, the presented results demonstrate that the real and the predicted CY response rates are clearly higher among tumor types known to be CY sensitive as compared to CY resistant tumor types.

Discussion

We have used patient tumor explants passaged as xenografts in nude mice in order to correlate tumor gene expression profiles with the test tumors' sensitivity to CY and have thereby identified a gene signature predicting tumor

response to CY. The reason for using this experimental set-up was that we wanted to avoid the limitations of clinical studies in determining the contribution of a single drug to therapeutic outcome. Nevertheless, at the same time we wanted to work in a biologically relevant system in order to be able to subsequently translate the results into the clinic.

The predictive gene signature we identified consists of 129 genes. The products of at least 18 of these genes interact with DNA and the products of 12 of these genes are already known to interact at least indirectly with CY. The signature was validated at two levels. First, using leave-one-out cross validation on the tumor training set, the CY response of training set tumors was correctly predicted in 15 out of 18 cases (83%) as compared to a response rate of 18 out of 49 (32%), following random testing. Second, for an independent set of 25 previously untested tumors with known gene expression profiles (validation set) CY sensitivity was predicted correctly for 6 out of 8 tumors (75%) and CY resistance for 15 out of 17 tumors (88%). For comparison, random testing of the same tumors resulted in a response rate of 8 out of 25 (32%). For the same 25 tumors, the median minimum T/C value for predicted responders was 1% as compared to 49% for predicted non-responders. Moreover, for tumor types considered as CY sensitive including, among others, small cell lung and breast cancer as well as melanoma, the combined real and predicted response rates for 37 tested and 26 untested tumors was 49%. For comparison, for tumor types considered as CY resistant such as colon and renal cancer, the combined real and predicted response rates for 37 tested and 75 untested tumors was 13%.

With regard to validation, it should be noted that if a LOOCV is performed with only one common gene signature determined collectively for all training set tumors, there is a high risk of overestimating the predictive power of that signature. By using the iterative leave-one-out process in determining the predictive genes (see Materials and Methods Section, above), we reduced that risk. Consequently, we obtained nearly the same error rates for LOOCV of the training set and the following prediction of the independent dataset, indicating that the obtained results can be generalised.

In conclusion, all results obtained for the CY predictive gene signature are consistent and plausible and generalization appears to be justified. The next step is the validation of the CY specific gene signature in the clinic.

Acknowledgements

Conflicts of Interest

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

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