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Microarray-based Expression of DNA Repair Genes Does not Correlate with Growth Inhibition of Cancer Cells by Natural Products Derived from Traditional Chinese Medicine

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Abstract. *Drug resistance represents a major obstacle in cancer chemotherapy. As chemically characterized compounds derived from plants used in traditional Chinese medicine (TCM) may have molecular targets different from those of standard antitumor drugs, they might be attractive candidates for novel therapeutics with improved pharmacological features. DNA repair is frequently involved in the development of resistance to established anticancer drugs, e.g. alkylating agents. Using a database of 531 chemically characterized TCM compounds from medicinal plants recently established by us, the IC_{50} values of 60 N.C.I. tumor cell lines for these 531 natural products were tested for correlation with the microarray-based mRNA expression of six genes involved in nucleotide excision repair (ERCC1, XPA, XPC, DDB2, ERCC4, ERCC5). No compound correlated with the expression of these genes, indicating that mRNA expression of these genes is not associated with resistance of the cell lines to these TCM compounds. The same is true for another six genes of the base excision repair pathway (MPG, APEX1, OGG1, XRCC1, LIG3, POLB). Microarray-based COMPARE analyses were performed to identify other candidate genes that are able to predict responsiveness of tumor cells to TCM-derived natural products. As an example, diallyl disulfide from garlic (Allium sativum L., Chinese name: dashuan) was chosen. Eighteen genes were identified whose mRNA expression predicted sensitivity or resistance to diallyl disulfide in hierarchical cluster analyses. Apart from some genes with still unknown function, genes were identified from different functional groups, e.g. signal transducers, regulators of GTPase activity, those associated with cytoskeleton formation and regulation, constituents of the ribosome. Remarkably, none of these genes have been described to be involved in DNA repair. In conclusion, our data indicate that TCM-derived natural products are worth being further investigated as novel compounds to eradicate tumors which reveal resistance to established anti-cancer drugs.*

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Drug resistance and severe adverse side effects are major obstacles of cancer chemotherapy (1-8). Therefore, new drugs with improved features are urgently required. Natural sources such as marine and terrestrial plants and animals are a fertile ground to find novel drugs with anti-tumor activity. The long-lasting experience of traditional folk medicines may facilitate the identification of novel agents. In China, herbs have been used as foods and medicine for centuries. In recent years, the active principles of medicinal herbs have been identified making the active chemical compounds accessible to pharmacological research (9-12). As compounds of traditional Chinese medicine (TCM) for cancer chemotherapy may have molecular targets different from those of standard anti-tumor drugs, they are attractive sources of novel drugs suitable to treat otherwise drug-resistant tumors with reduced side effects on normal organs.

In a recent pilot study, we found that only 2 out of 22 compounds of TCM were involved in *ABCB1* (*MDR1*)- or *ABCC1* (*MRP1*)-mediated multidrug resistance (13). This indicates that the efficacy of the majority of these cytotoxic natural products is not hampered by multidrug resistance. This encouraged us to further analyze the molecular pharmacology of TCM-derived drugs.

In the present investigation, we analyzed whether the microarray-based expression of DNA repair genes determines the level of resistance to TCM compounds, as previously described for established antitumor drugs (14-17). For this reason, we took advantage of a recently established

database of 531 natural products derived from TCM (18) and correlated the mRNA expression of DNA repair genes of 60 cell lines of the National Cancer Institute (N.C.I.), U.S.A., with the 50% inhibition concentrations (IC_{50}) of these cytotoxic compounds. Finally, we performed a microarray-based COMPARE analysis to identify candidate genes that are able to predict sensitivity and resistance of tumor cells to TCM-derived natural products.

Materials and Methods

Cell lines. The panel of 60 human tumor cell lines of the Developmental Therapeutics Program of the N.C.I., U.S.A., consisted of leukemia (CCRF-CEM, HL-60, K-562, MOLT-4, RPMI-8226, SR), melanoma (LOX-IMVI, MALME-3M, M14, SK-MEL2, SK-MEL28, SK-MEL-5, UACC-257, UACC-62), non-small cell lung cancer (A549, EKVX, HOP-62, HOP-92, NCI-H226, NCI-H23, NCI-H322M, NCI-460, NCI-H522), colon cancer (COLO205, HCC-2998, HCT-116, HCT-15, HT29, KM12, SW-620), renal cancer (786-0, A498, ACHN, CAKI-1, RXF-393, SN12C, TK-10, UO-31), ovarian cancer (IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SK-OV-3) cells, cells of tumors of the central nervous system (SF-268, SF-295, SF-539, SNB-19, SNB-75, U251), prostate carcinoma (PC-2, DU-145) and breast cancer (MCF-7, NCI/ADR-Res, MDA-MB-231, Hs578T, MDA-MB-435, MDA-N, BT-549, T-47D). Their origin and processing have been described elsewhere (19).

Sulforhodamine B assay. The determination of drug sensitivity in the N.C.I. cell lines by the sulforhodamine B assay has been reported (20). The IC_{50} values for standard anti-cancer drugs as well as of the 531 natural products have been deposited in the N.C.I.'s database (http://dtp.nci.nih.gov).

Statistical analyses. The mRNA expression values of 9,706 genes in 60 cell lines were selected from the N.C.I. database (http://dtp.nci.nih.gov). The mRNA expression was determined by microarray analyses (21, 22).

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, Cambridge, MA, U.S.A.). Kendall's τ-test determines the correlation of rank positions of values. Ordinal or metric scaling of data is suited for the test and transformed into rank positions. There is no condition regarding normal distribution of the data set for the performance of Kendall's τ-test.

COMPARE analyses were performed to produce rank-ordered lists of genes expressed in the 60 N.C.I. cell lines. The methodology has been described elsewhere in detail (23). Briefly, each gene of the Microarray Database of the N.C.I. is ranked for similarity of its mRNA expression to the IC_{50} values for natural products derived from TCM. To derive COMPARE rankings, a scale index of correlation coefficients (R-values) is created. In the standard COMPARE approach, greater mRNA expression in cell lines correlates with enhanced drug sensitivity, whereas in reverse COMPARE analyses greater mRNA expression in cell lines indicates drug resistance.

The χ^2 test was used as implement of the WinSTAT program (Kalmia) to proof bivariate frequency distributions for pairs of nominal scaled variables for dependencies.

Results

First, a recently established database of 531 chemically characterized compounds from medicinal plants used in TCM (18) was used to correlate the IC_{50} values of the 60 N.C.I. tumor cell lines for these 531 natural products with the microarray-based mRNA expression of six genes involved in nucleotide excision repair (*NER*; *ERCC1, XPA, XPC, DDB2, ERCC4, ERCC5*). These genes were represented by 18 clones. Interestingly, no IC_{50} value correlated to any of these genes with correlation coefficients of $R>0.6$ or $R<-0.6$. When this kind of analysis was applied to six genes of the base excision repair (BER) pathway (*MPG, APEX1, OGG1, XRCC1, LIG3, POLB*; represented by 19 clones), again no significant correlation to the IC_{50} values of the 60 N.C.I. cell lines for the 531 TCM compounds was found indicating the mRNA expression level of these 12 genes does not impact the cytotoxicity of the TCM compounds studied.

As a further step, we exemplarily selected one of the TCM compounds, diallyl disulfide, and performed COMPARE analyses of the IC_{50} values for this natural product and the microarray-based mRNA expression of 9,706 genes of the N.C.I. cell lines to produce scale indices of correlation coefficients. A standard COMPARE analysis was performed in which cell lines most inhibited by diallyl disulfide (lowest IC_{50} values) were correlated with the lowest mRNA expression levels of genes. The genes identified by setting a cut-off of $R>0.7$ are shown in Table I. These genes may be considered as possible candidate genes for determining cellular resistance to these compounds. Furthermore, reverse COMPARE analyses were carried out, which correlated the most inhibited cell lines with the highest gene expression levels (Table I). This approach provided genes that determine cellular sensitivity to diallyl disulfide (cut-off: $R<-0.7$). Apart from some genes with still unknown function, we identified genes from different functional groups to be correlated with cellular response to diallyl disulfide, *e.g.*, signal transducers (*IKZF1, SUB1, HCLS1, RASSF5*) and regulators of GTPase activity (*WAS, ARGGAP15, GIT2, GPSM3*). Furthermore, genes associated with cytoskeleton formation and regulation (*LCP1, WAS, ANXA2*) as well as constituents of the ribosome (*RPL5, RPL9*) among some other genes (*CD53, PTPN22, TJP1*) were associated with response to diallyl disulfide. Remarkably, none of these genes have been described to be involved in DNA repair.

The genes obtained by standard and reverse COMPARE analyses for diallyl disulfide were subjected to hierarchical cluster analysis to obtain a dendrogram where the cell lines are arranged according to their expression profile of these genes. The dendrogram for diallyl disulfide can be divided into four major cluster branches (Figure 1).

Then, the median $\log_{10}IC_{50}$ values for diallyl disulfide were then used as a cut-off threshold to define cell lines as

Table I. *Correlation of the constitutive mRNA expression of genes identified by COMPARE analysis with $log_{10}IC_{50}$ values for diallyl disulfide of 60 NCI cell lines.*

R-Value	Symbol	GenBank	Name	Function
Standard COMPARE:				
0.796	*IKZF1*	AI247840	IKAROS family zinc finger 1 (Ikaros) RNA	Transcription factor, zinc finger protein
0.792	*LCP1*	J02923	Lymphocyte cytosolic protein 1 (L-plastin)	Actin filament bundle formation
0.783	*HCLS1*	X16663	Hematopoietic cell-specific Lyn substrate 1	signal transduction
0.780	*CD53*	M37033	CD53	Cell differentiation antigen, surface glycoprotein
0.773	*WAS*	AI655719	Wiskott-Aldrich syndrome (eczema-thrombocytopenia)	Effector protein for Rho-type GTPases, regulates actin polymerization
0.765	*RPL5*	U14966	Ribosomal protein L5	Structural constituent of ribosome
0.758	*ARHGAP15*	AA714834	Rho GTPase activating protein 15	GTPase activator
0.753	*SUB1*	AI521453	SUB1 homolog (*S. cerevisiae*)	Transcription coactivator
0.752	*GIT2*	D63482	G protein-coupled receptor kinase interactor 2	GTPase activator
0.752	*PTPN22*	AF001846	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	Protein tyrosine phosphatase
0.742	*C9orf78*	AA516476	Chromosome 9 open reading frame 78	Unknown
0.741	*RASSF5*	AI990782	Ras association (RalGDS/AF-6) domain family 5	regulation of Ras apoptotic function, potential tumor suppressor
0.740	*GPSM3*	AJ243937	G-protein signaling modulator 3 (AGS3-like, *C. elegans*)	GTPase activator
0.734	not specified	D11327	Unknown	Unknown
0.733	*LOC440055*	AA977163	Similar to ribosomal protein S12	Unknown
0.732	*RPL9*	U09953	Ribosomal protein L9	Structural constituent of ribosome
Reverse COMPARE:				
–0.717	*TJP1*	R79560	Tight junction protein 1 (zona occludens 1)	Protein binding
–0.702	*ANXA2*	D00017	Annexin A2	Calcium-dependent phospholipid and cytoskeletal protein binding

being sensitive or resistant. As can be seen in Table II, the distribution of sensitive and resistant cell lines was significantly different between the branches of the dendrograms indicating that cellular response to diallyl disulfide is predictable by these genes.

Discussion

Recently, we established a database with more than 2,400 chemically characterized compounds derived from TCM, of which 531 exert considerable cytotoxicity against tumor cells (18). In the present investigation, the IC_{50} values of these 531 TCM compounds for 60 N.C.I. cell lines were tested for correlation with the microarray-based mRNA expression 12 DNA repair genes. No compound of this database correlated with the expression of these genes with a sufficiently high correlation factor (R>0.6 or R<–0.6) indicating that these genes may not play a role in sensitivity or resistance to these natural products derived from TCM. Of course, our results do not exclude that the mRNA expression of other DNA repair genes could be involved in cellular resistance to these TCM compounds. Furthermore, it has to be taken into consideration that the function of genes in conferring drug resistance might be independent of their mRNA expression levels. Nevertheless, our data indicate that cytotoxic compounds derived from TCM might be a valuable source for the development of novel treatment options for NER- or BER-proficient tumors which are otherwise resistant towards established anti-tumor drugs (14-17).

Table II. *Separation of clusters of 60 N.C.I. cell lines obtained by the hierarchical cluster analysis shown in Figure 1 in comparison to drug sensitivity. The median $log_{10}IC_{50}$ value (M) for diallyl disulfide was used as a cut-off to separate tumor cell lines as being 'sensitive' or 'resistant'.*

	Sensitive <–4.408*	Resistant >–4.408*
Cluster 1	6	0
Cluster 2	12	12
Cluster 3	6	18
Cluster 4	6	0

p=4.4x10^{-4}; *$\log_{10}IC_{50}$ (M)

The response of tumor cells to most established anti-tumor drugs is determined by multiple rather than by single factors. If not by the expression of NER and BER genes, the question arises which other genes might be involved in resistance to TCM compounds. We chose diallyl disulfide, a natural product from garlic (*Allium sativum* L., Chinese

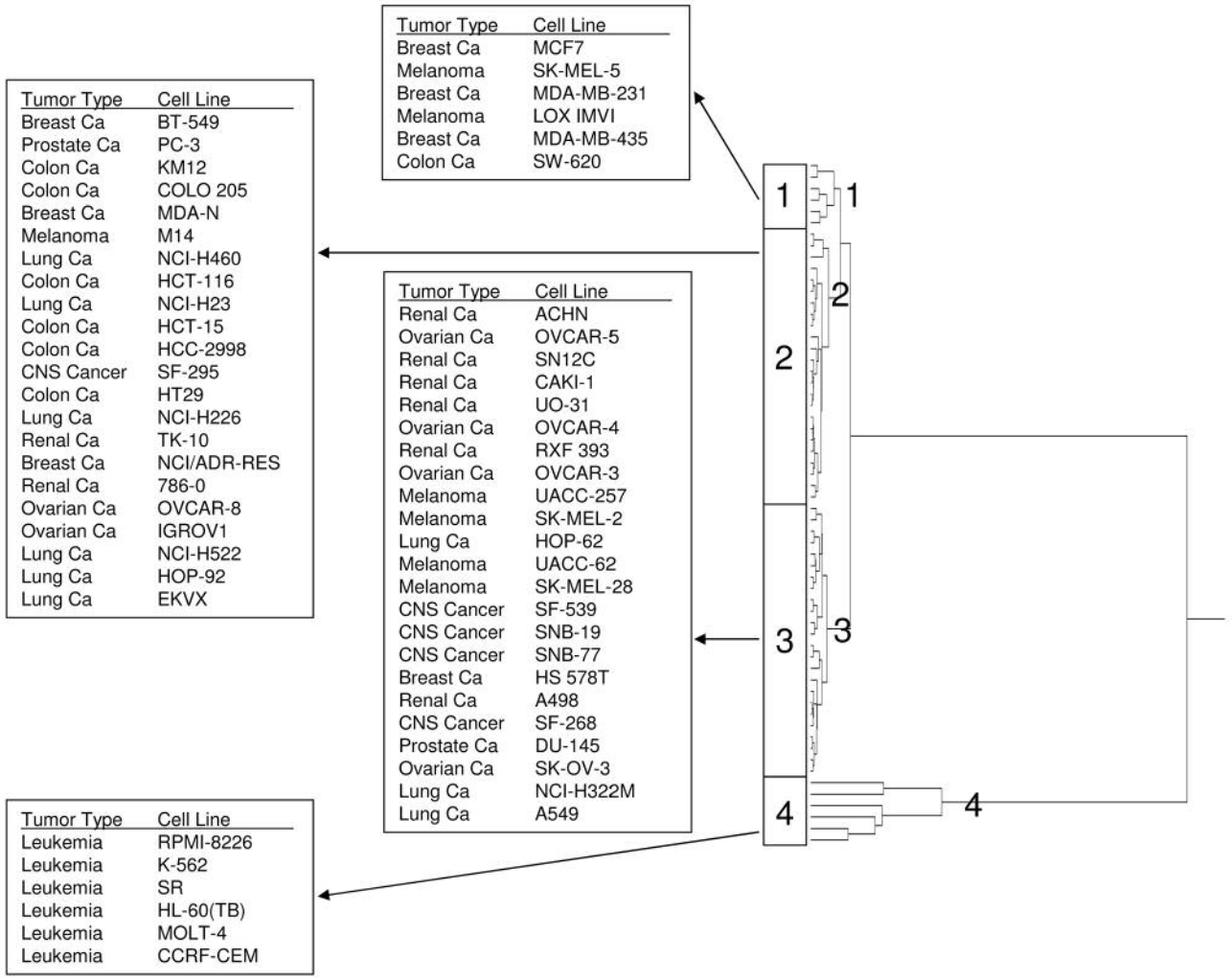

Figure 1. *Dendrogram of hierarchical cluster analysis (complete linkage method) obtained from mRNA expression of genes correlating with $log_{10}IC_{50}$ values for diallyl disulfide. The dendrogram shows the clustering of 60 cell lines of the N.C.I.'s screening panel.*

name: *dashuan*) as one example to address this question. This plant is used worldwide for nutritional purposes. In China, it has also been used for centuries for lowering blood pressure and in the treatment of heart diseases, as well as for the prevention of infections. There is a wealth of investigations on the cancer chemopreventive activity of garlic (24-26). Among other mechanisms, diallyl disulfide has been shown to induce apoptosis in cancer cells and inhibit tumor angiogenesis (27-30).

To gain insight into the molecular mechanisms involved in cellular response to diallyl disulfide, we performed COMPARE and hierarchical cluster analyses of microarray-based mRNA expression values for 9,706 genes of the 60 N.C.I. cell lines. Eighteen genes of different functional groups were found, *e.g.* signal transducers, regulators of GTPase activity and cytoskeleton formation as well as ribosome constituents. Although most of these genes have not yet been assigned to drug sensitivity or resistance, a few hints from the literature show associations with established anti-neoplastic drugs. Members of the Rho family of small GTPases are key regulators of actin reorganization, cell motility, cell–cell and cell–extracellular matrix (ECM) adhesion, as well as of cell cycle progression, gene expression and apoptosis. Rho proteins also affect cellular susceptibility to DNA-damaging agents, including anti-neoplastic drugs and ionizing radiation (31). This is a clue that the *WAS* and *LCP1* genes identified in the present study might also be involved in cellular resistance to cytotoxic agents. Furthermore, the ribosomal protein L5 (RPL5) has been found to be over expressed in doxorubicin-resistant cancer cells (32). Further studies are warranted to clarify the causative relevance of the genes identified in our study in the cellular response to diallyl disulfide. Interestingly, no DNA repair gene appeared in the list of genes that correlate with the cellular response to diallyl disulfide.

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