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Sarin, Navin, Engel, Florian, Kalayda, Ganna V, Frötschl, Roland, Cinatl, Jindrich, Rothweiler, Florian, Michaelis, Martin, Fröhlich, Holger and Jaehde, Ulrich (2017) *Knowledge-based approach to identify key determinants of cisplatin sensitivity.* International journal of clinical pharmacology and therapeutics, 55 . pp. 686-689. ISSN 0946-1965.

Knowledge-based approach to identify key determinants of cisplatin sensitivity

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Introduction

Platinum complexes are widely used in the treatment of many cancer entities, among them non-small cell lung cancer (NSCLC). The treatment outcome is often limited by the development of resistance due to multiple mechanisms [1, 2]. Previous results from our group have revealed that parental NSCLC cell lines are more sensitive to cisplatin-induced G_2/M cell cycle arrest and apoptosis than the respective cisplatin-resistant sub-line, even at cisplatin concentrations that induce comparable platinum-DNA adduct in the different models. This suggests that changes in cell signaling pathways involved in the regulation of the cell cycle and apoptosis play a role in the development of acquired resistance to cisplatin [Engel et al., manuscript in preparation].

Here, we performed a transcriptomics pathway analysis (microarray measurement of mRNA abundance) comparing the response of the A549 NSCLC cell line and its cisplatin-resistant sub-line A549^{r}CDDP^{2000} to cisplatin exposure. Moreover, a literature-based list of genes of interest (GOI), which may be related to cisplatin sensitivity, was compiled. The microarray results and the GOI list were then merged to identify candidates for future investigations.

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Material and methods

Cell culture

The human non-small cell lung carcinoma (NSCLC) cell line A549 was obtained from ATCC (Manassas, VA, USA) and its cisplatin resistant sub-line A549^{r}CDDP^{2000} was derived from the Resistant Cancer Cell Line (RCCL) collection (www.kent.ac.uk/stms/cmp/RCCL/RCCLabout.html). The sub-line had been established by adapting A549 cells to grow in the presence of increasing concentrations of cisplatin with a final concentration of 2,000 ng/mL cisplatin as described previously [3]. A549 cells were grown in IMDM medium (PAN-Biotech, Aidenbach, Germany) containing 4 mM L-glutamine, supplemented with 10% fetal calf serum, 100 IU/mL penicillin and 0.1 mg/mL streptomycin. The medium of the A549^{r}CDDP^{2000} cells additionally contained 2 µg/mL cisplatin. Cells were cultivated as monolayers in a humidified atmosphere at 37 °C and 5% CO_2. For all experiments, cells were allowed to attach overnight, experienced 4 hours of serum starvation and were subsequently treated with cisplatin for 24 hours in IMDM medium without any supplement. The cells were treated with the respective EC_{10} of cisplatin, i.e. 11 µM for the parental and 34 µM cisplatin for the resistant cell line. In the following, equimolar treatment refers to treatment of both cell lines with 11 µM cisplatin and equitoxic treatment refers to treatment of the sensitive cell line with 11 µM and the resistant cell line with 34 µM cisplatin.

Microarray

Total RNA was isolated using the my-Budget RNA Mini Kit (Bio-Budget, Krefeld, Germany) according to the manufacturer's

instruction. Isolated RNA was stored at −80 °C until the analysis was performed.

The transcriptome was then analyzed using the One-Color Whole Genome Array SurePrint G3 Human GE V2 8×60K Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instruction. Briefly, total RNA was transcribed to cDNA using AffinityScript-RT, oligo dT-promoter primer and T7 RNA polymerase and labelled using the one-color RNA spike-in kit (positive controls) including cyanin 3-CTP (Cy3) dye. After purifying the labeled/amplified cRNA using the RNeasy® Mini Kit (Agilent Technologies), cRNA was quantified spectrophotometrically using NanoDrop™ ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). 40 µL of equivalent amounts of Cy3-labeled cRNA in 10× blocking agent and 25× fragmentation buffer, diluted with 2× GEx HI-RPM hybridization buffer were loaded on the gaskets of the microarray slide and kept at 65 °C for 17 hours with 10 rpm of agitation. After washing twice with different washing buffers, the microarray was read out with the SureScan™ Microarray Scanner System (Agilent Technologies) to obtain immunofluorescence intensity. Raw data were analyzed by the Feature Extraction software (Agilent Technologies). The analysis of the obtained microarray data was performed with GeneSpring GX Vers. 12.6 (Agilent Technologies).

Results

Pathway analysis

After checking the internal quality controls of the microarray and quantile normalization, differentially expressed genes (DEG) were identified with a fold change cutoff at 2.0 and a corrected p-value cutoff at 0.05 (unpaired t-test, false discovery rate) for the three treatment conditions: A549 untreated vs. A549 11 µM; A549^{r}CDDP^{2000} vs. A549^{r}CDDP^{2000} 11 µM and A549^{r}CDDP^{2000} untreated vs. A549^{r}CDDP^{2000} 34 µM cisplatin. The DEG lists for each of the three treatment conditions were analyzed in a pathway analysis with a hypergeometric method (GeneSpring GX Vers. 12.6, Agilent Technologies) based on the WikiPathways

database to identify pathways that were differentially regulated by cisplatin treatment in the two cell lines. Pathways were categorized as differentially regulated by p-values ≤ 0.01. Usually p-values ≤ 0.05 are used for that kind of analysis. In our case we decided to apply the more stringent cutoff because no correction for multiple testing was included in the calculation algorithm of the pathway analysis. The identified pathways were then used to compile a genes of interest (GOI) list as described in the following section.

Compilation of the genes of interest (GOI) list

Pathways including genes that encode gene products that were previously reported to be involved in the cellular response to cisplatin [4, 5, 6, 7] were selected from the list of pathways. Subsequently, genes belonging to these pathways were compiled in a list of 1,036 GOI.

Identification of candidates

The genes of the GOI list were then used to identify a set of candidates for the biological validation of the microarrays. Therefore, a Venn diagram was created to identify genes, regulated upon different treatment conditions as indicated by the microarray and overlapping with the GOI list (Figure 1). Numbers in the different fields of the diagram indicate the number of genes simultaneously up- or down-regulated under the two indicated conditions. The gene lists were created with genes simultaneously regulated with a fold change ≥ 2 and a p-value cutoff in an unpaired t-test of ≤ 0.05.

Three sections of the Venn diagram were analyzed in detail. The central section represents genes of interest regulated under all treatment conditions. This section consists of the two genes: mouse double minute 2 homologue (MDM2), a proto-oncogene targeting tumor suppressor p53, and cyclin-dependent kinase inhibitor 1A (CDKN1A, p21), a regulator of cell cycle, both p53 target genes involved in apoptosis regulation. Both are simultaneously regulated in sensitive and resistant cells. This suggests that there is a direct consequence of cisplatin treatment independently of the cellular resistance status.
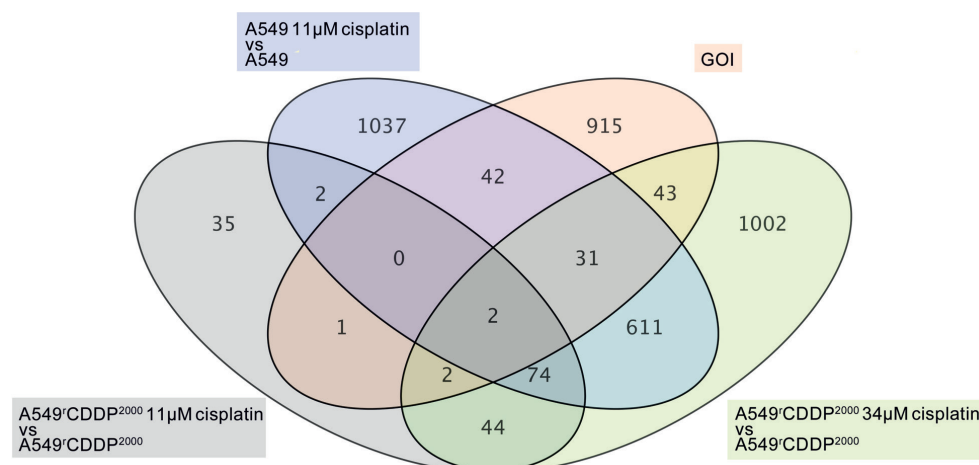
Figure 1.   Venn diagram showing the number of simultaneously up- and down-regulated genes under different treatment conditions and overlap with the genes of interest (GOI) list.

The second section is the overlap of the GOI genes with the genes found differentially expressed between untreated and 11 μM cisplatin-treated A549^{r}CDDP^{2000} cells. SLC12A3, a Na$^+$/Cl$^-$ symporter, is the only gene in this overlap. As this gene regulation only occurs in resistant cells after low-dose cisplatin treatment in contrast to the sensitive cells, SLC12A3 may be involved in the mechanism of cellular tolerance to cisplatin.

Moreover, an overlap of 43 genes was found for the comparison of the GOI list with the genes that were differentially regulated in A549^{r}CDDP^{2000} cells upon treatment with 34 μM cisplatin: APAF1, BCL2, BCL2A1, BIK, BIRC3, CASP10, CAV1α, CAV1β, CDKN2D, CXCL1, CXCL1 isoforms, DUSP5, DUSP7, EGF, ERBB3, FOSL1, GSN, HBEGF, HRAS, IGF2, IGFBP3, IL8, MAP2K3, MAP3K12, MCM6, MCM9, MEF2C, NFE2L2, NFKB2, NRG1, NRG1 isoforms, PLAT, PMAIP1, PRKCD, PTPRU, RASGRP1, RPS6KA1, RPS6KA3, RPS6KA5, SPRY2, TNFRSF10A, TRAF1, WNT4. Since these genes were not found differentially regulated in response to 11 μM cisplatin, their differential expression may contribute to the cisplatin resistance phenotype, and these genes represent candidates for future studies. Notably, several of these genes are involved in cell growth and proliferation, like EGF, ERBB3, HRas or several mitogen-activated kinases, or play a role in apoptosis and cell cycle control, like BCL2, CASP10, or CDKN2D.

Conclusions

Here, we have identified a set of candidate genes whose gene products may modulate cell sensitivity to cisplatin and that warrant further investigation in subsequent studies. Although our method has its limitation due to the focus on already published data in the selection of pathways and genes, it provides a possibility to reduce the massive amount of data derived from a whole genome array. So far unknown (till now not published) affected pathways, which could additionally account for chemoresistance were thus excluded from our analysis. Nevertheless, this is the first step towards a systems pharmacological approach.

Conflict of interest

There are no conflicts of interest to declare.

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