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ORIGINAL ARTICLE

Identification of pharmacodynamic biomarkers and common molecular mechanisms of response to genotoxic agents in cancer cell lines

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Abstract

Purpose Genotoxic agents (GAs) including cisplatin, doxorubicin, gemcitabine, and topotecan are often used in cancer treatment. However, the response to GAs is variable among patients and predictive biomarkers are inadequate to select patients for treatment. Accurate and rapid pharmacodynamics measures of response can, thus, be useful for monitoring therapy and improve clinical outcomes.

Methods This study focuses on integrating a database of genome-wide response to treatment (The NCI Transcriptional Pharmacodynamics Workbench) with a database of baseline gene expression (GSE32474) for the NCI-60 cell lines to identify mechanisms of response and pharmacodynamic (PD) biomarkers.

Results and conclusions Our analysis suggests that GA-induced endoplasmic reticulum (ER) stress may signal for GA-induced cell death. Reducing the uptake of GA, activating DNA repair, and blocking ER-stress induction cooperate to prevent GA-induced cell death in the GA-resistant cells. ATF3, DDIT3, CARS, and PPP1R15A appear as possible candidate PD biomarkers for monitoring the progress of GA treatment. Further validation studies on the proposed intrinsic drug-resistant mechanism and candidate genes are needed using in vivo data from either patient-derived xenograft models or clinical chemotherapy trials.

Keywords Genotoxic agents · Pharmacodynamic biomarkers · Response mechanisms · ER stress

Introduction

Genotoxic agents (GAs) are key members of the chemotherapy repertoire, because cancer cells are often highly proliferative. GAs target DNA by various mechanisms and different targets. For example, cisplatin forms intra-strand crosslinks in DNA, doxorubicin, and topotecan inhibit topoisomerases II and I, respectively, and gemcitabine blocks nucleotide metabolism: all result eventually in failed DNA replication, disturbed DNA integrity, cell cycle inhibition, and induced cell death [1, 2]. However, the response to GAs is inconsistent and varies between tumor types and even within the same tumor type [3, 4]. Insensitivity of some cancer cells towards GAs, thus, remains a major obstacle in cancer treatment. Such insensitivity or resistance can be due to various mechanisms including diminished agent accumulation (efflux) and activation, elevated DNA repair or DNA damage tolerance, enhanced activation of anti-apoptotic proteins, and

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blocked apoptosis pathways [3, 5, 6]. Resistance mechanisms developed by genetic or epigenetic alterations protect cancer cells from GAs cytotoxic action [4]. For example, mutation and expression of DNA repair and apoptosis genes, such as BRCA1, BRCA2, and TP53, are considered key in predicting a GA response [4, 5]. However, the status of such genes/proteins only modestly correlates with response [7], indicating that the response to GAs is complex and that response after treatment requires careful monitoring.

Early tumor assessment after treatment may enable the adjustment of dose and schedule of the drug, and improve clinical outcomes and ameliorate side effects [8–10]. Conventional monitoring methods to judge response, which are often based on changes in tumor size, can be subjective and time consuming [11]. In addition, early changes in tumor size are often not correlated with biologic changes in cancer cells, causing misleading predictions of drug response [11]. Pharmacodynamic (PD) biomarkers may prove valuable for rapid and accurate evaluation of tumor response in the clinic [9, 10]. Gene expression-based biomarkers have the potential advantage of providing quantitative results with a wide dynamic range [12]. In addition, changes in gene expression after treatment provide information about cellular mechanisms of drug action [12].

The study presented here focuses on identification of genes that may be potential PD biomarkers of GA response or elucidate response mechanisms, by analyzing gene expression data from the NCI Transcriptional Pharmacodynamics Workbench (NCI TPW) (https://tpwb.nci.nih.gov/ [13]). The NCI TPW is a publicly available database of drug-induced transcriptional profiles measured in the NCI-60 cell line panel and represents an extensive compilation of directly measured longitudinal transcriptional responses to 15 anticancer agents. The cells were exposed to these agents at a "low" and "high" concentration, approximating the clinical C_{max} [14] and the average concentration resulting in 50% inhibition of the cell lines. Human tumor cell lines are traditionally valuable tools to identify genetic variants impacting drug response. Gene expression changes following treatment with four GAs (cisplatin, doxorubicin, gemcitabine, and topotecan) were analyzed. The main hypotheses is that since all four GAs interrupt DNA replication and induce DNA damage, there may be key PD biomarkers and centralized mechanisms of action of GAs. For the PD biomarker study, data available in the NCI TPW were used to collect the lists of genes, whose change in expression after treatment correlated with drug response based on growth inhibition of 50% (log10 GI_{50}, referred as GI50 from this point forward) for each GA. The response and resistance mechanisms of GAs were investigated using differential gene expression profiles between 'sensitive' and 'resistant' cell lines before and after treatment.

Methods

The 100 genes with the largest absolute correlation between 24-h post-treatment gene expression, and GI50, for the NCI-60 cell lines treated with a lower concentration of each GA were obtained from the NCI TPW [13]. The data were collected from the NCI TPW database (https://tpwb.nci.nih.gov/) using the "Correlation Analysis" option for each of the GA treatments. The cell lines most sensitive and resistant to each GA were classified based on GI50 values provided in the TPW. Baseline gene expression data for the NCI-60 cell lines were obtained from the Gene Expression Omnibus (GSE32474) [15] and were analyzed to find genes differentially expressed between sensitive and resistant cell lines using GEO2R analysis tool (www.ncbi.nlm.nih.gov/geo/geo2r/). Genes which were differentially expressed for multiple GAs were identified using a Venn diagram (Venny 2.1).

For the Receiver Operating Characteristic (ROC) analysis, cell lines were clustered into three groups by dividing the difference between the highest and lowest GI50 values by three for each GA forming high (resistant), intermediate, and low (sensitive) GI50 groups (Fig. 3). The outliers of high GI50 values in the doxorubicin and topotecan treatments were not considered for determining the threshold for dividing the above three groups. The ROC curve was constructed by classifying the resistant and sensitive groups of cell lines based on the changes in RNA expression of the candidate genes after treatment from the TPW. The area under the curve (AUC) values in the ROC curve were calculated using the *R* with "rms" and "ModelGood" packages.

Results

Identification of response mechanisms and PD biomarkers of GAs

To investigate cellular response mechanisms after exposure to GAs, 100 genes with the largest absolute correlation between change in gene expression after treatment and sensitivity (measured by GI50) in the NCI-60 cell lines [13] were selected. Gene lists were generated separately for treatment with concentrations of cisplatin (3 μM), doxorubicin (0.1 μM), gemcitabine (0.2 μM), and topotecan (0.01 μM), respectively, for 24 h. To identify centralized cellular mechanisms after exposure to a GA, genes common across multiple agents were included in our study. No genes were identified in common for all 4 drugs. However, 26 negatively correlated genes and 4

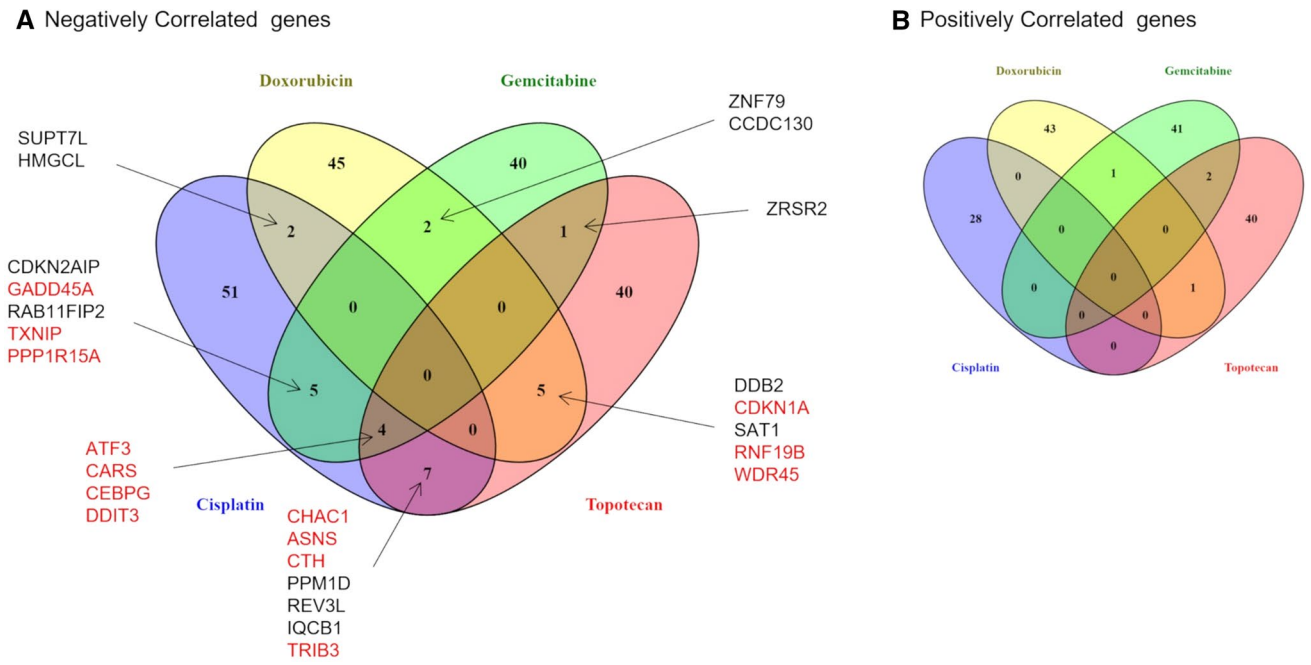

Fig. 1 Distribution of the 100 genes with the largest absolute correlation between changes in gene expression and GI50 of cisplatin, doxorubicin, gemcitabine, and topotecan, respectively, in the NCI-60 lines treated with a low concentration for 24 h. The negative correlation means that those genes were generally up-regulated after treatment in the low GI50 concentration lines, but down-regulated or not changed in the high GI50 concentration lines. The positive correlation means that those genes were down-regulated in the resistant cell lines after treatment, and up-regulated in the sensitive cell lines. Cisplatin: 3000 nM, doxorubicin: 100 nM, gemcitabine: 200 nM, topotecan: 10 nM. ER-stress-related genes are represented with red color

positively correlated genes were identified in common for at least two GA drugs, which are shown in the Venn diagram (Fig. 1). Negative correlation is an association between those genes generally up-regulated after GA treatment in cell lines with low GI50 (referred to as 'sensitive cells'), but down-regulated or unchanged in cell lines with high concentration GI50 (referred to as 'resistant cells'). Annotation of the 26 up-regulated genes (negative correlation) found that most of them were endoplasmic reticulum (ER) stress-related genes including ATF3, CARS, CEBPG, DDIT3, GADD45A, TXNIP, PPP1R15A, CHAC1, ASNS, CTH, TRIB3, CDKN1A, RNF19B, and WDR45 (Fig. 1a) [16–19]. These results indicate that GAs may induce ER stress contributing to cell arrest or death in sensitive cells. In contrast, GA-induced ER stress may be blocked in resistant cells. Based on published information, the potential relationships between ER-stress genes are summarized in Fig. 2, where the genes highlighted in bold were up-regulated in the sensitive cell line cohort as identified above and in Fig. 1a.

The potential of the genes identified above to serve as PD biomarkers was analyzed in cell lines for early clinical assessment after GA treatment. The cell lines in the resistant cell line group (high concentration GI50) and the sensitive cell line group (low concentration GI50) were used for the

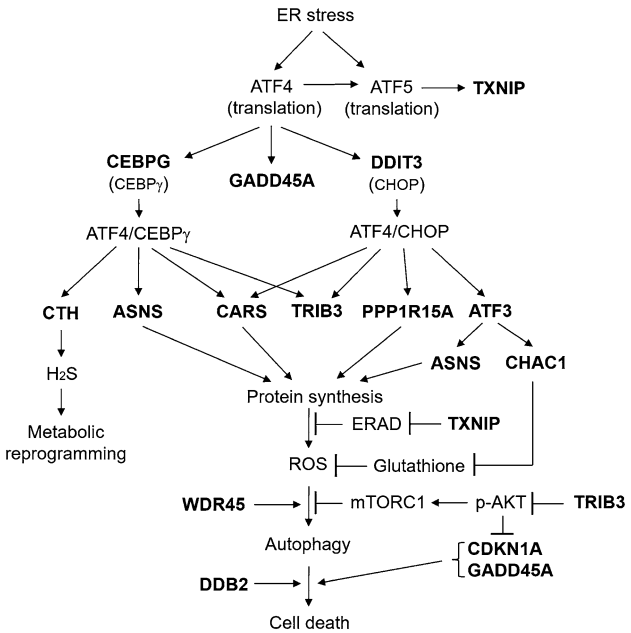

Fig. 2 Summary of the functional relationships between the ER-stress-related genes. Genes in bold are the negatively correlated genes in Fig. 1. *ERAD* ER-associated degradation, *ROS* reactive oxygen species

ROC analysis to identify PD biomarkers. Since the change in RNA expression of each gene in the cell lines varied depending upon GA treatment, the analysis was performed separately for each GA and the mean AUC of each gene was calculated (Table 1). The AUC values for topotecan were, generally, lower, because the range of GI50 values was relatively narrow. Based on the mean AUC values threshold above 80, ATF3, DDIT3, CARS, and PPP1R15A emerged as potential PD biomarker candidates. When the potential PD biomarkers were evaluated as a group, the AUC values increased, indicating that the 4-gene group was more reliable than the single genes as a monitor of response (Table 2).

Identification of resistance mechanisms of GAs

The causes of drug resistance can be classified as preexisting (intrinsic resistance) or drug-induced (acquired resistance) [20]. The main genetic causes of intrinsic resistance include mutation, deletion, and overexpression of drug response-related genes. Understanding intrinsic resistance mechanisms is important for selecting effective drugs before treatment. To identify intrinsic GA-resistance mechanisms using expression data, the "common" sensitive and resistant cell lines across all four GAs have been redefined. Starting from the sensitive and resistant cell lines for each single GA in Fig. 3, "common" resistant and sensitive cell lines in at least two GAs were identified using a Venn diagram (Fig. 4, Supplementary Table 1). Then, analysis for differentially expressed genes in the "common" resistant lines compared to the "common" sensitive lines (Fig. 4, Supplementary Table 1) was performed using baseline gene expression data obtained using Affymetrix human genome U133 plus 2.0 arrays with the robust multichip analysis (GSE32474) [15]. The top 250 significant probe sets were identified using the GEO2R analysis tool (www.ncbi.nlm.nih.gov/geo/geo2r/). Among the above, there are 46 and 118 protein-encoding genes in the sensitive lines that were expressed higher and lower, respectively, than in the resistant lines

Table 1 Area under the curve (AUC) values (%) of ER-stress-related genes in the receiver operator characteristic curve (ROC) constructed in logistic regression for evaluating them as a pharmacodynamic biomarker of genotoxic agents

	Cisplatin	Doxorubicin	Gemcitabine	Topotecan	Mean AUC
ATF3	82	91	94	78	**86**
CARS	97	67	91	69	**81**
CEBPG	90	45	91	74	75
DDIT3	80	95	96	80	**88**
GADD45A	79	79	90	68	79
TXNIP	73	80	89	67	77
PPP1R15A	80	89	89	63	**81**
CHAC1	91	74	77	68	78
ASNS	88	47	82	79	74
CTH	79	58	83	68	72
TRIB3	74	52	76	70	68
CDKN1A	69	83	67	73	73
RNF19B	69	83	76	71	75
WDR45	75	86	60	78	75

Mean AUC values greater than 80 are highlighted in bold

Table 2 Area under the curve (AUC) values (%) of the combined ER-stress-related genes in the receiver operator characteristic curve (ROC) constructed in logistic regression for evaluating them as pharmacodynamic biomarkers in combination of genotoxic agents

	Cisplatin	Doxorubicin	Gemcitabine	Topotecan	Mean AUC
A+B	82	95	95	83	89
A+C	97	91	95	80	91
A+D	84	98	94	80	89
B+C	97	98	96	80	93
B+D	82	98	95	79	89
C+D	97	88	94	70	87
A+B+C	96	98	95	83	93
A+B+D	82	98	96	83	90
B+C+D	97	99	96	79	93
A+C+D	97	97	96	81	93
A+B+C+D	96	99	97	83	94

A ATF3, *B* DDIT3, *C* CARS, *D* PPP1R15A

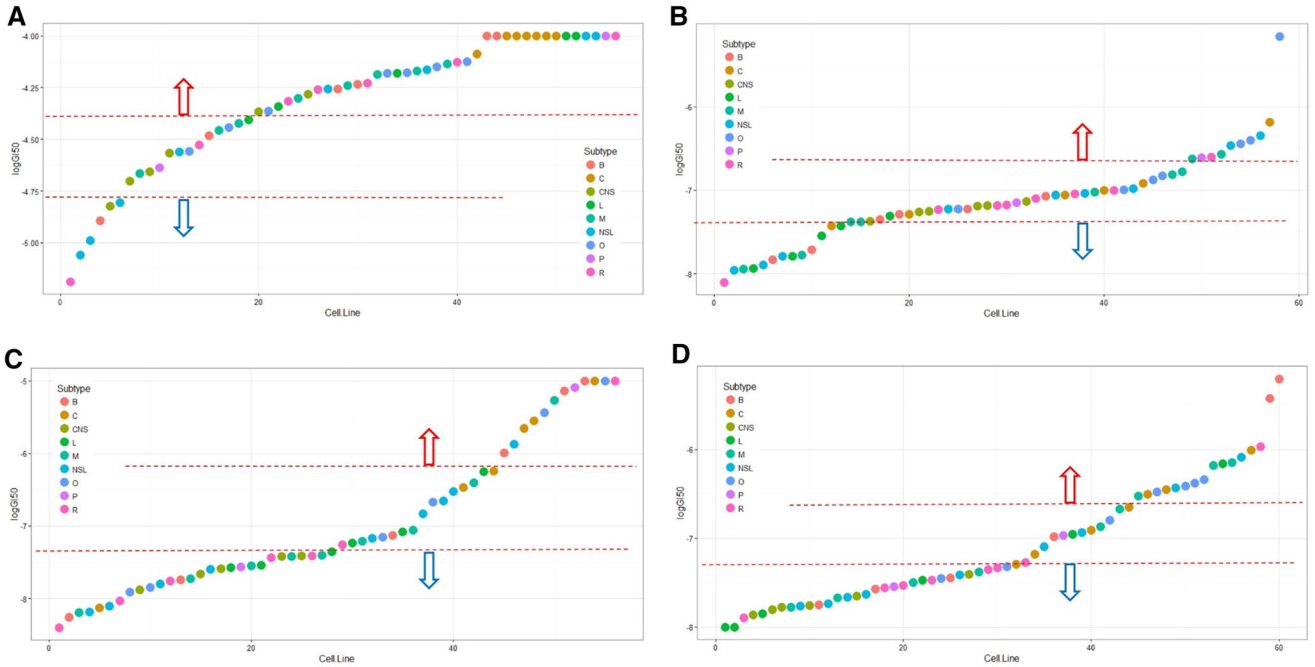

Fig. 3 NCI-60 lines GI50 response to cisplatin (**a**), doxorubicin (**b**), gemcitabine (**c**), and topotecan (**d**). The lines were clustered into three groups (high, intermediate, and low GI50 groups) by dividing the difference between the highest and lowest log10 GI50 value by 3 for each drug. The outliers of high GI50 concentrations in B and D were not considered for this calculation. High log10 GI50 lines (⇑) were represented as resistant cells and low log10 GI50 lines (⇓) were represented as sensitive cells. *B* breast, *C* colon, *CNS* central nervous system, *L* leukemia, *M* melanoma, *NSL* non-small cell lung, *O* ovarian, *P* prostate, *R* renal

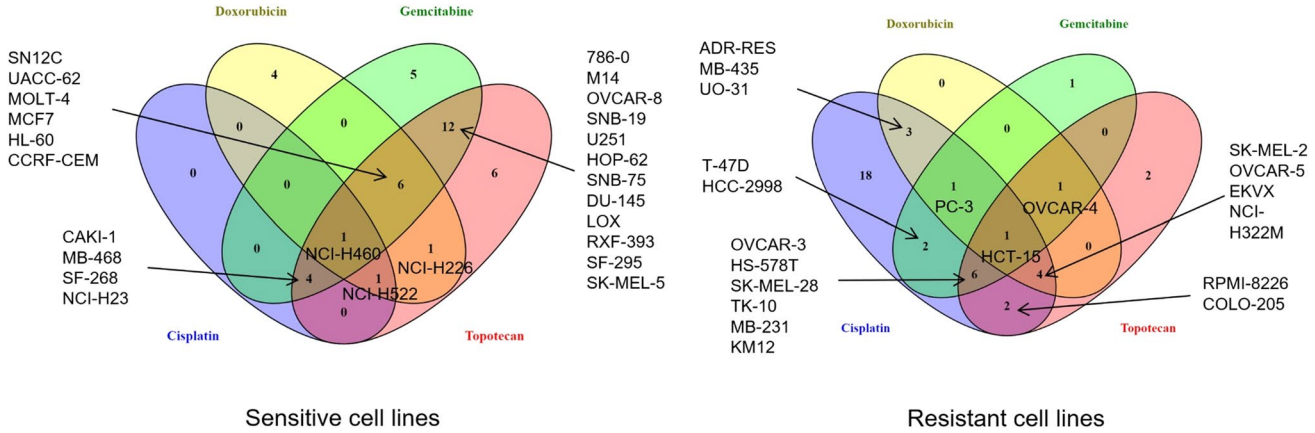

Fig. 4 Cancer cell lines sensitive or resistant to cisplatin, doxorubicin, gemcitabine, and topotecan, respectively, based on Fig. 3

(Supplementary Table 2). Gene functional analysis was carried out using DAVID Bioinformatics Resources (https://david.ncifcrf.gov/) with those genes. Among the 118 genes highly expressed in the resistant lines, 21 genes were involved in cell:cell junctions including tight junctions and adherens junctions (Table 3, Supplementary Fig. 1) [21, 22], indicating that cell junction proteins may protect the tumor from GAs. Moreover, ER-stress-, DNA repair-, and PI3K/AKT-related genes were also identified as differentially expressed, and those more highly expressed in the resistant cell lines (Table 3) were investigated for a potential role in GA-resistance mechanisms. Specifically, TNK1, CBLC, and SFN have been reported to activate DNA repair [23–25]. ST14, LCN2, CDH1, PROM1, and EPCAM have been reported to activate PI3K/AKT [26–30] and MST1R (RON) has been reported to activate both DNA repair and PI3K/AKT pathway [31, 32]. ATP2C2 and AGR2 were reported to inhibit ER-stress induction [33, 34], and SPINT1 and

Table 3 Cell junction-related genes and ER-stress/DNA damage/CHOP inhibiting genes expressed more highly in the resistant cells than in the sensitive cells

Gene	Probe set ID	Functional pathways in which the gene is involved	Fold change
EPCAM	201839_s_at	Cell:cell junctions, PI3K/AKT	17.76
CDH1	201131_s_at	Cell:cell junctions, PI3K/AKT	6.79
SFN	33323_r_at	Cell:cell junctions, DNA repair	5.71
CLDN3	203953_s_at	Cell:cell junctions	4.77
KRT18	201596_x_at	Cell:cell junctions	4.36
CLDN4	201428_at	Cell:cell junctions	4.35
CLDN7	202790_at	Cell:cell junctions	3.03
TMC5	240304_s_at	Cell:cell junctions	2.78
TMC4	226403_at	Cell:cell junctions	2.57
LAD1	203287_at	Cell:cell junctions	2.41
PLS1	205190_at	Cell:cell junctions	2.4
EPB41L4B	223427_s_at	Cell:cell junctions	2.3
MARVELD3	239148_at	Cell:cell junctions	2.01
DLG3	212729_at	Cell:cell junctions	1.76
EPS8L1	91826_at	Cell:cell junctions	1.69
MISP	212925_at	Cell:cell junctions	1.65
TJP3	213412_at	Cell:cell junctions	1.58
F11R	224097_s_at	Cell:cell junctions	1.55
EVPL	204503_at	Cell:cell junctions	1.52
CRB3	232609_at	Cell:cell junctions	1.39
SH3D19	1558647_at	Cell:cell junctions	1.38
AGR2	209173_at	Inhibit ER stress	8.86
ATP2C2	214798_at	Inhibit ER stress	1.35
SPINT2	210715_s_at	Block CHOP expression	6
SPINT1	202826_at	Block CHOP expression	4.27
LCN2	212531_at	PI3K/AKT	4.06
PR0M1	204304_s_at	PI3K/AKT	3.21
UCA1	227919_at	Inhibit cell death	2.79
MACC1	1566764_at	Inhibit cell death	2.76
ST14	202005_at	PI3K/AKT	2.63
MST1R	205455_at	PI3K/AKT, DNA repair	1.93
CBLC	220638_s_at	DNA repair	1.8
TNK1	217149_x_at	DNA repair	1.22

2 were reported to block CHOP expression [35]. Finally, MACC1 and UCA1 were reported to inhibit cell death [36, 37]. These reports suggest that cancer cells harboring active DNA repair and inactive ER-stress induction systems may be protected from GA-induced cell death.

Central nervous system (CNS) cancer cell lines were found to be exclusively included in the sensitive group, while colon cancer cell lines were only in the resistant group (Supplementary Table 1). Neurons are known to be sensitive to ER stress [16]. RNA expression of ER-stress genes between CNS and colon cancer clinical samples were compared using baseline gene expression data available in the cancer genome atlas (TCGA) to begin to validate the findings in tissue samples. All cell junction-, ER-stress-, and DNA repair-related genes, which were more highly expressed in the resistant cell lines, were also more highly expressed in colon cancer samples (Supplementary Figs. 3, 4), suggesting that GA-mediated severe ER stress might be critical to induce GA-mediated cell death in cancer.

Discussion

Understanding resistance mechanisms of cancer cells to GAs continues to be an important focus. Abnormal p53 function and enhanced DNA repair have been broadly accepted as mechanisms of resistance to GAs [7, 38]. Activation of the unfolded protein response (UPR) following ER stress has also been identified as a mechanism of resistance (reviewed in [39]). However, response to GAs varies not only between tumor types, but also between individual tumors of the same type regardless of p53 status and DNA repair systems (Supplementary Table 1) [40–42]. The study presented here sought to identify other broad GA-resistance mechanisms along with potential PD biomarkers for assessing treatment response.

GA response data for the NCI-60 lines indicated that many pro-apoptotic ER-stress-related genes such as ATF3, DDIT3 (CHOP), GADD45A, TXNIP, PPP1R15A (GADD34), CHAC1, and TRIB3 were up-regulated in sensitive compared to resistant cell lines. Severe ER stress induces pro-apoptotic proteins, such as CHOP (DDIT3), and later activates autophagy-induced cell death [43]. Although GA–DNA interactions are considered key leading to cell death, other DNA damage-independent mechanisms are important, and our data suggest that ER stress could be one. ER stress potentially leads to misfolded proteins and is regulated by the Unfolded Protein Response (UPR) which is an adaptive response that can increase cell survival [44, 45]. However, in the case of irreversible ER stress, UPR switches to induction of apoptosis in damaged cells [46, 47]. Therapy-induced ER stress contributes to changes in drug sensitivity, although it is not completely clear what triggers the switch from an adaptive response to apoptosis [39, 48]. A UPR-induced protein, CHOP (DDIT3) initiates apoptosis through regulation of BH3 proteins [49] and also through augmented ROS production, ATP depletion, and reactivated protein synthesis [18]. Sensitive cell lines activated the UPR response to GAs more robustly than the GA-resistant cell lines suggest that the UPR response might be leveraged to increase drug sensitivity in non-responsive tumors. Thus, identifying whether the genotoxic response leads to apoptosis through

modulation of the BH3 protein response, change in ROS production, or ATP depletion would provide critical information. However, the UPR activation in sensitive cells may be an attempt by the cell to protect against inevitable cell death. The lack of ER-stress response via UPR in resistant cells may reflect innate resistance that prevents induction of ER stress. To address this, the endogenous gene expression profiles of GA-resistant cells were used to identify genes differentially expressed at baseline compared to sensitive cells. Our analysis showed that many cell junction protein-encoding genes were more highly expressed in the resistant cells (Table 3). Since cell junction proteins regulate drug delivery, they are important for drug efficacy and response [21] and poor drug delivery may be an important factor. Moreover, UPR induced the ER chaperone BiP in an epithelial colon cancer cell, but a cell death protein CHOP in a mesenchymal cell [50]. These reports support our results identifying a common theme for activity and resistance to GA's involving ER-stress induction and cell junction protein-encoding gene expression. When these potential resistance genes identified in the NCI-60 cell lines were evaluated across a cohort of tumor tissue representing the more resistant cell lines (colon) compared to a more sensitive tumor tissue cohort (CNS), all of the genes were found to be more highly expressed in the resistant cohort, lending further support to the hypothesis.

The previous studies show some cell-surface proteins encoded by the genes identified may support chemo-resistance. EPCAM (epithelial cell adhesion molecule), the gene most differentially expressed (Table 3), was shown to contribute to chemo-resistance in multiple cell types, including prostate, breast, and colon cancers [27, 51, 52]. In addition, several of the cell-surface genes identified have been associated with epithelial-to-mesenchymal transition (EMT), and recent data have identified an intriguing role for EMT in chemo-resistance in cancer [53, 54]. EMT has been linked to increased ER-stress response which enhances tumor cell survival, indicating a possible protective mechanism [55]. Claudin proteins including claudin-3, -4, and -7 (CLDN3, 4, and 7) were reported to be overexpressed in many cancers, and by leading to EMT, were associated with cisplatin resistance in ovarian cancer [56]. The cadherin encoded by CDH4 has also been associated with EMT and resistance to cisplatin [57]. Cytokeratin 18 (KRT18) is an epithelial cell-specific intermediate filament protein that is lost during EMT and its loss is reported to decrease cisplatin sensitivity [58]. However, recent data show KRT18 critically contributes to initiating TGF-β1-induced EMT [59]. Thus, our data support a role for highly expressed genes involved in EMT, and also expression of cell surface and cell:cell junction genes, to potentially contribute to inherent cell resistance.

In addition to cell junction protein-encoding genes, genes blocking DNA damage, ER stress, and apoptosis are also more highly expressed in the resistant cells (Table 3). DNA repair processes in cancer cells are, generally, considered a response to genotoxic damage [7, 38]. DNA repair genes, including TNK1, CBLC, SFN, and MST1R, were more highly expressed in the resistant cells, and all have been reported as participating in resistance. The PARP enzyme TNK1 and an E3 ubiquitin ligase CBLC were reported to be involved in non-homologous end joining (NHEJ) DNA repair and in homologous recombination (HR) DNA repair, respectively [23, 24]. An epithelial cell-surface protein MST1R (RON) was reported to be involved in NHEJ DNA repair [32] and to be related to cisplatin resistance of ovarian cancer cells [60], gemcitabine resistance in pancreatic cancer cells [61], and was considered poor prognostic marker in gastric, bladder, colorectal, breast, and non-small-cell lung cancers [31]. SFN (14-3-3σ) was reported to interact with DNA repair protein human exonuclease 1 (hEXO1) to regulate DNA repair, resulting in resistance to DNA-damaging drugs [25], and was a prognosis marker for poor survival of colorectal cancer patients [62].

GA-induced ER stress may be controlled in the resistant cells; ATP2C2 (SPAC2) was reported to maintain low cytosolic and high luminal free Ca^{2+} concentration [33] and AGR2 was found to be involved in the UPR to minimize ER stress and cell death [34]. Finally, cell death may be blocked by MACC1 and UCA1. It was reported that MACC1 decreased cisplatin sensitivity in epithelial ovarian cancer cells [36] and UCA1 (a long non-coding RNA) increased cisplatin and gemcitabine resistance of bladder cancer cells [37, 63].

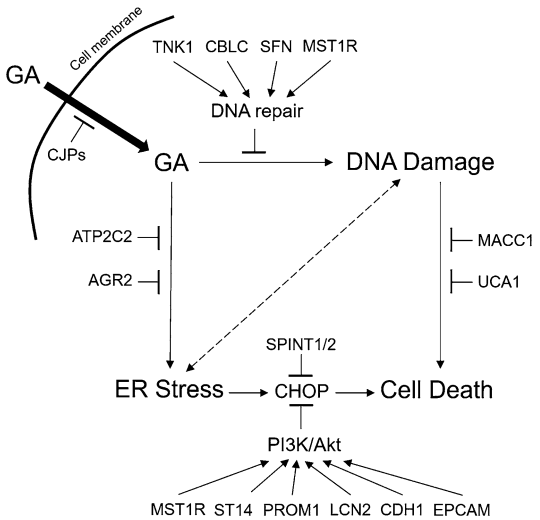

Fig. 5 Suggested resistance mechanisms in the genotoxic agent (GA)-resistant cancer cells. The genes are more highly expressed in the GA-resistant cells than in the GA-sensitive cells. *CJP* cell junction protein (see Table 3). For additional details and supporting literature reference, please refer to the main text

In summary, our analysis suggests that ER-stress induction is a key factor of GA-induced cell death, although the precise mechanisms of GA induction of ER stress remain under investigation and likely vary between drugs. Our results indicate that resistant cells are protected from the ER-stress-induced apoptosis, by inherent resistance mechanisms. One hypothesis is that decreased uptake of GAs, efficient DNA repair, and blocking ER-stress induction may cooperate to reduce GA-induced DNA damage in the GA-resistant lines (Fig. 5). The cooperation between DNA damage and ER stress is supported by the previous studies reporting that DNA damage-induced apoptosis was facilitated by ER protein MG23 [64], DNA repair was inhibited by ER stress [65], and ER chaperone protein BiP was up-regulated by impaired excision repair of DNA [66]. Finally, the results of our analysis suggest that ATF3, DDIT3, CARS, and PPP1R15A are potential candidate PD biomarkers for monitoring response to GA treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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