Changes in leukocyte gene expression profiles induced by antineoplastic chemotherapy

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Abstract. In the present study, we studied changes in gene expression induced by chemotherapy (CT) on normal peripheral blood leukocytes (PBLs), at baseline and following three CT cycles, in order to identify which genes were specifically affected and were potentially useful as biomarkers for a personalised prognosis and follow-up. A PBL subtraction cDNA library was constructed from four patients undergoing CT with paclitaxel and carboplatin (PC). mRNA from the PBLs was isolated prior to the patients receiving the first cycle and following the completion of the third cycle. The library was screened and the expression of the identified genes was studied in PBLs obtained from patients suffering from cancer prior to and following three cycles of PC and a reference group of patients undergoing treatment with Adriamycin-cyclophosphamide (AC). From the 1,200 screened colonies, 65 positive clones showed varied expression intensity and were sequenced; 27 of these were mitochondrial DNA and 38 clones (27 different) were coded for cytosolic and nuclear proteins. The genes that were studied in patients undergoing CT were ATM (ataxia-telangiectasia mutated gene), eIF4B (translation initiation factor 4B), MATR3 (Matrin 3), MORC3 (microrchidia 3), PCMTD2 (protein-L-isoaspartate O-methyltransferase), PDCD10 (programmed cell death gene 10), PSMB1 (proteasome subunit type β), RMND5A (required for meiotic nuclear division 5 homologue A), RUNX2 (runt-related transcription factor 2), SACM1L (suppressor of actin mutations 1-like), TMEM66 (transmembrane protein 66) and ZNF644 (zinc finger protein 644). Certain variations were observed in the expression of the genes that are involved in drug resistance mechanisms, some of which may be secondary to non-desirable effects and others of which may cause the undesired effects of CT. The expression of genes with a dynamic cellular role showed a marked positive correlation, indicating that their upregulation may be involved in a specific pattern of cell survival versus apoptosis in response to the cell damage induced by CT. Whether these CT-induced changes are random or directed in a specific selection-evolution manner needs to be elucidated.

Introduction

Anticancer chemotherapy (CT) functions by producing metabolic alterations, changing the structure and function of DNA, affecting mitotic microtubules or triggering apoptotic pathways in the target cells. As well as beneficial effects in cancer cells, CT results in non-desirable cytotoxic effects in normal healthy tissues. Bone marrow is most susceptible to the non-specific effects of CT; damage may result in short-term toxicity, such as myelosuppression and anaemia, or in long-term problems such as myelodysplastic syndromes or secondary leukaemia forms.

To investigate the changes in gene expression and the possible pathways involved in CT-induced cytotoxicity in normal blood cells, we studied peripheral blood leukocytes (PBLs) prior to and following three cycles of CT.

Most cytotoxic anticancer drugs target DNA directly through reactive metabolites or indirectly through incorporation into DNA (nucleotide analogues) or the blockade of DNA-metabolising enzymes, including polymerases and topoisomerases (1,2). This DNA damage triggers signalling pathways that ultimately lead to cell death (1,2).

Paclitaxel is a tubulin-binding agent that stabilises microtubules (α-β-tubulin heterodimers) in the microtubule-GDP complex, thereby inhibiting the dynamic role of the complex and consequently arresting mitosis (1,2). Platinum chemotherapeutic agents, including carboplatin, halt the cell cycle by forming stable complexes with DNA. The efficacy of chemotherapeutic agents in most types of cancer is limited by the development of resistance. Resistance to paclitaxel suggests the aberrant expression of β-tubulin isotypes, usually βIII-tubulin, or microtubule-regulating proteins (3). Resistance to platinum is commonly developed by the DNA nucleotide excision repair pathways as repair or mismatch repair, such as excision repair cross-complementation group 1 enzyme (ERCC1) (4).

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Doxorubicin interacts with DNA by intercalating between two base pairs, blocking the progression of topoisomerase II and thereby preventing the process of replication (2). Resistance to doxorubicin is multifactorial, including increased levels of factors involved in reducing the intracellular drug concentration by increasing drug extrusion through membrane-associated pumps, the altered expression of topoisomerase II, increased ability of the cell to repair DNA damage and changes in gene expression that reduce the apoptotic response (5,6).

Cyclophosphamide mainly acts through its metabolite, phosphoramide mustard, which is an irreversible interstrand DNA crosslinker that causes cell death. Cytochrome P450 (CYP), glutathione S-transferase (GST) and ALDH protect the cells in tissues with rapid cell turnover against the toxic effects of phosphoramide mustard (7) by converting aldophosphamide to molecules that do not produce the toxic metabolites.

The aim of this study was to identify a group of genes in PBLs whose expression is modified by CT.

Materials and methods

Patients. This study was approved by the Ethics Committee of La Laguna University and HUNSC and all the subjects signed informed consent documents prior to entering the study. The study subjects were Caucasian, of European origin and recruited from the reference medical area of the hospital. The patients in this study were administered CT only (not radiotherapy or any other adjuvant therapy), and this was their first CT. Two groups were analysed: patients undergoing carboplatin-paclitaxel (PC) treatment and, as a reference, patients treated with doxorubicin (Adriamycin)-cyclophosphamide (AC).

Leukocyte isolation. PBLs were obtained immediately prior to patients undergoing the first cycle and following the third cycle (at the time the 4th cycle was administered). A volume of 1X PBS (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl) was added to an identical volume of freshly collected blood and mixed gently. The mix was placed over 5 ml Ficoll-Hypaque (d=1.077 g/ml) (Sigma, St. Louis, MO, USA) and centrifuged for 30 min at 600 x g. The intermediate layer (mononuclear white cells) was aspirated and washed three times in three volumes of HBSS (5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM D-glucose, 0.02% phenol red) and centrifuged at 300 x g for 10 min. The pellet containing the cells was resuspended in 1 ml of PBS. The trypan blue test and cell counting in a Neubauer chamber were performed. The amount of collected cells ranged from 3 to $4x10^6$ cells.

PBL mRNA extraction, double stranded (ds) cDNA synthesis and PCR amplification. The collected PBLs from 5 ml of blood were spun at 300 x g for 5 min and mRNA was obtained from the cell pellet using the Total SV RNA Isolation system (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. cDNA was synthesised using the iScript cDNA Synthesis kit (Bio-Rad, Mannheim, Germany) for the real-time quantitative RT-PCR (qRT-PCR) assay and the Super SMART PCR cDNA Synthesis kit (Clontech Laboratories, Mountain View, CA, USA) for the subtraction library according to the manufacturer's instructions.

ds-cDNA subtraction and library construction. The subtraction library was constructed using the PCR Select cDNA Subtraction kit (Clontech Laboratories) according to the manufacturer's instructions, with ds-cDNA pools obtained from the white cells of the four patients immediately prior to undergoing the first cycle and at the time the 4th cycle of PC was administered, using equal amounts of cDNA from each patient. The pool of differentially expressed cDNA was cloned into the pBSK+ vector in competent *E. coli*, XL-2-Blue cells.

Dot-blot screening. Probes were used to subtract the cDNA from the PBLs obtained pre- and post-treatment and were digoxigenin-dUTP labeled using the DIG DNA labeling kit (Roche Diagnostics, Mannheim, Germany). Colonies from the library were seeded in plates containing LB-ampicillin (0.1 mg/ml), X-Gal (40 μg/ml) and IPTG (0.2 mM) solid medium and incubated at 37˚C overnight. The colonies were selected and placed into 96-well plates in liquid LB-ampicillin (0.1 mg/m) medium and incubated at 37˚C overnight. A volume of 1 μl from each cultured well was placed in duplicate onto two nylon membranes (Roche Diagnostics) and incubated at 37˚C overnight over LB-ampicillin (0.1 mg/ml) solid medium plates. Duplicate membranes were probed with both probes (8) and positive clones were re-checked by double-dot-blotting of the PCR amplified product using T3 and T7 flanking sequence primers (8).

DNA sequencing and sequence identification. DNA sequencing was performed at La Laguna University Sequencing Facility. DNA for sequencing was prepared using the Qiaprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequence alignments of the sequences with those in the GenBank, EMBL, DDBJ and PDB databases were performed using the BLASTN and BLASTX algorithms (9).

qRT-PCR. mRNA was extracted from the PBLs to perform qRT-PCR for the selected genes (Table I) and the corresponding cDNAs were synthesised as described above. PCR assays were carried out using the Bio-Rad CFX96 Real-Time PCR system (Hercules, CA, USA). The specific primers for each gene are shown in Table I. PCR was performed using the SYBR-Green Supermix (Bio-Rad). The amplification reactions were performed in a 10-μl final volume containing 2X SYBR-Green Supermix and 10 μM of each primer. Each sample was analysed in triplicate and multiple water blanks were included. β-actin was amplified as a reference for mRNA quantification. The relative gene expression was presented as arbitrary units of the ratio of $Ct_{gene}/Ct_{\beta-actin}$.

Statistical analysis. Statistical analysis was performed using the SPSS software and Pearson's correlation coefficient. P<0.01 was considered statistically significant. The Kolmogorov-Smirnov analysis of these data showed that the expression values of these genes did not have a normal Gaussian distribution. Thus, in order to use the Pearson's correlation coefficient, we obtained the decimal logarithm of each expression value. A Kolmogorov-Smirnov test of the logarithm values showed a normal distribution.

Table I. Primers and annealing temperatures used for qRT-PCR of selected genes.

Gene	Primer	Sequence (5'→3')	Tm (°C)
ATM	ATM-F	AACGAGCCGATCCACAACC	63.4
	ATM-R	CTCATGTAGTCCACAACAGCAAGC	63.3
eIF4B	eIF4B-F	GGCAAAAAGGATCAAGACTCC	60.1
	eIF4B-R	CCATCAACAGAGAGAGCAGC	58.7
MATR3	MATR3-F	CCTAGTCTCAACCCAGTGC	55.7
	MATR3-R	CATCCCTACACCTTTCTCC	54.5
MORC3	MORC3-F	TCCACCTCAGTCTGAACC	54.1
	MORC3-R	TCAAACTGACTACTCAATCTCC	53.7
PCMTD2	PCMTD2-F	ATGCAAAGCAGAAACTGG	54.8
	PCMTD2-R	CGATCATACTGAGAACAATCC	54.7
PDCD10	PDCD10-F	CGATCATACTGAGAACAATCC	50.2
	PDCD10-R	ATACGAAGAAGGGACTCC	51.4
PSMB1	PSMB1-F	AGACTTGGGGATGGAACC	57.2
	PSMB1-R	TCACTCAATCGAGTATCAGAAGC	58.6
RMND5A	RMND5A-F	AGACCCAAGTCAGAAGGAACC	59.6
	RMND5A-R	GGAGCTGTTTTGAGCTATAAGC	57.5
RUNX2	RUNX2-F	GCCTCTGCTGTTATGAAAAACC	60.1
	RUNX2-R	ATGGTCAAGGTGAAACTCTTGC	60.5
SACM1L	SACM1L-F	GATTTGACCCATACTTTGC	53
	SACM1L-R	TGACCATTCCATACAAACC	54
TMEM66	TMEM66-F	TATCCTCCATTTTCCCACC	57.2
	TMEM66-R	CAAAACCAGAAGTTGCACC	56.7
ZNF644	ZNF644-F	GGACTTGACTATGCACTCAGC	57.6
	ZNF644-R	TGTAAACCTCGTCAGCACC	57.2
β-actin	ACT-F	CTTCCTTCCTGGGCATGG	61.6
	ACT-R	GCCGCCAGACAGCACTGT	63.7

qRT-PCR, real-time quantitative RT-PCR; Tm, annealing temperature; ATM, ataxia-telangiectasia mutated gene; eIF4B, translation initiation factor 4B; MATR3, Matrin 3; MORC3, microrchidia 3; PCMTD2, protein-L-isoaspartate O-methyltransferase; PDCD10, programmed cell death gene 10; PSMB1, proteasome subunit type β; RMND5A, required for meiotic nuclear division 5 homologue A; RUNX2, runt-related transcription factor 2; SACM1L, suppressor of actin mutations 1-like; TMEM66, transmembrane protein 66; ZNF644, zinc finger protein 644; ACT, actin; F, forward; R, reverse.

Results

Construction and screening of the subtracted cDNA library. We constructed a cDNA subtraction library from the PBLs obtained from patients immediately prior to undergoing the first cycle of CT and at the time the 4th cycle was administered (after completing 3 cycles). Screening of 1,200 colonies yielded a total of 65 positive clones of variable expression intensity. The sequencing and identification of the clones following CT treatment revealed that 27 were mitochondrial DNA and 38 (38 clones but 27 different genes) were coded for cytosolic and nuclear proteins (Tables II and III).

We classified the identified genes into seven groups according to the function of the protein they encode: protein synthesis activators (either at the transcriptional or translational level), DNA repair mechanisms, signal transduction pathways (apoptosis-survival), protein repair enzymes, cellular multidynamic systems, integral parts of metabolic pathways and unknown function (Table II).

Genes and proteins analysed in cancer patients undergoing CT. Certain genes were selected for the specific study of variations in their expression levels in two series of patients undergoing CT (Table II). The selected genes were ATM (ataxia-telangiectasia mutated gene), eIF4B (translation initiation factor 4B), MATR3 (Matrin 3), MORC3 (microrchidia 3), PCMTD2 (protein-L-isoaspartate O-methyltransferase), PDCD10 (programmed cell death gene 10), PSMB1 (proteasome subunit type β), RMND5A (required for meiotic nuclear division 5 homologue A), RUNX2 (runt-related transcription factor 2), SACM1L (suppressor of actin mutations 1-like), TMEM66 (transmembrane protein 66) and ZNF644 (zinc finger protein 644). To assess the specificity of carboplatin and paclitaxel on the regulatory response, a group of patients undergoing treatment with doxorubicin (Adriamycin) and cyclophosphamide was analysed concomitantly. Quantitation of the gene expression was carried out using qRT-PCR relative to actin.

Table II. Identified genes coding for cytosolic and nuclear proteins whose expression levels varied following treatment with paclitaxel and carboplatin chemotherapy.[a]

Identified gene	Clones	Diff signal int
Transcription and translation factors		
eIF4B	1	Medium
H2AFZ	1	Low
RUNX2	1	High
ZNF644	1	Medium
DNA repair		
ATM	2	Medium
Signal transduction		
MORC3	7	High
PDCD10	1	High
Protein remodelling		
PCMTD2	1	Low
PSMB1	3	High
Cellular multidynamic systems		
ADD3	1	Low
MATR3	1	Low
RMND5A	1	Medium
SACM1L	1	Low
Metabolic pathways		
COQ3	1	High
CSNK1A1	1	High
FAR1	1	Low
FTL	1	High
GMDS	1	Medium
SORL1	1	Medium
Unknown		
ANKRD10	1	Medium
Chromosome 8 clone RP11-320N21	1	High
Chromosome 1 clone RP11-545E24	2	Low
Chromosome 5 clone RP11-1C1	2	Low
Clone DNA16445 SELT (UNQ150)	1	High
F8A1	1	Medium
TMEM66	1	Low
TMEM209	1	Low

[a]The genes are classified according to the functionality of the proteins they encode. Diff signal int, different signal intensity; eIF4B, translation initiation factor 4B; H2AFZ, H2A histone family member Z; RUNX2, runt-related transcription factor 2; ZNF644, zinc finger protein 644; ATM, ataxia-telangiectasia mutated gene; MORC3, microrchidia 3; PDCD10, programmed cell death gene 10; PCMTD2, protein-L-isoaspartate O-methyltransferase; PSMB1, proteasome subunit type β; ADD3, adducin 3; MATR3, Matrin 3; RMND5A, required for meiotic nuclear division 5 homologue A; SACM1L, suppressor of actin mutations 1-like; COQ3, coenzyme Q3 homologue; CSNK1A1, casein kinase 1, alpha 1; FAR1, fatty acyl CoA reductase 1; FTL, ferritin, light polypeptide; GMDS, GDP-mannose 4,6-dehydratase; SORL1, sortilin-related receptor, L; ANKRD10, ankyrin repeat domain 10; RP11, retinitis pigmentosa; F8A1, coagulation factor VIII-associated 1; TMEM, transmembrane protein.

Table III. Identified leukocyte mitochondrial genes whose expression levels varied following paclitaxel and carboplatin chemotherapy.

Identified gene	Clones	Diff signal int
16S ribosomal RNA gene	3	High
Isolate TV15 mitochondrion	1	Medium
Haplogroup J1b2 mitochondrion	4	Medium
Haplogroup U5b1 mitochondrion	2	High
Haplogroup U5b2 mitochondrion	1	High
Haplogroup K2a mitochondrion	2	Medium
ATP synthase, H$^+$ transporting, mitochondrial F1 complex, O subunit	1	Medium
Haplogroup H6* mitochondrion	2	High
Isolate L332 mitochondrion	1	High
Haplogroup H2a1 mitochondrion	1	High
Isolate MTDNA381 mitochondrion	1	High
Isolate ST024 mitochondrion	1	Medium
Haplotype U4a mitochondrion	1	High
Isolate Koryak 28 mitochondrion	1	High
Isolate MTDNA538 mitochondrion	1	Medium
Haplotype H5 mitochondrion	3	High
Isolate Tub28 mitochondrion	1	Medium

Diff signal int, different signal intensity.

Analysis of variation in the expression of selected genes induced by CT. The relative gene expression prior to and following CT with PC (Table IVA) or AC (Table IVB) was evaluated. In almost all of the genes studied there was a significant variation of expression, with up- and downregulation in patients treated with PC and AC. Only a few cases (one or two per group) of certain genes (Tables IVA and B) did not show significant variation in their expression levels. Column 'N' in Tables IVA and B indicates the number of cases in which the expression of each gene was increased or decreased significantly and the column 'Ratio' shows the mean of the increased or decreased ratio between the relative gene expression prior to and following CT (Pre-/Post-CT) for each patient. Genes with the greatest increases in expression induced by PC were RMND5A (7-fold), PDCD10 (3.9-fold) and MATR3 (4.5-fold). The increases in gene expression induced by AC therapy ranged between 1.4- and 2.1-fold. Decrease of the expression of the studied genes was shown in fewer cases at levels that range about a half of the previous expression to CT.

Results of the analysis of correlation among the genes with up- or downregulated expression are shown in Fig. 1.

Discussion

Areas of progress in the fields of cancer biology and CT currently include strategies for identifying genes that are differentially expressed in different situations, including normal and cancer cells or, as in this study, prior to and following CT. Several methods have been used for these analyses, including SAGE (serial analysis of gene expression) (10), the differential display

Table IV. Mean and typical error of relative gene expression, prior to and following chemotherapy.

A, Relative gene expression prior to and following chemotherapy with paclitaxel and carboplatin

Gene	Expression	N	Pre-CT	Post-CT	Ratio
ATM	Increase	6	866±371	2301±1000	2.8±0.20
	Decrease	2	2479±839	1316±303	0.6±0.05
	No change	0			
eIF4B	Increase	3	11730±3958	20092±6688	1.8±0.18
	Decrease	5	14588±6386	5358±1578	0.6±0.08
	No change	0			
MATR3	Increase	2	1041±188	4358±1108	4.5±1.90
	Decrease	5	8357±2090	3673±1848	0.5±0.10
	No change	1	881	882	
MORC3	Increase	4	166±22	1733±1354	2.1±0.80
	Decrease	2	1502±458	835±330	0.5±0.05
	No change	2	2235±1319	2485±1513	
PCMTD2	Increase	5	656±226	2276±1006	2.7±0.98
	Decrease	1	891	700	0.8
	No change	2	819±519	884±594	
PDCD10	Increase	6	924±313	10828±6560	3.9±0.71
	Decrease	2	7067±2439	4756±3138	0.6±0.25
	No change	0			
PSMB1	Increase	6	788±378	3137±1178	3.3±0.64
	Decrease	0			
	No change	2	3898±2078	4172±2566	
RMND5A	Increase	5	816±294	13569±8201	7±3.07
	Decrease	2	6091±1403	3172±560	0.5±0.05
	No change	1	1756	1495	
RUNX2	Increase	4	399±187	1081±447	2.1±0.24
	Decrease	2	3277±108	1236±413	0.4±0.10
	No change	2	584±479	498±408	
SACM1L	Increase	5	352±157	742±284	1.7±0.19
	Decrease	3	6006±2751	1524±481	0.4±0.11
	No change	0			
TMEM66	Increase	3	2900±1496	16501±5145	3±0.25
	Decrease	3	26174±7780	12398±4596	0.5±0.17
	No change	2	3626±1691	3905±1900	
ZNF644	Increase	4	221±106	1040±377	2.9±0.29
	Decrease	4	2026±1062	1012±467	0.6±0.08
	No change	0			

B, Relative gene expression prior to and following chemotherapy with Adriamycin and cyclophosphamide

Gene	Expression	N	Pre-CT	Post-CT	Ratio
ATM	Increase	4	1589±453	2385±697	1.5±0.11
	Decrease	4	2655±460	1235±139	0.5±0.08
	No change	2	2554±951	2349±853	
eIF4B	Increase	4	7622±1114	16094±4490	2±0.39
	Decrease	5	11250±1730	7462±1817	0.6±0.09
	No change	1	9430	8304	
MATR3	Increase	4	2766±590	4154±1027	1.5±0.07
	Decrease	5	3789±405	2154±347	0.6±0.07
	No change	1	1357	1276	

Table IV. Continued.

Gene	Expression	N	Pre-CT	Post-CT	Ratio
MORC3	Increase	3	842±72	1302±127	1.6±0.12
	Decrease	5	1135±70	786±112	0.7±0.06
	No change	2	1090±106	1066±59	
PCMTD2	Increase	3	791±71	1247±318	1.5±0.27
	Decrease	3	864±380	405±173	0.5±0.09
	No change	4	993±256	976±250	
PDCD10	Increase	7	4348±1448	10148±7747	2±0.46
	Decrease	2	2908±111	1873±64	0.6±0.00
	No change	1	2628	2967	
PSMB1	Increase	6	5489±3056	15822±12499	1.7±0.41
	Decrease	4	2966±481	1877±362	0.6±0.09
	No change	0			
RMND5A	Increase	4	920±150	1741±288	1.9±0.14
	Decrease	4	3032±1351	1518±446	0.6±0.12
	No change	2	807±131	852±98	
RUNX2	Increase	4	632±96	1241±187	2.1±0.33
	Decrease	4	1441±306	755±177	0.5±0.05
	No change	2	444±180	437±155	
SACM1L	Increase	3	953±99	1497±127	1.6±0.25
	Decrease	6	1124±251	631±141	0.6±0.09
	No change	1	584	584	
TMEM66	Increase	2	6941±2765	9935±3096	1.5±0.15
	Decrease	6	11805±1983	6396±775	0.6±0.04
	No change	2	7143±2734	7808±3201	
ZNF644	Increase	2	1072±239	2148±1179	1.9±0.65
	Decrease	6	1016±244	484±62	0.5±0.07
	No change	2	773±99	802±80	

Every measure was the result of triplicate samples. N indicates the number of cases in which the expression of each gene was increased, decreased or not significantly altered following CT. Ratio shows the mean and typical error of the increased or decreased quotient between relative gene expression prior to and following CT (Pre-/Post-CT) for each patient. CT, chemotherapy; Pre-CT, prior to CT; Post-CT, following CT; PC, paclitaxel and carboplatin; AC, Adriamycin and cyclophosphamide; ATM, ataxia-telangiectasia mutated gene; eIF4B, translation initiation factor 4B; MATR3, Matrin 3; MORC3, microchidia 3; PCMTD2, protein-L-isoaspartate O-methyltransferase; PDCD10, programmed cell death gene 10; PSMB1, proteasome subunit type β; RMND5A, required for meiotic nuclear division 5 homologue A; RUNX2, runt-related transcription factor 2; SACM1L, suppressor of actin mutations 1-like; TMEM66, transmembrane protein 66; ZNF644, zinc finger protein 644.

of mRNA by PCR (11) and the generation and screening of cDNA subtraction libraries (12). We selected the last method in order to obtain cDNA clones that could be used to analyse or produce recombinant proteins and subsequent antibodies for lesser known genes and proteins. This study specifically concerns the construction and screening of the library, the description of differential genes and their analysis in two groups of patients undergoing CT. Thus, in addition to patients receiving PC, we extended our study to women suffering from breast cancer who were undergoing doxorubicin (Adriamycin) and cyclophosphamide treatment in the adjuvant setting in order to differentiate genes whose expression varies as a general response to CT from those which are more specifically correlated with PC treatment.

The ATM gene is distinctive as mutations in this gene are associated with the ataxia telangiectasia disorder. ATM is a central transducer of DNA damage signals and ensures the normal repair of DNA ds breaks (13). ATM belongs to the PI3/PI4-kinase family and is a cell cycle checkpoint kinase that functions as a regulator of a wide variety of downstream proteins, including the tumour suppressor proteins p53, BRCA1, CHK2, RAD17 and RAD9 and the DNA repair protein NBS1. The ATM and ATR proteins are closely related and control the cell cycle checkpoint signalling pathways that are required for cellular responses to DNA damage and for genome stability (14). We suggest that leukocytes upregulate the ATM gene during CT in order to repair DNA that has been damaged by drugs.

eIF4B stimulates the helicase activity of the DEAD box protein translation initiation factor 4A (eIF4A) to unwind the inhibitory secondary structure of the 5' untranslated region of eukaryotic mRNAs. Increasing the expression of the eIF4B gene might be a general response to CT in order to accelerate the synthesis of proteins involved in drug resistance or

Gene		ATM	EIF4B	MATR3	MORC3	PCMTD2	PDCD10	PSMB1	RMNS5A	RUNX	SACM1L	TMEM66
EIF4B	PC	ns										
	AC	ns										
MATR3	PC	ns	+									
	AC	+	ns									
MORC3	PC	+	ns	ns								
	AC	ns	ns	ns								
PCMTD2	PC	ns	ns	ns	+							
	AC	+	ns	ns	ns							
PDCD10	PC	ns	ns	ns	+	+						
	AC	ns	ns	ns	ns	-						
PSMB1	PC	+	ns	ns	+	ns	+					
	AC	-	ns	ns	ns	-	+					
RMNS5A	PC	ns	ns	ns	+	+	+	ns				
	AC	ns	ns	+	ns	ns	ns	ns				
RUNX2	PC	+	ns	ns	+	ns	+	+	+			
	AC	ns	ns	ns	ns	ns	ns	ns	+			
SACM1L	PC	+	ns	ns	+	ns	ns	+	+	+		
	AC	+	ns	+	ns	ns	ns	ns	ns	ns		
TMEM66	PC	+	ns	ns	+	ns	+	+	+	+	+	
	AC	ns	+	ns	ns	ns	ns	ns	+	+	ns	
ZNF644	PC	+	ns	ns	+	ns	+	+	+	+	+	+
	AC	ns	+	ns	ns	ns	+	+	ns	ns	ns	+

Figure 1. Correlations between the studied genes. +, positive correlation, P<0.01; -, negative correlation, P<0.01; ns, no significant correlation; PC, paclitaxel and carboplatin; AC, Adriamycin and cyclophosphamide; ATM, ataxia-telangiectasia mutated gene; eIF4B, translation initiation factor 4B; MATR3, Matrin 3; MORC3, microrchidia 3; PCMTD2, protein-L-isoaspartate O-methyltransferase; PDCD10, programmed cell death gene 10; PSMB1, proteasome subunit type β; RMND5A, required for meiotic nuclear division 5 homologue A; RUNX2, runt-related transcription factor 2; SACM1L, suppressor of actin mutations 1-like; TMEM66, transmembrane protein 66; ZNF644, zinc finger protein 644.

metabolic changes involved in cell survival, but the helicase activity of eIF4B suggests that its upregulation is more specifically correlated with carboplatin treatment (15).

MATR3 is a nuclear matrix protein that has been reported to interact with other nuclear proteins to anchor RNAs to the nuclear matrix, modulate the activity of proximal promoters and to be the main PKA substrate following the activation of the NMDA receptor (16). Two transcript variants encoding the same protein have been identified for this gene. Matrin3 has been identified as a Ca^{2+}-dependent CaM-binding protein and a downstream substrate for caspases (17). MATR3 acts as a co-factor of the HIV-1 Rev protein during nuclear export and the translation of unspliced and partially spliced viral mRNA (18).

MORC3, also termed KIAA0136, ZCWCC3 and NXP-2, encodes a member of the MORC protein family, which is characterised by conserved structures consisting of a GHL (Gyrase B, Hsp90 and MutL)-ATPase domain, a zinc finger-type CW domain (four cysteines and two tryptophans), a nuclear localisation signal (NLS) and coiled-coil domains at the carboxy-terminus, as mentioned in (19). MORC3 is ubiquitously expressed in humans, localises to the nuclear matrix, has RNA binding activity and regulates p53 activity and localisation to promyelocytic leukaemia-nuclear bodies (PML-NBs) (19). The upregulation of MATR3 and MORC3 may indicate an attempt to drive an injured cell, through the caspase and p53 pathways, to apoptosis.

PCMTD2 is also termed PIMT (protein-L-isoaspartate O-methyltransferase). The formation of L-isoaspartyl (isoAsp) peptide bonds is a major source of protein damage *in vivo* and *in vitro*. PIMT is a protein repair enzyme that converts

L-isoaspartyl residues in damaged proteins to normal L-aspartyl residues. In rat nucleosomes, the histone H2B was found to accumulate isoAsp in the Asp25-Gly26 bond in the N-terminal tail. In chicken nucleosomes, isoAsp accumulated mainly in histone H2A and, to a lesser extent, in histone H2B (20-22).

PDCD10 is also known as cerebral cavernous malformation-3 and CCM3. This gene encodes an evolutionarily conserved protein which is associated with cell apoptosis and has multiple alternatively spliced variants encoding the same protein (23). The protein interacts with the serine/threonine protein kinase MST4 to modulate the extracellular signal-regulated kinase (ERK) pathway. The protein also interacts with and is phosphorylated by serine/threonine kinase 2 in a signalling pathway, which is critical for vascular development (24). Gene chip data have suggested that PDCD10 is involved in tumour signalling, as it was shown to be upregulated in pancreatic adenocarcinoma (25), metastatic colon cancer cells resistant to cisplatin-induced apoptosis and other cancer cell lines (26). Thus, our data suggest that the interaction pathway PDCD10-MST4-ERK-MAPK, one of the most significant regulators of cell proliferation, is also affected by the two CT therapies investigated in the present study.

The PSMB1 proteasome is a multicatalytic proteinase complex with a highly ordered ring-shaped core structure. This gene encodes a member of the proteasome B-type family, also known as the T1B family, with a 20S core β subunit. The PSMB1 subunit is critical for the degradation of several proteins, more specifically those involved in cell proliferation and cancer (27).

RMND5A, also known as CTLH, RMD5, p44CTLH, FLJ12753, FLJ13910, FLJ21795 and MGC78451, forms

complexes in cells with RanBPM, ARMC8α, ARMC8β, Muskelin and p48EMLP. RMND5A is a key component of the CTLH complex (C-terminal to lissencephaly type-1-like homology motif). This complex is involved in microtubule dynamics, cell migration, nucleokinesis and chromosome segregation (28). Therefore, the variations in the expression of the RMND5A gene may be due to the effects of paclitaxel.

RUNX2 is a member of the RUNX family of transcription factors, which are essential for osteoblast growth and differentiation and chondrocyte maturation. RUNX2 encodes a nuclear protein with a Runt DNA-binding domain that controls the expression of target genes involved in cell fate decisions (29). Runx-2 isoforms are produced by transcript variants caused by alternative promoters and alternative splicing. In the osteosarcoma cell line Saos2, Runx2 has been shown to regulate apoptosis via modulation of the Bcl2 family of proteins (30).

The SACM1L gene encodes a phosphoinositide phosphatase that is an integral membrane protein of the endoplasmic reticulum (ER) and the Golgi apparatus representing a class of phosphoinositide degradation enzymes (31,32). The integral membrane Sac1 phosphatases are a major class of this type of enzyme. Additionally, SAC1 accumulates in the Golgi complex of quiescent cells and suppresses anterograde trafficking by depleting Golgi PI(4)P (33).

For the purposes of our study, the reported case of a mixed lineage leukaemia MLL-rearrangement with the suppressor of actin mutations 1-like (MLL-SACM1L) rearrangement is significant. This rearrangement was observed in bone marrow cells during CT for acute lymphoblastic leukaemia with hyperdiploidy and was continuously detected over seven years without exhibiting clonal expansion (34).

TMEM66, also known as XTP3, FOAP-7, HSPC035, MGC8721 and FLJ22274, is regarded as a potential marker for prostate cancer, included into a group of secreted proteins (35,36) and counted among others in a screening for proapoptotic genes (37). No specific function has yet been attributed to the protein product of this gene.

ZNF644 (38) is a large multizinc finger homologue of the KIAA1221 and WIZ proteins (39). It has been reported that these three proteins co-purified with CDYL, which is associated with histone methylase G9a and mediates a REST tumour suppression function. The specific function of ZNF644 is currently unknown.

All the studied genes in the patients from the PC group showed a positive correlation with each other, with the marked exception of eIF4B and MATR3. Most of these correlations were not found in the AC patient group; furthermore, some of the correlations were negative in this group. Notably, MORC3 and ZNF644 in the PC patients had a positive correlation with the rest of the genes, whereas, in the AC patients, MORC3 did not show a correlation with any gene and ZNF644 was correlated with only a few genes.

A high correlation of the levels of expression of most of the identified genes coding for cytosolic and nuclear proteins were included in the cellular multidynamic system and signal transduction groups (Table II). The balance in the variation in their expression drives the cell to life versus apoptosis as a response to cellular damage induced by CT. This expression pattern corresponds to the pattern for CT-surviving cells, assuming that cells that did not react by modifying the expression of these genes, thereby inducing apoptosis and cell death. This hypothesis is further supported by the different expression patterns observed in the leukocytes obtained from AC-treated patients. The few up- or downregulations the PC- and AC-treated groups have in common are likely to be due to the similar modes of action of carboplatin and Adriamycin and the manner in which cells react to the two drugs.

A notable differential positive correlation between the PC and AC groups is that of PCMTD2 with PDCD10 and the non-significant PCMTD2 with PSMB1 in PC-treated patients. In the case of AC-treated patients, the expression of the PCMTD2 gene showed a negative correlation with the expression of PDCD10 and PSMB1. As such, the expression of an apoptosis regulatory gene was correlated with a protein repair gene and not a proteasome subunit gene in the PC-treated group. However, the same apoptosis regulatory gene in AC-treated patients was negatively correlated with the protein repair gene and the proteasome subunit gene. This further supports the theory that the increased expression of genes that are involved in protein repair and damaged protein degradation following PC treatment is indicative of an attempt to rescue the cells from the effects of PC-induced cytotoxicity.

In the PC-treated patients, the amount of circulating platelets and the expression levels of the ATM, MORC, PSMB1, RUNX TMEM and ZNF genes showed a negative correlation ($P<0.05$). No other correlation between groups of patients with up- or downregulated genes and clinical parameters, including anaemia, fever, secondary neuropathy or mucositis, has been demonstrated for any of the studied parameters.

The results indicate a heterogeneous response of white cells (Tables IVA and B) resulting in different populations that have the up- or downregulation of the expression of certain genes in common. The contradictory responses of cells which contemporaneously increased the expression of genes involved in the survival and apoptotic responses may be due to the critical moment at which cells are committed to evolving and surviving or being eliminated by apoptosis.

This study was performed with a reduced sample of patients, as a pilot study, to identify genes whose expression levels are affected by CT. Now that the changes in gene expression are known, further studies should be performed with a larger cohort of patients and a longer follow-up period to ascertain whether certain genes are associated with specific clinical or analytical manifestations.

The patients treated with PC CT had stage III-IV non-small cell lung cancer or gynaecological cancer. This group of patients have a high incidence of chronic anaemia, reducing the possibility of identifying the effects of the changes in gene expression on the development of bone marrow toxicity.

Certain genes that showed altered patterns of expression in the leukocytes may be involved in the generation of the non-desirable effects of CT in normal blood cells. In the short-term, it should be determined whether there is any association between these changes and immune deficiency, which leads to infectious complications in a reversible or permanent manner. This is critical in establishing prognostic significance in terms of immune surveillance and the recurrence rate.

If these variations in gene expression originate in stem cells, they may be important for CT-associated anaemia or, in the long-term, for myelodysplastic syndromes. If these

CT-induced alterations in gene expression also occur in tumour cells, we should expect them to contribute, in heavily treated patients, to an increased genomic instability and rapid disease progression. More studies are necessary to determine whether these changes in gene expression are correlated with the short- and/or long-term toxicities of anticancer CT. Additionally, whether these changes are random or directed in a specific selection-evolution manner also needs to be elucidated.

Acknowledgements

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