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Research article

Analysis of baseline and cisplatin-inducible gene expression in Fanconi anemia cells using oligonucleotide-based microarrays

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Abstract

Background: Patients with Fanconi anemia (FA) suffer from multiple defects, most notably of the hematological compartment (bone marrow failure), and susceptibility to cancer. Cells from FA patients show increased spontaneous chromosomal damage, which is aggravated by exposure to low concentrations of DNA cross-linking agents such as mitomycin C or cisplatin. Five of the identified FA proteins form a nuclear core complex. However, the molecular function of these proteins remains obscure.

Methods: Oligonucleotide microarrays were used to compare the expression of approximately 12,000 genes from FA cells with matched controls. Expression profiles were studied in lymphoblastoid cell lines derived from three different FA patients, one from the FA-A and two from the FA-C complementation groups. The isogenic control cell lines were obtained by either transfecting the cells with vectors expressing the complementing cDNAs or by using a spontaneous revertant cell line derived from the same patient. In addition, we analyzed expression profiles from two cell line couples at several time points after a 1-hour pulse treatment with a discriminating dose of cisplatin.

Results: Analysis of the expression profiles showed differences in expression of a number of genes, many of which have unknown function or are difficult to relate to the FA defect. However, from a selected number of proteins involved in cell cycle regulation, DNA repair and chromatin structure, Western blot analysis showed that p21$^{waf1/Cip1}$ was significantly upregulated after low dose cisplatin treatment in FA cells specifically (as well as being expressed at elevated levels in untreated FA cells).

Conclusions: The observed increase in expression of p21$^{waf1/Cip1}$ after treatment of FA cells with crosslinkers suggests that the sustained elevated levels of p21$^{waf1/Cip1}$ in untreated FA cells detected by Western blot analysis likely reflect increased spontaneous damage in these cells.

Background

The autosomal recessive disorder Fanconi anemia (FA) is clinically characterized by bone marrow failure, predisposition to cancer and various developmental abnormalities [1]. FA is genetically heterogeneous, and thus far eight complementation groups have been described. Six of the identified FA genes (*FANCA*, *-C*, *-D2*, *-E*, *-F*, and *-G*; [2–8]) encode proteins that are unique and lack apparent homology to other proteins or to each other. In addition, there are no conserved motifs present in these FA proteins, hampering understanding of their molecular function. Recently, biallelic mutations were found in the *BRCA2* gene in patients belonging to complementation groups FA-D1 and one FA-B patient [9]. At the cellular level, FA is characterized by increased spontaneous genomic instability and hypersensitivity to DNA crosslinking agents, e.g. cisplatin and mitomycin C (MMC) [reviewed in [1]]. Multiple studies have also shown defects in FA cells related to the interferon-signaling pathway. Compared to control cells, FA cells express constitutively high levels of ISGF3 gamma, IRF-1, p21$^{waf1/Cip1}$ and MxA [10,11].

Studies on the FA proteins have shown that five FA proteins, FANCA, FANCC, FANCE, FANCF, and FANCG, bind to each other to form a nuclear core complex [Reviewed in [12] and [13]]. FANCD2 is a nuclear protein that requires activation by mono-ubiquitination. In mutant FA cells that lack one of the FA core complex proteins, FANCD2 is not activated by ubiquitination, suggesting that these FA proteins exert a common molecular function in the nuclear compartment of the cell [14]. Whether BRCA2 is involved in the same pathway or exerts a separate function is currently unknown [15].

Crosslinking agents are widely used for the treatment of various types of cancer and are thought to exert their cytotoxic effect predominantly through irreversible binding with DNA. How the FA proteins are related to this cytotoxic effect, e.g. by functioning in processes such as DNA repair, cell cycle control or protection, is unknown. Upon treatment with a discriminating dose of crosslinking agent that will only transiently arrest the growth of wild type cells, FA cells arrest in the late S or early G2-phase of the cell cycle, and ultimately undergo cell death [16–21]. In contrast to normal cells, FA cells fail to inhibit replicative DNA synthesis after treatment with crosslinking agents. While normal cells will arrest in S-phase, FA cells continue replication and subsequently arrest at a later cell cycle check point [22,23]. This hypersensitivity to crosslinking agents is the hallmark of the FA phenotype.

Many studies have indicated that *in vitro* treatment of cells with cisplatin affects the expression of specific genes involved in various molecular processes such as transcription, DNA repair, apoptosis, and cell cycle regulation. Examples are induction of c-jun, c-fos, ercc1, gadd45, gadd153, and p21$^{waf1/Cip1}$ [24–28]. Some of these genes respond within hours after treatment of cells, e.g. c-jun, whereas others show maximum changes in expression after 24 to 72 hours, e.g. p21$^{waf1/Cip1}$, gadd45 and gadd153.

Recently developed techniques, cDNA microarrays and oligonucleotide expression probe arrays, enable the systematic analysis of expression of thousands of genes in a single experiment [reviewed in [29]]. These techniques are useful to evaluate possible differences in gene expression profiles between FA and control cells, either at baseline or after crosslinker treatment. To study this, there are a number of experimental approaches to consider. For example, one might compare FA cells with cells from healthy controls. This might necessitate studying a large number of both types of cells in order to reduce false positives due to genetic differences in the cell lines that are unrelated to FA. Another approach would be to study cell lines derived from FA patients that are compared with the same cell lines corrected by transfection with the complementing cDNA. The use of such isogenic cell lines would presumably reduce the number of false positives and therefore reduce the number of cell lines that need to be studied. However, this might also result in a non-physiologic expression of the FA protein in transfected cells, which might influence the expression profiles. Therefore, we used the latter approach in combination with a unique cell line couple, one FA-like cell line and one wild-type, which are both derived from the same (mosaic) patient [30]. This cell line couple has the advantage of being both isogenic and expressing physiologic levels of the FA gene.

The aim of this study was to compare the expression profiles of FA cells defective in one of the FA core complex proteins with isogenic control cells at baseline, as well as in response to treatment with discriminating concentrations of crosslinking agents. Differences in expression profiles of known genes might shed light on the cellular function of the FA proteins.

Methods

Cell lines and cell culture

Lymphoblastoid cell lines that were used in this study are derived from three individuals, one FA-A (HSC72) and two FA-C (HSC536 and VU450) patients. The isogenic control cell lines included an *in vivo* revertant cell line derived from a mosaic patient, VU450R and the non-reverted cell line VU450, or were derived by transfecting the cells with either empty vector or with vectors expressing the complementing cDNAs, HSC72 with pDR2 or pDR2-FANCA-flag [31] and HSC536 with pDR2 or pDR2-flag-FANCC [17]. The VU450R cell line is an ideal isogenic control, since reversion by recombination resulted in endogenous wild-type FANCC expression [30]. Cells were

cultured at 37°C in 5% CO_2 in RPMI1640 medium (Life Technologies, Gaithersburg, MD) containing 10% newborn calf serum (Hyclone Laboratories, Lorgan, UT) and supplemented with 200 μg/ml hygromycin for transfected cells.

Cisplatin treatment

Optimal discriminating doses of cisplatin (Pharmachemie b.v., NL) for each cell couple were determined using growth inhibition tests [32,33]. Cells were pulse treated for 1 hour with different concentrations (0, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 μM) of cisplatin. Cells were subsequently washed and allowed to grow further until three cell divisions were reached in the untreated samples. For further analysis, concentration of cisplatin was such that the highest differential effect between the FA and control cells was obtained, i.e. growth inhibition of >50% in FA cells and <10% in control cells: for the VU450 cell couple, 2.5 μM; HSC536 cell couple, 1 μM; and HSC72 cell couple, 5 μM. Approximately 10^8 cells were pulse treated with cisplatin for one hour, washed and cultured further in separate portions of 2.5×10^7 cells in fresh medium.

RNA isolation

Cells were harvested at various time points after treatment. Total RNA was extracted using TRIzol LS reagent (Life Technologies, Gaithersburg, MD) followed by a second round of RNA purification using RNAeasy columns (QIAGEN, Valencia, CA). Gene expression profiles were determined from the three cell couples without treatment, from the HSC72 cell couple 1, 3, 7 and 24 hours after cisplatin treatment, and from the VU450 cell couple 1 and 7 hours after treatment.

Genechip expression analysis

Gene expression profiles were determined using the Human Genome U95A probe arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. These genechips are oligonucleotide-based and represent ~12,000 human sequences that were previously characterized in terms of function or disease association. Double stranded cDNA was synthesized from each total RNA sample (10 μg) using T7-(dT)24 primer (GENSET Corp) and SuperScript Choice system (Life Technologies, Rockville, MD). From these cDNA samples, target samples were prepared using *in vitro* cRNA transcription with biotinylated nucleoside triphosphates and BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY). The cRNA samples were fragmented and hybridized to U95A probe arrays. Scanning of the arrays was performed with the HP Gene Array Scanner and data quantified using Genechip Analysis Suite 3.3 (Affymetrix, Santa Clara, CA).

Data analysis

For analysis of the data, the data from all untreated cell couples were combined and used as baseline for normalization. All data sets were subsequently calculated relative to the normalized data. Expression levels of beta-actin and GAPDH (relative intensity) ranged between 0.8 – 1.2 in all data sets. Genechip Analysis Suite 3.3 (Affymetrix, Santa Clara, CA) and GeneSpring 3.2.2 software (Silicon Genetics, Redwood, CA) was used for analysis of the data. A gene was considered as being up- or down-regulated only if the average difference (AD) was > 10 (with target intensity for scaling = 100) and if gene was called present. Genes considered of potential interest were: genes >2-fold up- or down-regulated in all pairs for non-treated samples when comparing FA *v* control, and >3-fold up- or down-regulated at any time point after cisplatin treatment in either cell couple. A further selection from the latter set of genes was made by scrutinizing for consistency in changes of expression among cell line couples and in response to cisplatin treatment (as indicated in table 2).

Immunoblotting

Protein extracts were prepared from cells as described previously [34]. Protein concentration in the extracts was determined using a Bio-Rad Protein Assay (Hercules, CA), and 10 μg protein was loaded on SDS-polyacrylamide gels. Gels were transferred to Immobilon-P membrane (MILLIPORE, Bedford, MA) and blocked in TBST with either 5% dry milk or 5% BSA for 1 hour at room temperature. Incubation with primary antibody was according to the manufacturer's protocols. The following primary antibodies were used for immunoblotting: mouse anti-p300 (NM-11) and rabbit anti-p21$^{waf1/Cip1}$ (Zymed Laboratories, San Francisco, CA), mouse anti-CDC25a (abcam, UK), mouse anti-PMS2 (E-19), rabbit anti-RGS2, rabbit anti-BAF170 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-p16 (G175-405; PharMingen), and mouse anti-β-tubulin (Boehringer Mannheim).

Results

Expression analysis of untreated cells

Expression data from three different cell line couples, FA cell lines (HSC72, HS536, and VU450) and their isogenic controls (HSC72 + FANCA, HSC536 + FANCC, and VU450 revertant), were analyzed. In addition, for two cell line couples (HSC72 and VU450), expression profiles were determined after treatment with discriminating concentrations of cisplatin. In general, approximately half of the genes present on the probe arrays were identified as being expressed in the lymphoblastoid cell lines. Some low abundant mRNAs were not detected, as evidenced by the fact that of the four FA genes present on the arrays (FANCA, FANCC, FANCD2 and FANCG), only one (FANCG) was detected, in both FA and control cell lines.

Table 1: Differentially expressed genes in untreated FA cells.

Description	GenBank accession no.	Fold change		
Up regulated in FA cells		*VU450*	*HSC536*	*HSC72*
- GLUT1 C-terminal binding protein, GIPC	AF089816	5	2	17
- homeo box B7, HOXB7	M16937	5	61	3
- plasma glutamate carboxypeptidase, PGCP	W29330	5	3	2
- proteinase inhibitor, SERPINB7	U71364	7	2	6
- E1A binding protein p300, EP300	U01877	2	2	2
Down regulated in FA cells				
- selectin L, SELL	M25280	2	16	5
- small inducible cytokine A4, SCYA4	J04130	2	3	41
- TXK tyrosine kinase, TXK	L27071	10	3	3
- solute carrier family 16, SCL16A5	U59299	2	2	2
- cyclin-dependent kinase inhibitor 2A, p16	U26727	2	203	5

As shown in Table 1, comparison of the expression profiles from the three FA cell lines and their isogenic controls showed that 10 genes were identified as being more than 2-fold differentially expressed in all cell couples. Although we can not exclude that some of these genes may have a physiological effect, many of the genes in Table 1 are difficult to relate to the molecular defect in FA cells, i.e. genomic instability and cross linker sensitivity. There is still a reasonable chance that the majority of these genes were obtained by chance since only three cell line couples were used. Therefore, we further analyzed those most likely to be related to the FA phenotype, i.e. p300 and p16 that are involved in cell cycle regulation. However, comparison of protein expression by Western blotting showed no consistent differences in either p16 or p300 protein levels (Figure 1).

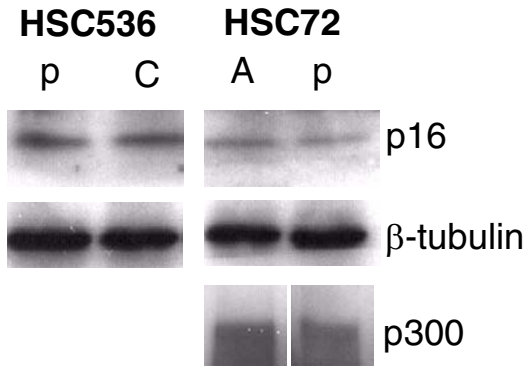

Figure 1
p16 and p300 protein expression in FA and control cell lysates. 10 μg protein from indicated cell extracts were immunoblotted with p16, β-tubulin, and p300 specific antibodies, as indicated. Extracts were from HSC536 stably transfected with empty vector (p; lanes 1) or with vector expressing *FANCC* (C; lanes 2), and from HSC72 stably transfected with vector expressing *FANCA* (A; lanes 3) or with empty vector (p; lanes 4). β-tubulin was used as additional loading control.

Expression analysis of cisplatin-treated cells

To test the possibility that the hypersensitivity of FA cells to crosslinking agents is reflected by a differential response at the level of gene expression, we first determined the concentration of cisplatin that best discriminated between the FA and control cell line couples. These concentrations were 2.5 μM and 5 μM cisplatin for the VU450 and HSC72 cell couples, respectively (Figure 2 and data not shown).

Using specific criteria for differences in response (see methods section), analysis of the expression profiles from untreated versus 1 and 7 hours after cisplatin treatment for the VU450 cell couple and from untreated versus 1, 3, 7, and 24 hours after treatment for the HSC72 cell couple, resulted in 59 genes that were initially identified (Table 2). The selection criteria were mild, in order to avoid loss of interesting data, thus increasing the risk of identifying genes by chance. This is reflected by the facts that many genes do not show a clear pattern of response to cisplatin treatment and that all genes are in the low intensity range. However, some of the genes in Table 2 are of possible interest in the context of FA and crosslinker treatment, particularly those involved in cell cycle regulation (cdc25A and RGS2), DNA repair (PMS2), and chromatin structure (BAF170). Unfortunately, protein expression analysis us-

Table 2: Differentially expressed genes between FA and control cells after cisplatin treatment.

Gene Description	GenBank accession no.	Change*
Membrane protein		
C-type lectin, AICL	X96719	Up 1 h in WT
Integral membrane protein, LIG-1	W25875	Up 1 h in FA
Integral membrane protein, SIGMAR1	U79528	NC
Growth factor		
Insulin growth factor binding protein 2, IGFBP-2	S37730	NC
Endothelial cell growth factor, platelet-derived, ECGF1	M63193	Down 24 h in WT
IL18R1/IL-1RRP	U43672	NC
BMP-4	U43842	Down 1 h in FA
IL10R	U00672	NC
Notch ligand, JAGL1	AF003837	NC
Signal transduction		
Adapter molecule in signal transduction, DOC1	U53445	Up 24 h in FA
SRC family tyrosine kinase, FYN	M14333	Down 24 h in FA
Protein tyrosine phosphatase, PTPG	U46116	Down >7 h in FA
Regulator of G-protein signaling, G0S8/RGS	L13463	Up 1 h in FA
Regulator of G-protein signaling, GIPC/RGS19IP1	AF089816	Up 24 h in WT
Mitogen-activated protein kinase kinase kinase kinase 4, MAP4K4	AB014587	NC
Serine/threonine kinase, KIAA0623	AB014523	NC
Related to the N-terminus of TRE, RNTRE	D13644	NC
Related to intracellular calcium signaling, HOMER-1B	Y17829	NC
MEK6/MKK6	U39657	NC
Zinc finger protein, LOC58500	X16282	NC
LYT10/NF-kB2	U20816	NC
Dual specific tyrosine kinase, DYRK2	Y13493	NC
Phospholipase C beta 2, PLCB2	M95678	NC
Containing Pleckstrin homology domain, KIAA0763	AB18306	NC
Vesicular Rab-GAP/TBC-containing protein, AD3	AB024057	NC
Calcium-binding protein, S100C/Calgizzarin	D38583	NC
Transcription factor		
Transcript homolog, MEST/PEG1	D78611	NC
Myotubularin related protein 2, MTMR2	AB028996	NC
Homeobox protein related to skeletal development, MSX1	M97676	NC
Transcription factor, MRG1	U65093	NC
Homeobox, PRH/PRHX	L16499	NC
MSX2/MSH/HOX8	D89377	NC
Transcriptin activating factor, CREBP	L05515	NC
Transcription coactivator, TCFEC/TFECL	D43945	NC
Putative transcription regulator, CARM-1	AI660225	Down 7 h in FA
Cell cycle		
CDC25A	M81933	NC
Apoptosis		
Similar to rat CIPHAR-1, DKFZp564O0823	AL080121	NC
BCLX	Z23115	NC
Protease inhibitor, PLANH2/PAI2	Y00630	NC
Vanin-2, VNN2	D89974	Down 24 h in WT
DNA repair/Chromosome regulator		
Similar to DNA helicase, FLJ10738	W28620	NC
HPARG	AF005043	NC
BAF170	U66616	NC
PMS2	U13696	NC
Intracellular processing		
Ubiquitin protein ligase, E6-AP	AF002224	Up >1 h in WT
Putative splicing factor, DOM3Z	AF059252	Down >7 h in FA
CBP2/TAFI	M75106	NC
Uronyl 2-sufotransferase	AB020316	NC
Miscellaneous		
Procollagen-proline, 2-oxoglutarate 4-dioxygenase, P4HA2	U90441	NC

Table 2: Differentially expressed genes between FA and control cells after cisplatin treatment. ***(Continued)***

Unknown, FLJ21174	AA149307	NC
Unknown, expressed in macrophage	X89059	Up >1 h FA
Clathrin light chain b	X81637	NC
Related to lipodystrophy, LPIN2	D87436	NC
ME1	U43944	NC
Unknown, KIAA0090	D42044	Down 24 h in WT
Unknown, DKFZp564I122	AL080062	NC
Unknown, KIAA0241	D87682	NC

* NC indicates that the observed changes were not consistent. When consistent changes were observed indicated are up or down regulation, followed by time point(s) after treatment at which changes of expression were observed, and cell type; WT indicates observed in corrected cells, FA indicates observed in FA cells.

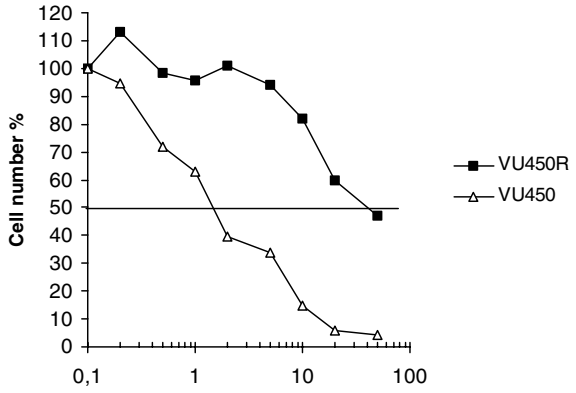

Figure 2
Cisplatin induced growth inhibition. VU450 cell lines were one hour pulse treated with indicated concentrations of cisplatin. Dotted line indicates the dosage used for gene expression experiments. VU450R; reverted (crosslinker resistant) cell line and VU450; FA (crosslinker sensitive) cell line.

ing Western blotting did not confirm any consistent changes in protein expression after cisplatin treatment (Figure 3A).

Of the genes with relatively high signal intensities, the cell cycle inhibitor p21$^{waf1/Cip1}$ was increased in FA cells (HSC72) 24 hours after treatment but not in control cells (HSC72 + FANCA). However, this increase was < 2-fold and therefore excluded in the initial analysis. Western blot analysis of p21$^{waf1/Cip1}$ protein expression showed a strong increase in FA cells at 24 hours after cisplatin treatment, while p21$^{waf1/Cip1}$ was only slightly increased in the corrected cells (Figure 3B). In addition, p21$^{waf1/Cip1}$ appeared to be somewhat higher expressed in FA cells even without treatment, which was not observed in the expression array analysis.

Discussion

The FA proteins have a caretaker function and a defective FA pathway results in chromosomal instability, a phenotype that is aggravated by exposure of cells to cross-linking agents [1]. Here we have tried to identify differences in gene expression between FA and control cells that might shed light on the molecular role of the FA proteins. Although recent findings of BRCA2 mutations in a subset of FA patients and studies on the FANCD2 protein suggests a link between the FA pathway, BRCA1, and DNA repair [9,14], the exact function of the FA core complex remains elusive.

We examined samples using oligonucleotide arrays harboring probes directed to approximately 12,000 different transcripts. Various types of crosslinking agents are used in FA research, such as mitomycin C, diepoxybutane, psoralen with UV, and cisplatin [1]. In the present study, we used cisplatin because the effect of this agent on gene expression has been well described and there is no need for metabolic activation of the drug (unlike mitomycin C). Relatively late effects, such as cell cycle arrest and apoptosis, have been documented for FA [16–23]. Our main interest, however, was to determine the effect on early response genes that preceded cell cycle arrest. Therefore, cells were pulse treated for one hour with cisplatin and samples taken shortly thereafter. The concentration of cisplatin used was chosen to achieve the highest discrimination between FA and control cells in growth inhibition assays. Although this cytotoxic dosage is relatively low compared to those used in prior studies of cisplatin-induced gene expression changes, we reasoned that a higher dosage would lead to indiscriminate growth inhibition in both FA and control cells [17,18,20]. In addition, these concentrations were found to be sufficient for inactivating tyrosine phosphorylation of CDC2, 24 hours post treatment, as assessed by Western blotting (data not shown).

The profiling analysis revealed differences in expression of a number of genes, many of which are difficult to relate to

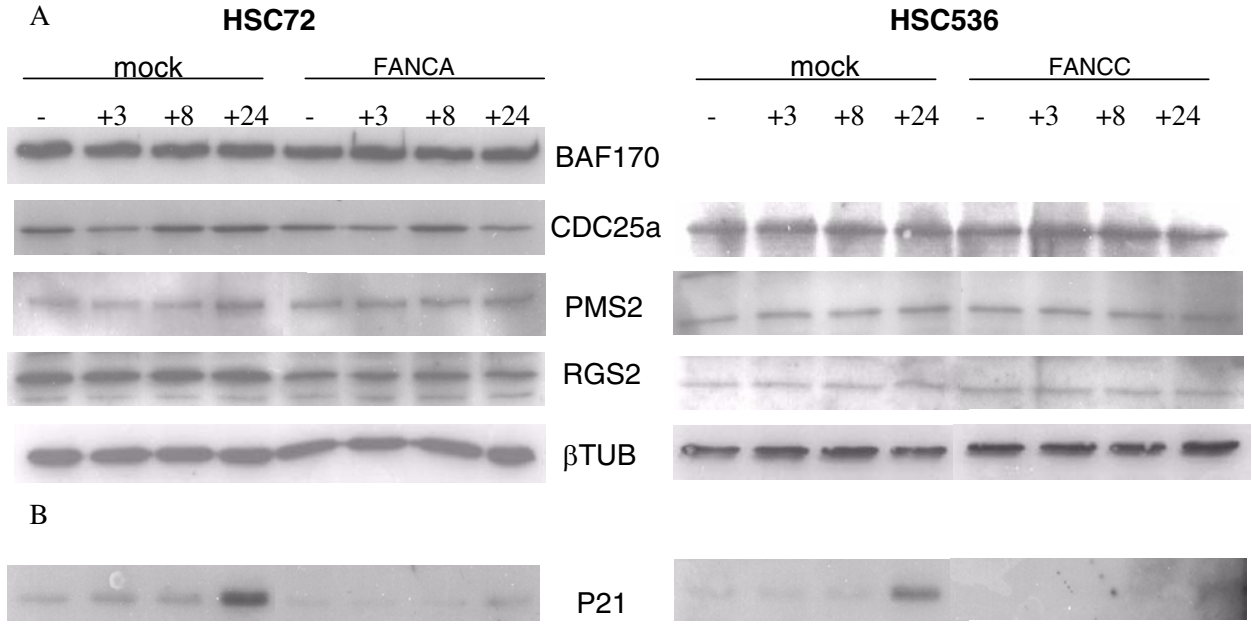

Figure 3
Protein expression in FA and control cells treated with cisplatin. 10 μg protein from indicated cell extracts were immunoblotted with specific antibodies directed against indicated proteins; A) BAF170, CDC25a, PMS2, RGS2 and β-tubulin; B) p21$^{waf1/Cip1}$. Extracts were from HSC72 and HSC536 cells transfected with empty vector or with vector expressing correcting cDNA as indicated. Cells were one hour pulse treated with discriminating concentrations of cisplatin; HSC72 with 5 μM and HSC536 with 1 μM. β-tubulin was used as additional loading control.

the FA pathway or are unlikely to be expressed in B-lymphocytes. Only a few genes are related to processes that are possibly linked to the FA defect, such as those involved in cell cycle regulation, chromatin structure, and DNA mismatch repair. However, using Western blot analysis with antibodies directed to the respective gene products, we were unable to confirm differences in protein levels for all these genes. Genes that previously have been shown to respond to cisplatin and that were present on the arrays, i.e. c-jun, c-fos, ercc1, gadd153, gadd45, pcna and p21$^{waf1/Cip1}$, were not changed under the conditions used, except for an increase in expression of p21$^{waf1/Cip1}$. Although the change in our experiments was < 2-fold, there was a clear induction of p21$^{waf1/Cip1}$ expression in FA cells 24 hours post treatment that was not seen in the control cells. Microarray based experiments to discover cisplatin induced differences in gene expression have been performed previously both using cell lines [35] and tissues from rats [36]. These experiments are however difficult to relate to our experiment due to much longer exposure time to similar concentrations of cisplatin used [35] or due to the differences in experimental approach [36]. Although in those experiments many genes were found to be differentially expressed, also p21$^{waf1/Cip1}$ was found to be induced by cisplatin treatment. The reason for not detecting other cisplatin-induced genes in our experiments might be the relatively low concentrations of cisplatin used. This suggests that p21$^{waf1/Cip1}$ (compared to other genes) regulation is very sensitive to cisplatin, at least in lymphoblastoid FA cells. p21$^{waf1/Cip1}$ is known to be a key regulator of G1 cell cycle arrest, but has more recently also been implicated in G2 cell cycle regulation [37]. This observation fits well with the previously observed upregulation of p53 and cyclin B proteins, inactivation of CDC2, and cell cycle arrest that follows exposure to low doses of cross-linking agents [17–19].

Earlier studies reported that FA lymphoblasts express increased levels of transcripts from genes connected to the interferon pathway, IRF-1 and MxA [10,11]. Although the approaches used were different, these observations are not confirmed by our data. Both genes were detected as being expressed but showed no significant differences between FA and controls. In addition, the constitutive elevated expression of p21$^{waf1/Cip1}$ observed in FA cells [9] was not found at the level of mRNA in our experiments. However, we did observe a slight increased expression of p21$^{waf1/Cip1}$ protein in FA cells. This might be explained by post-

transcriptional regulation of p21*waf1/Cip1* or by a relatively low sensitivity of the expression arrays, incapable of detecting minor increases in mRNA. Interestingly, the observed elevated levels of p21*waf1/Cip1* expression in FA cells is reminiscent of a number of other DNA repair defective cell types, i.e. those defective in ATM, ERCC1, BRCA1, and BRCA2 [38–42].

Except for p21*waf1/Cip1*, these experiments did not reveal differences in gene expression that could explain the basic defect in FA, when looking at baseline or when looking for genes that preceded cisplatin-induced cell cycle changes. There are several possible explanations possible for this. First, no significant changes at the transcriptional level are present in FA cells. It might well be that phenotypic differences of FA cells are predominantly reflected at the post-translational level. The recent finding of FANCD2 mono-ubiquitination suggests that regulation by protein modification is an important step in the FA pathway [14]. Second, subtle changes in gene expression might remain undetected. The up- or down-regulation of an entire functional pathway may have large effects but be difficult to trace using these types of experiments, particularly when our knowledge of components involved in the various functional pathways is limited. Third, expression of certain genes may be different but not detected either because of lack of sensitivity or absence of the oligonucleotide sequence for those specific genes in the arrays used. Concerning sensitivity, it might be that the response of cells to cross-linker damage is related to signals at a certain stage of the cell cycle (e.g. during replication), resulting in a dilution of the response when using an asynchronous population of cells. The p21*waf1/Cip1* results showed that differences in expression of transcripts with high intensity on the arrays were detected even though this was a single data point and the difference was < 2-fold. Therefore, differences < 2-fold in genes with low intensity signals are presumably present but missed because of the increasing levels of noise in that region of intensity, making it difficult to discriminate between true and false positives in a limited number of samples.

Conclusions

Analysis of the expression data resulted in the identification of 10 genes (Table 1) that were found to be consistently differentially expressed between FA and control cells, as well as 59 genes (Table 2) that exhibited different response patterns between FA and control cells after treatment with cisplatin. However, subsequent analysis of selected genes at the protein level by Western blot analysis did not confirm the observed differences in expression. Several limitations of the experimental approach were discussed that might explain these results.

The observed strong cisplatin-induction of p21*waf1/Cip1* in FA cells, but not in control cells, suggests that FA cells respond similarly as non-FA cells but in a hypersensitive manner. According to experiments from other investigators [20] cell cycle checkpoints function properly, suggesting that cross-linking agents evoke more damage or create damage that remains unrepaired in cells with a defective FA pathway. Therefore, the observed increase in basal p21*waf1/Cip1* levels in FA cells by Western blot analysis most likely reflects a physiologic response to the presence of spontaneous damage in a subpopulation of these cells.

Competing interests

None declared.

Authors' contributions

QW and JdW performed all experiments, isolated, purified RNA from the samples for microarray, performed initial analysis and Western blotting. AM analysed the microarray data in further detail. HJ and JL participated in its design and co-ordination.

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