

Gene Expression Profiling of Xeroderma Pigmentosum

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Abstract

Xeroderma pigmentosum (XP) is a rare recessive disorder that is characterized by extreme sensitivity to UV light. UV light exposure results in the formation of DNA damage such as cyclobutane dimers and (6-4) photoproducts. Nucleotide excision repair (NER) orchestrates the removal of cyclobutane dimers and (6-4) photoproducts as well as some forms of bulky chemical DNA adducts. The disease XP is comprised of 7 complementation groups (XP-A to XP-G), which represent functional deficiencies in seven different genes, all of which are believed to be involved in NER. The main clinical feature of XP is various forms of skin cancers; however, neurological degeneration is present in XPA, XPB, XPD and XPG complementation groups. The relationship between NER and other types of DNA repair processes is now becoming evident but the exact relationships between the different complementation groups remains to be precisely determined.

Using gene expression analysis we have identified similarities and differences after UV light exposure between the complementation groups XP-A, XP-C, XP-D, XP-E, XP-F, XP-G and an unaffected control. The results reveal that there is a graded change in gene expression patterns between the mildest, most similar to the control response (XP-E) and the severest form (XP-A) of the disease, with the exception of XP-D. Distinct differences between the complementation groups with neurological symptoms (XP-A, XP-D and XP-G) and without (XP-C, XP-E and XP-F) were also identified. Therefore, this analysis has revealed distinct gene expression profiles for the XP complementation groups and the first step towards understanding the neurological symptoms of XP.

Introduction

The structural integrity of chromosomal DNA is of paramount importance for the survival of a cell, an organism and indeed an entire species [1]. The faithful passage of this nucleotide blueprint requires its stable transition between successive cellular generations [2]. Both replicating and non-replicating DNA are

vulnerable to various forms of errors and lesions that constitute or lead to gene mutations [3]. To counteract the effects of mutagenic and carcinogenic agents all organisms are equipped with a sophisticated network of DNA repair systems that are essential for genetic stability [4]. The identification of several cancer syndromes in humans where the causative factor is a genetic mutation in a component of one or more of

the DNA repair systems has highlighted the importance of maintaining DNA integrity.

Exogenous DNA mutagenesis is a consequence of external agents impacting on the integrity of cellular DNA. These agents can be divided into three classes: ultraviolet (UV) irradiation, ionising irradiation and alkylating agents. DNA repair is an important molecular defence system in which mutations caused by these agents in actively transcribed genes are preferentially repaired. All damaged DNA is repaired before DNA replication to prevent a mutation being transmitted to daughter cells [5]. DNA repair can occur by one of three cellular responses that involve direct reversal, excision or tolerance of the DNA damage.

UV irradiation primarily results in DNA damage in the form of 6,4 photoproducts and cyclobutane pyrimidine dimers which require excision from the surrounding DNA. Nucleotide excision repair (NER) involves an enzyme system that hydrolyses two phosphodiester bonds on either side of a lesion, creating an oligonucleotide surrounding the damage. NER has two distinct subpathways, global genomic repair (GGR) and transcription-coupled repair (TCR). GGR can operate at any location in the genome, and its efficiency varies depending on the type of lesion. In contrast, TCR specifically removes lesions that block RNA polymerases on the transcribed strands of active genes [6, 7] and eliminates different lesions at similar rates [8].

Since 1991 extensive research has been conducted on NER in humans. A wealth of information has become available since the cloning of the human repair genes, XPA to XPG and ERCC1 [9]. The discovery of defective NER in individuals with the rare heritable diseases xeroderma pigmentosum (XP), trichothiodystrophy (TTD) and Cockayne's syndrome (CS) and the subsequent isolation of UV-sensitive mutants of rodent cell lines led to the cloning of the human repair genes. Before XP was identified there was relatively little known about NER and DNA repair in general. As information regarding XP is accumulated, more is being discovered about the NER proteins and their involvement in DNA repair and other processes. The role of UV-induced DNA damage in skin cancer and the importance of adequate repair systems to remove the damage are clearly illustrated in patients with XP [10, 11].

XP is a recessively inherited genetic disorder, which occurs at a frequency of 1:250,000 in the United States, but has a higher frequency in Japan and Mediterranean areas [12]. XP patients exhibit a 1000

times greater susceptibility to uniformly distributed melanomas, basal cell carcinomas and squamous cell carcinomas in sunlight-exposed areas of skin compared to unaffected individuals [10, 11, 13]. XP cells cannot efficiently carry out NER, and as a result there is an accumulation of errors as the damaged DNA tries to replicate itself [14].

XP includes seven genetic subgroups known as complementation groups (XP-A to XP-G) that represent different genes in the NER pathway (Table 1). The functions of most of the XP and CS proteins associated with NER have been identified and mutations located in each of the genes have begun to be correlated with cellular functions and severity of the disease [15]. Currently, individuals are diagnosed with XP based on clinical criteria and identification of mutations in one of the NER genes. TTD and CS are different to XP as they do not have the higher rate of skin cancers and are characterized by growth and mental retardation. Mutation detection in these individuals is time-consuming and costly as many require multiple genes to be screened before a mutation is identified. In addition to the high skin cancer incidence in XP, progressive neurological degeneration in the form of primary neuronal degeneration and mental retardation occurs in a significant number of patients, which is a result of mutations in specific XP genes.

Recent advances in biotechnology have seen the introduction of technologies that allow large numbers of genes and proteins to be analysed. This technology is a useful tool when studying complex disorders such as XP, which are believed to involve changes in expression of many genes or proteins. One such example is microarrays that can detect expression levels of up to 30,000 genes at the same time. A number of studies have shown that gene expression profiling can identify sets of genes that allow identification of disease subtypes. For example, two distinct forms of diffuse large B-cell lymphoma were identified using gene profiling of 96 malignant and normal samples [16]. Similarly, Sorlie and colleagues [17] found that in a subset of patients, classification of breast cancer tumours by gene expression profiles could be used as a prognostic marker with respect to overall and relapse-free survival. Oestrogen receptor (ER) positive tumours could also be divided by gene expression profiles into two distinct subgroups with differing prognosis. The identification of distinct profiles for each XP complementation group would expedite the diagnostic process for new cases of XP and further our understanding of some of the biological processes associated with NER deficiency.

Table 1. Genes with brain-related function or expression that were significantly altered in association with neurological symptoms in XP

Acc. No.	Name	Function	P value
AA464744	KIAA1111	brain cDNA clone	0.00004
R79082	protein tyrosine phosphatase	cellular signalling pathways	0.0004
N35888	phosphomannomutase 2 (PMM2)	brain specific glycosylation factor	0.0007
R93912	glycogen synthase kinase 3 beta (GSK3 beta)	signal cascade of neuronal apoptosis	0.0008
N70841	GABA-B Receptor 1a	neurotransmitter receptor	0.0008
T64482	esterase D/formylglutathione hydrolase	expressed predominantly in brain	0.0008
AA463251	nucleosome assembly protein (NAP)	induces neuronal apoptosis	0.0008
AA495858	hydroxymethylbilane synthase	cell cycle and differentiation in glioma cells	0.0023
AA460927	translin	dendritic RNA binding complex	0.0034
H10965	peroxisomal biogenesis factor 12 (PEX12)	catalyses metabolism, peroxisome biogenesis disorder (symptoms include neurodevelopmental delay)	0.0041
N33331	peroxisome proliferator activated receptor (PPAR)	anti-inflammatory and decreases free radical formation	0.0062
AA456830	diacylglycerol kinase (DAGK)	myelination	0.0064
AA491225	myotubular myopathy 1 (MTM1)	expressed in brain, family members involved in neuromuscular disorders	0.0064
AA504333	endothelial-monocyte activating polypeptide II (EMAPII)	brain inflammatory response	0.0069
AA644088	cathepsin C	upregulated after brain ischaemia	0.0071
H19522	exostoses-like protein 1 (EXTL1)	tumour suppressor, highly expressed in brain	0.0076
	transducer of erbB2 (Tob)	tumour suppressor, highly expressed in brain	0.0076
AA489331	adenosine deaminase	metabolism of adenosine	0.0083

XP is a well characterized DNA repair disorder with expected outcomes of gene expression for the NER pathway after UV light irradiation. However, there is an unknown biological basis for neurological symptoms appearing in particular complementation groups of XP. Recent studies in neurological [18] and psychiatric disorders [19-21] have reported altered expression of genes with neurological expression and/or function in peripheral tissue, suggesting that identification of differential gene expression between complementation groups with the presence/absence of neurological symptoms in peripheral tissues such as skin fibroblasts may determine the genes or biological mechanisms involved in these previously unexplained clinical characteristics of XP.

In this study, 6000 gene cDNA microarrays were used to identify altered gene expression in control and XP fibroblast cell lines after UV light exposure. NER

gene expression was altered in all of the XP cell lines compared to the control cell line, and distinct gene expression profiles for each XP complementation group were identified. Finally, genes involved in the neurological symptoms present in XPA, XPD and XPG complementation groups were also identified. This may shed some light on the biological cause of these previously unstudied symptoms of XP.

Materials and methods

Control and XP fibroblast cell lines

The control fibroblast cell line was obtained from the Molecular Genetics Laboratory, Hunter Area Pathology Service, John Hunter Hospital, Newcastle, Australia. The XP fibroblasts were obtained from the NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research. The following XP cell lines had

known mutations: XPA (GM00544B) 2bp deletion A468-A469; XPC (GM00030A) 83bp insertion at 462; and XPD (GM00435) C to T substitution C2047T. XPE (GM01389A), XPG (GM3021B) and XPF (GM03542C) cell lines had a clinical history consistent with previously reported cases for these complementation groups. All the fibroblast cell lines were incubated with 1 x DMEM (10% FCS) at 37°C (5% CO₂). To identify differential gene expression in the control and XP fibroblasts, each cell line was exposed to UV light to induce NER. A cell survival study, involving 2 J/m² UV light exposure over a time course followed by trypan blue exclusion to identify the percentage of cell survival, identified 1 minute of 2 J/m² UV light exposure sufficient to induce NER but not immediate cell death. Half of the control and XP cells were exposed to 2 J/m² UV light for 1 min in PBS when 100% confluent, and were allowed to recover for 1 hour.

RNA extraction

RNA was extracted from approximately 1 x 10⁷ fibroblasts using TRIzol reagent as per manufacturer's instructions. Analysis and quantification of the RNA samples were performed by spectrophotometry (Cary 50 Bio UV-Visible spectrophotometer, Varian) using a ratio of A₂₆₀/A₂₈₀ as described previously [21]. RNA integrity was assessed by agarose/formaldehyde gels as described previously [21].

Microarray procedure

Total RNA (25 µg) was used for indirect labelling of first-strand cDNA with Cy3 and Cy5 using the CyScribe cDNA Post-Labeling Kit (Amersham Pharmacia) as per manufacturer's instructions. The labelled cDNA was hybridized on ResGen 6K cDNA microarrays (Clive and Vera Ramaciotti Centre for Gene Function Analysis) and washed as per the CyScribe cDNA Post-Labeling Kit (Amersham Pharmacia) manufacturer's instructions. The microarrays were then scanned with an Axon 4000B microarray scanner and the image captured using GenePix Pro 3.0 software.

Microarray data analysis

The images were captured and analysed using GenePix Pro 3.0 and results tables containing fluorescence ratios were obtained. The control and XP fibroblast cell lines were all performed in duplicate with reverse labelling of the second microarray. All the reverse labelled microarray results were inverted to ensure all results were analysed as a ratio of treated/untreated. Areas or spots on the arrays that were affected by

background were flagged and excluded from further analyses. GeneSpring 5.0 (Silicon genetics, USA) was used to normalize per spot and per array (Lowess curve) and to a 50th percentile.

The relatedness of each XP complementation group and the control fibroblast cell line was tested by performing unsupervised cluster analysis using distance and correlation of all of the duplicate microarrays. The number of genes expressed in control and XP fibroblasts after UV light exposure was measured using a level of detection two-fold greater than the surrounding background. The number of genes in each cell line with greater than 1.5 fold alteration after UV light exposure was also measured. Statistically significant gene lists were created comparing the control fibroblasts and XP complementation groups using a non-parametric test (Wilcoxon-Mann-Whitney test) with a p-value cutoff of 0.05. Using these lists a dendrogram (experiment tree) was created using standard correlation to show the relationships between the expression profiles of the complementation groups. A second dendrogram (gene tree) was created to show relationships between the expression levels of genes across all the complementation groups. Percentage expected NER activity for each XP complementation group was then used to determine the accuracy of the clustering pattern. Significantly altered gene expression based on presence/absence of neurological symptoms was identified using the non-parametric test (Wilcoxon-Mann-Whitney test) with a p-value cutoff of 0.05. Using this list a second dendrogram was created to show the relationships between the profiles of the XP complementation groups.

Relative real-time PCR

To confirm altered gene expression of STAT1 and LSD1 identified by microarray analysis cDNA relative semi-quantification was performed by real-time PCR as described previously [21].

Results

Gene expression profiling

The control fibroblasts expressed more genes than any of the XP fibroblast cell lines after UV light irradiation (Fig. 1), indicating that altered expression due to NER deficiency was being detected. Gene expression alterations greater than 1.5 fold, specific to each XP complementation group, were also identified (Fig. 2). Although there was greater expression in the control fibroblasts after UV light treatment, the number of genes altered by greater than

1.5 fold was greater in some XP complementation groups, in particular XPD. Conversely, the XPA fibroblasts showed a low number of genes differentially expressed by greater than 1.5 fold (Fig. 2), which is reflective of the low number of genes expressed by the XPA fibroblast cultures after UV light exposure (Fig. 1).

To ascertain the relatedness of each XP complementation group and the control, standard correlation and distance were used to create a dendrogram to show relationships between gene expression profiles. Similarity of gene expression profiles is measured by distance on a dendrogram. Distance is measured by the vertical height of the branches of the dendrogram; therefore the closer the relatedness of one profile to another, the shorter the vertical branch. A second dendrogram (gene tree) was created using standard correlation and distance to show relationships between the expression levels of genes across the groups. Unsupervised clustering analysis was used to identify the relatedness of the gene expression profiles for the control fibroblasts and the XP complementation groups (Fig. 3). The relatedness of the control fibroblasts and XP complementation groups, with the exception of XPD, correlated directly with the expected

percentage of NER activity present in each cell line. XPE has approximately 45% normal NER activity and was the most similar to the control profile as measured by distance on the dendrogram (i.e. there is vertical distance between all the other XP profiles and the branch joining control

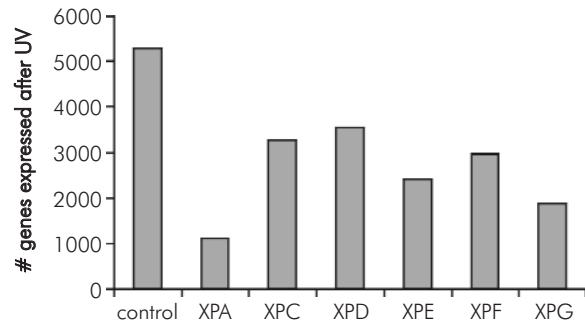


Fig. 1. Number of genes expressed in control and XP complementation group fibroblasts after 2 J/min UV light treatment. Control and XP fibroblast cell line cultures were exposed to UV light. The number of genes expressed in each cell line was determined by the fluorescence of expressed genes being two-fold above surrounding background on the duplicate microarrays. A higher number of genes were expressed in the control fibroblasts after UV light exposure when compared to the XP complementation groups

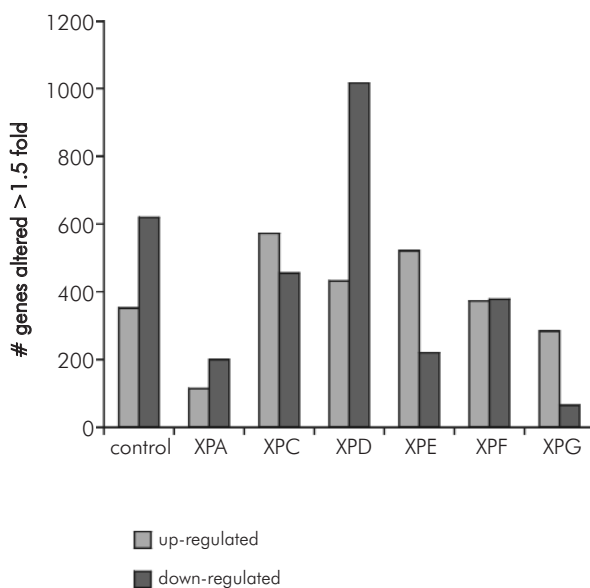


Fig. 2. Number of genes altered greater than 1.5 fold in control and XP complementation group fibroblasts after 2 J/min UV light treatment. Control and XP fibroblasts cell line cultures were exposed to UV light. Numbers of differentially expressed genes were determined by identifying genes with a greater than 1.5 fold change in expression after UV light exposure. Similar numbers of genes were differentially expressed in the control and XP complementation groups with the exception of XPD which had a considerably higher number of genes down-regulated by greater than 1.5 fold and XPA which had a low number of differentially expressed genes, reflective of the low number of genes expressed in the XPA fibroblasts after UV light exposure

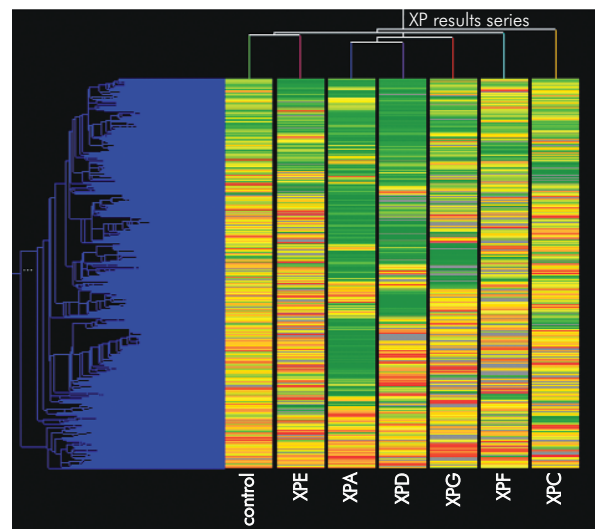


Fig. 3. Cluster analysis of control and XP complementation groups after UV light treatment. A comparison of control and XP complementation group with gene expression. Control and XP fibroblasts cell line cultures were exposed to UV light. Each column represents gene expression for each of the cell lines after UV light exposure. Down-regulation is represented as green, up-regulation as red and normal expression as yellow. The dendrogram on top represents the relationship between and within the 7 cell lines. The dendrogram on the side represents relatedness of genes based on expression levels. The relatedness of the control fibroblasts and XP complementation groups with the exception of XPD correlated directly with the expected percentage of NER activity present in each cell line

and XPE). XPF and XPC have approximately 20% normal NER activity and were the next closest to the control profile respectively (represented as the next two shortest branches of the tree). XPG was closely related to XPF by distance and has approximately 10% normal NER activity. Finally XPA and XPD were closely correlated and the furthest in distance from the control profile. XPA has less than 2% of normal NER activity and XPD has approximately 30%.

The unsupervised clustering showed that XPA, XPD and XPG profiles had a high correlation, suggesting that an alternative parameter to percentage of expected NER deficiency was influencing the profiles. To investigate the cause of the XPD profile not correlating with NER activity, and the presence of the XPA, XPD and XPG cluster, the presence/absence of neurological symptoms was used to identify significantly altered genes. 383 genes were significantly altered in the XP groups with neurological symptoms (XPA, XPD, XPG) when compared to the XP groups without the symptoms (XPC, XPE, XPF). 18 highly altered ($P < 0.01$) genes have previously been reported to have brain-related function or expression (Table 1).

Cluster analysis of XP complementation groups exhibiting neurological symptoms using this list also identified distinct profiles associated with the symptoms (Fig. 4). The profiles for XPA, XPD and XPG (Fig. 4 red branches) were closely related as depicted by small joining branches, and distinctly different to control, XPC, XPE and XPF (Fig. 4 blue branches).

Semi-quantitative real-time PCR

Semi-quantitative real-time PCR using SybrGreen was used to confirm altered gene expression identified

by microarray analysis. Initially, the expression of two housekeeping genes, 18s ribosomal RNA and β -Actin, were measured for each of the UV-treated and untreated control and XP fibroblasts cell lines. Two genes, signal transducer and activator of transcription 1 (STAT1) and lysine specific demethylase 1 (LSD1), having consistently altered expression across the cell lines from the microarray analysis, were chosen for confirmation. STAT1 and LSD1 exhibited similar fold change expression in the microarray and real-time PCR analysis (Table 2) after UV light treatment. LSD1 expression was not expressed at a detectable level in the control fibroblasts after UV light exposure. These results confirm that the microarray results were reflective of expression levels in the fibroblast cell lines before and after UV light treatment.

Discussion

Exposure to UV light initiates differential expression of a high numbers of genes in fibroblasts compared to, for example, cardiac myocytes [22]. The present study exemplifies the use of fibroblasts to study gene expression profiles in disorders with reduced NER activity as a high number of genes were expressed in the control fibroblasts after UV light exposure and a diminished number of genes were observed in the NER-deficient XP fibroblasts. The distinguishing feature of XP compared to other NER-deficient disorders is DNA repair or DNA replication deficiencies involving most of the genome [23]. For this reason, after UV light irradiation, it is to be expected that whole genome expression in XP fibroblasts will be considerably lower than in unaffected fibroblasts, due to the high levels of damaged DNA. This was confirmed in this study by the decrease in overall expression in the XP complementation groups compared

Table 2. Control and XP complementation groups microarray and relative real-time PCR fold change expression levels after UV light irradiation

Cell Line	LSD1		STAT1	
	Microarray Fold Change	Real-Time PCR Fold Change	Microarray Fold Change	Real-Time PCR Fold Change
control	-4.6	N/D	-1.9	-5.4
XPA	-3.1	-2.2	-7.2	-3.1
XPC	-5.8	-2.2	-1.8	-5.2
XPD	-12.7	-8.5	-11.6	-3.4
XPE	-5.0	-4.5	-2.6	-4.6
XPF	-2.8	-2.8	-2.2	-9.3
XPG	-5.5	-1.0	-1.5	-3.6

N/D = not detected

to the control after UV light treatment. The overall decrease in numbers of genes expressed was not reflected, however, in the number of genes altered in each XP complementation group compared to control. This result may be due to the NER deficiency resulting in altered expression of other DNA repair mechanisms, cell-cycle genes and apoptotic pathways.

Although all the XP complementation groups have some level of NER deficiency, they have distinct characteristics such as differing levels of UV light sensitivity, skin cancer susceptibility and neurological symptoms. Distinct gene expression profiles were identified for each complementation group, with percentage NER activity showing a relationship to relatedness of profiles. The XPE profile was the most similar to the control profile, which is to be expected as XPE has relatively high levels of NER activity and the least severe symptoms [24]. The remaining XP complementation groups followed the pattern of the lower the percentage NER activity the further the profile from control, with the exception of XPD. XPD showed a similar profile to XPA, which has a <2% NER activity, and was equal furthest from control.

The presence of neurological symptoms in XPA, XPB, XPD and XPG has rarely been investigated. In this study, a list of genes significantly different in XPA, XPD and XPG compared to control, XPC, XPE and XPF was generated and cluster analysis performed to identify distinct gene expression profiles related to the presence of neurological symptoms. Many of the altered genes have neurological functions and have previously been reported to be expressed in brain tissue and, although not one of the aims of this study, further analysis of the data may identify underlying causes for the symptoms. This current study suggests that deficiencies in NER probably result in the accumulation of DNA damage in neurological tissue, probably as a result of endogenous insult.

Finally, relative real-time PCR was used to confirm the altered expression of two genes in the control and XP cell lines. STAT1 and LSD1 showed similar expression between the microarrays and real-time PCR, suggesting that the microarray data is semi-quantitative, and more importantly is identifying altered regulation of genes in each sample tested.

In conclusion, the data herein suggests that cluster analysis can identify distinct gene expression patterns between XP complementation groups. Relative real-time PCR confirmation has also shown that the data are representative of gene expression in the control and XP cell lines before and after UV irradiation. The

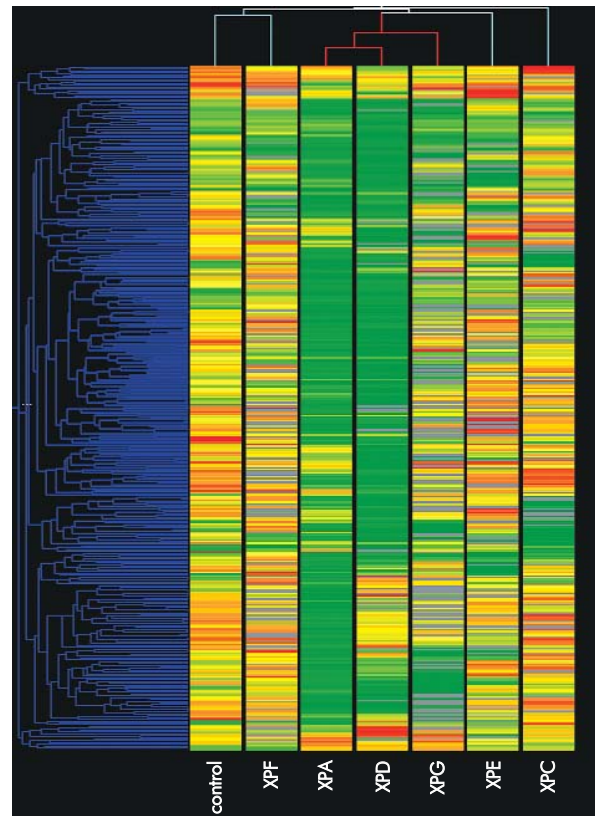


Fig. 4. Cluster analysis of neurological symptoms present in control and XP complementation groups. A comparison of control and XP complementation group with presence/absence of neurological symptoms and gene expression. Control and XP fibroblasts cell line cultures were exposed to UV light. Each column represents expression in each of the cell lines of 383 genes significantly differentially expressed in XP complementation groups with and without neurological symptoms. Down-regulation is represented as green, up-regulation as red and normal expression as yellow. The dendrogram on top represents the relationship between and within the two groups; blue branches represent absence and red branches represent presence of neurological symptoms. The dendrogram on the side represents relatedness of genes based on expression levels. A cluster of red branches is clearly depicted in the top dendrogram indicating a distinct gene expression profile for the presence of neurological symptoms in XP

identification of distinct gene expression profiles for XP complementation groups will assist in diagnosis of the disease as well as further our understanding of the biology of XP. In addition, the understanding of the biological cause of neurological symptoms in XP will be furthered with the instigation of gene expression profiling in XP complementation groups.

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