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Identification of Estrogen-responsive Genes in p53$^{+/-}$ Knockout and Isogenic Wild-type Parent Strain Mice by CDNA Macroarray Analysis

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To identify putative genetic targets for p53 *in vivo*, in this research applied the cDNA macroarray gene expression profiles associated with apoptosis by comparing p53$^{+/-}$ knockout mice and wild-type mice on the uterus of female mice. p53$^{+/-}$ knockout mice and wild-type mice were treated with DES (500 μmole kg^{-1}) or vehicle i.p once daily for 4 days. Total RNAs were obtained from uterus of control and DES-treated. The signal intensities of individual gene spots on the membrane were quantified and normalised to the expression level of the GAPDH gene as an internal control. Present results demonstrated that sixteen genes; bad, bax, bcl-2, bcl-w, bcl-x, caspase-3, caspase-7, caspase-8, c-myc, E124, GADD45, mdm2, NKκb1, p53, p21, Rb and trail were up-regulated and six genes; caspase-1, caspase-2, DR5, E2F1, FasL and iNOS did not changed in response to DES treatment in wild-type mice compared to p53$^{+/-}$ knockout mice. Most genes are involved in cell cycle regulation, signal transduction, apoptosis, or transcription. The greatest changes were seen in bad, bcl-x, mdm2, p53 and p21 gene expression in wild-type mice compared to p53$^{+/-}$ knockout mice. In comparing p53 and p21 gene expression in wild-type mice and p53$^{+/-}$ knockout mice, there was a 2.1-fold versus 8.3-fold; 16-fold versus 5.5-fold an increment in induction, respectively. RT-PCR was used to confirm the biggest changes of p21, p53 and bax genes. Using this approach, present study identified apoptosis associated genes regulated in response to DES and have revealed putative differences between the isogenic parent strain and p53$^{+/-}$ knockout mice, which will contribute to a better understanding of toxicity/carcinogenicity mechanisms in this model.

Key words: p53$^{+/-}$ knockout, gene expression, apoptosis, macroarray, carcinogenesis

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INTRODUCTION

Diethylstilbestrol (DES) is a synthetic stilbene oestrogen receptor agonist that was used to treat women considered at risk of miscarriage; it is estimated that two to three million pregnant women were treated with DES[1]. In 1971 DES was banned from use during pregnancy after clear evidence of an increase in adenocarcinomas of the vagina and cervix (at puberty) of daughters exposed to DES *in utero*[2]. In addition, women treated with DES also exhibited an increased risk for development of breast cancer. DES is classified as a 'Group 1' human carcinogen by the International Agency for Research on Cancer and was selected by the International Life Sciences Institute Alternatives to Carcinogenicity Testing programme as a representative non-mutagenic (i.e. Ames/Salmonella-negative) carcinogen acting through a receptor-mediated mechanism[3]. However, although DES is a known carcinogen in humans and rodents the cellular and molecular mechanisms by which it induces cancer have not been fully elucidated, although both genotoxic and epigenetic effects have been characterised[4]. The $p53^{+/-}$ hemizygous knockout mouse model has one copy of the wild-type allele of the p53 tumour suppressor gene and one copy of a null allele that is not transcribed, offering a single target for mutagens, a condition analogous to humans with some heritable forms of cancer (e.g., Li Fraumeni syndrome)[5]. The $p53^{+/-}$ mice have a low incidence of spontaneous tumours up to nine months but rates increased substantially thereafter[6]. Although more sensitive and showing a shorter latent period for tumours, the single functional gene in the $p53^{+/-}$ mouse model allows some nucleotide excision repair of DNA damage relative to homozygous $p53^{-/-}$ knockout mice, but the reduced ability of $p53^{+/-}$ mice to repair DNA damage is an additional possible origin for the earlier and more frequent appearance of tumours in these mice, compared to wild-type mice[7]. However, a more complete picture of the molecular basis for tumour susceptibility is required in this model, including the characterisation of the molecular response to model carcinogens. cDNA gene expression profiles using macroarray analyses, RT-PCR and densitometric analysis were also performed in order to relate the changes of gene expression profile following DES treatment. Recent findings on gene expression profile changes after DES administration on this model was documented[8]. This study aimed to investigate the gene expression profiles in the target organ of reproductive especially uterus, any differences in gene expression could be reflective the way genes control the cell in response to toxicity/carcinogenicity in this models.

MATERIALS AND METHODS

Experimental design: Twenty Female C57BL/6J wild-type mice and $p53^{+/-}$ knockout mice (n=5/group, 23-26 g), were treated with 500 μmole kg^{-1} b.w i.p, once daily for 4 days. Control animals were treated with the trioctanoin vehicle only. Animal studies were performed in accordance with the UK "Animals (Scientific Procedures) Act". All animals were sacrificed 24 h after the last dose, uterus organs harvested and frozen immediately at -80°C. Samples (n=4/group) were pooled to yield a representative for analysis. Total RNA was isolated using RNA Stat-60. The purity of RNA was measured spectrophotometrically (Shimadzu-MPS 2000, UK) and value of the ratio between 260:280 nm was within 1.7-2.1. Quality of RNA preps was checked on electrophoresis 1% native agarose gels and stained with EtBr. The RNA was used only when both 18S and 28S bands were intact (Fig. 1).

Macroarray analysis: Analysis differential expression levels of multiple genes involved in murine cell apoptosis was determined using cDNA expression arrays (mGEA1012020, SupperArray Inc.Bethesda Maryland, USA). cDNA probes were synthesized from total RNA (from control versus DES-treated mice; wild-type versus p53 knockout mice) using reverse transcriptase. The cDNA probes with ^{32}P-labelling, which represent an abundance of the mRNA population, were hybridised with gene-specific cDNA fragments spotted on the membranes. The relative expression level of each gene is determined by comparing the signal intensity of each gene in the membrane after normalisation to the signal of housekeeping gene, GAPDH by phoshorimager. The level of radiation intensity was captured by the screen and it was the analysed using the OptiQuant Phosphorimager software (Packard Instrument, Company USA). All experiments were done in four independent array membranes (n=4/arrays).

PCR analysis: The primer sequences (and expected sizes of the PCR products) were: p53 (AF161020), 5'-GATGACTGCCATGGAGGACT-3' and 5'-CTCGGGTGGCTCATATAAGGTA-3' (664 bp); p21(U24173), 5'-CGGTGGAACTTTGACTTCGT-3' and 5'-CACAGAGTGAGGGCTAAGGC-3' (423bp); bcl-x (L35049), 5'-TTGGACAATGGACTGGTTGA-3' and 5'-ACCCCAGTTTACTCCATCCC-3' (457 bp) and GAPDH (M32599), 5'-ACCCCAGTTTACTCCATCCC-3' and 5'-TGTTCCGGGTGGTTCTGCAG-3' (500bp). All the primers were purchased from Gibco BRL,UK. PCR amplifications were performed for: 30 cycles of 94°C for

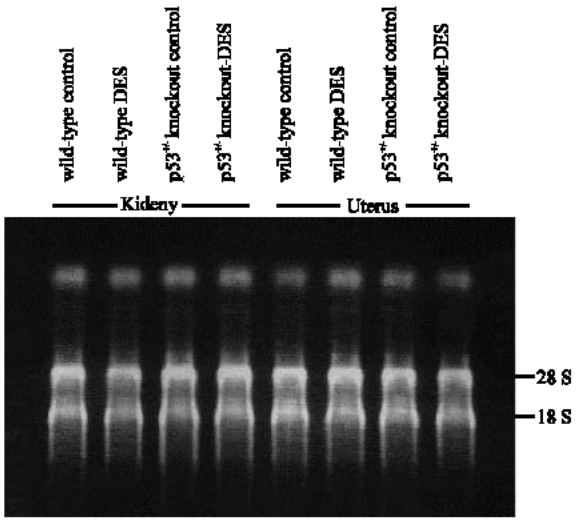

Fig. 1: Total RNA was extracted and quality of RNA preps from kidneys and uterus organs were run on a 1% native agarose gels electrophoresis staining with EtBr. The locations of the 28 S and 18 S ribosomal RNAs are marked on the right

1 min, 58°C for 40 sec and 72°C for 1 min. PCR products were subjected to electrophoresis on 3% agarose gels and stained wit EtBr. The intensity of the bands was quantified by densitometric scanning followed by LabWorks Image Acquisition and analysis software (UVP, Cambridge, UK).

Statistical analysis: Results are expressed as mean±S.E. The statistical evaluation of the results was performed by one-way ANOVA analysis, followed by student's t-test. Data are presented as means with 95% confidence intervals.

RESULTS

This study demonstrated here the differential expression of 23 specific genes associated with apoptosis from uterus samples. The signal intensities of individual gene spots on the membrane were quantified using OptiQuant phosphorimaging. The expression of all the genes on each array was normalised on the expression level of the GAPDH gene as an internal control. To identify genes with significantly different expression between normal and treated-DES, rational control/treated cut off values were determined (Table 1) selected of mRNA's significant up-regulated and down-regulated in wild-type mice and $p53^{+/-}$ knockout mice following DES treatment ($p<0.05$) using cDNA macroarray hybridisation analysis. The expression of two housekeeping genes,

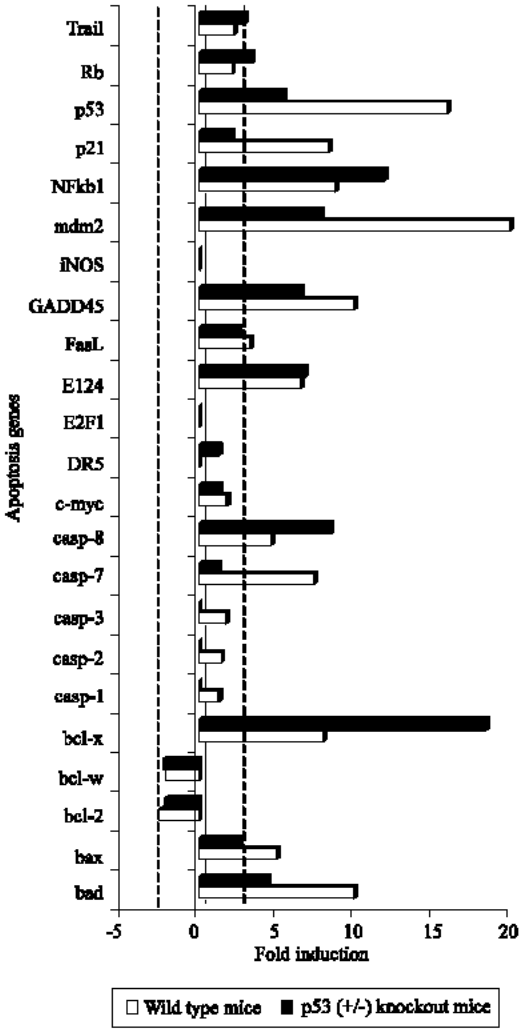

Fig. 2: Fold induction or repression of expression of 23 genes associated with apoptosis from uterus organs in response to DES treatment in $p53^{+/-}$ knockout mice and wild-type mice, as compared to control (vehicle-treated animals)

GAPDH and β-actin, was stable and did not differ in both control and treated samples. Figure 2 shown the histogram of 23 genes associated with apoptosis in the uterus organs. For the purpose of this study, only those genes, which displayed either greater than or equal to a two-fold up-or down regulation, have been considered. Overall array results shown there is similarity trends of fold induction of cDNA gene expression profiles between wild-type mice and $p53^{+/-}$ knockout mice, due to DES treatment.

Majority of genes expressed slightly higher in wild-type mice compared to $p53^{+/-}$ hemizygous knockout mice. Our results demonstrated that sixteen genes; bad,

Table 1: Induction or repression (greater than two-fold) of genes in response to DES treatment in wild-type mice and $p53^{+/-}$ knockout mice

Gene bank	Gene Name	$p53^{+/-}$ knockout mice	wild-type mice
L 37269	Bad	10.0*	4.4
L22472	Bax	5.0*	6.5
L31532	Bcl-2	-2.6	-1.9
L35049	bcl-x	-2.1	-2.9
AF067834	caspase-8	4.7*	8.4
U06948	FasL	2.2	2.0
U41751	E124	6.6	6.8
L28177	GADD45	10.0*	6.6
U40145	mdm2	20.0*	7.8
M57999	Nfκb1	8.8	11.9*
U24173	p21	8.3*	2.1
K01700	p53	16.0*	5.5
M26391	Rb	2.1	3.4
M86183	Trail	2.2	2.9

*$p<0.05$ significantly different comparing wild-type mice with $p53^{+/-}$ knockout mice; negative values indicate repression of that gene

bax, bcl-x, caspase-3, caspase-7, caspase-8, c-myc, E124, GADD45, mdm2, NFκb1, FasL, p53, p21, Rb and trail were up-regulated, two gene; bcl-2 and bcl-w were down regulated and five genes; caspase-1, caspase-2, DR5, E2F1 and iNOS did not changed in response to DES treatment in wild-type mice compared to $p53^{+/-}$ knockout mice. Results also revealed statistically significant differences for bad, p21, p53 and caspase-8 of the uterus samples when compared to wild-type mice and $p53^{+/-}$ knockout mice ($p<0.05$). The greatest changes were seen in bad, bcl-x, mdm2, p53 and p21 genes expression in wild-type mice compared to $p53^{+/-}$ knockout mice. In comparing p53 and p21 genes expression in wild-type mice and $p53^{+/-}$ knockout mice there was a 2.1 fold vs. 8.3 fold; 16 fold vs. 5.5 fold increase in induction, respectively.

To examine the reliability and reflection changes of present arrays data, selected a candidate genes (p53, p21 and bcl-x), which have been significantly big changes in arrays analysis and examined their expression levels by RT-PCR analysis. We used the same RNA samples that had served for the cDNA macroarray analysis. The three up regulated gene (p53, p21 and bcl-x) showed elevated expression in wild-type mice compared to $p53^{+/-}$ knockout mice. Densitometric analysis (Fig. 3) of p53, p21 and bcl-x also revealed that all mRNA's were present at much higher levels in treated samples compared to controls in both models. Results show that, indeed, the activation of p53 mRNA in wild-type treated group was accompanied by an increase in expression of p21 mRNA and pro-apoptotic gene, bcl-x.

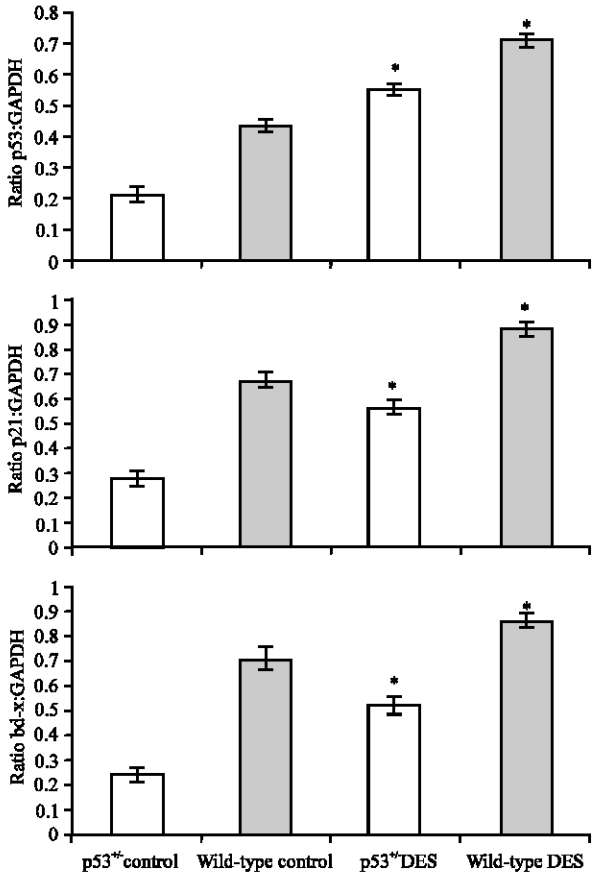

Fig. 3: Densitometric analysis of p53, p21 and bcl-x mRNA bands resulting from electrophoresis RT-PCR analysis of uterus samples. p53, p21 and bcl-x mRNA higher expressed in both DES-treated animals. Values are means±sp from triplicate experiment. * Significant different when compared to control with treated animals, $p<0.05$ by Student's t-test

DISCUSSION

This study reported the transcriptional changes of gene expression on the impact of genetic manipulation in $p53^{+/-}$ knockout mice and wild-type mice following DES-treatment. A relatively new technique were applied to differential gene expression through cDNA expression arrays and RT-PCR analysis in order to confirm the reliability and reflection changes of present arrays data. Results are consistent with a previous report, that the up-regulation of p53 and p21 mRNA indicated the possible activation of p53. After DNA damage, cells are observed to undergo cell cycle arrest and/or apoptosis. There is evidence that genotoxic insult damage leads to a p53 dependent increase in p21 mRNA and protein expression that results in cell cycle arrest at the border the G_1/S phases. Induction of the G_1 cell cycle checkpoint depends on post-translational modification, stabilization and translocation of the p53 protein to the nucleus where it activates transcription in conjunction with other co-activators. Activated transcription of p21

inhibits cyclin-dependent kinases resulting in hypophosphorylated Rb and G_1 arrest.

Present arrays data also revealed an increase of other transcriptional targets of p53 that include mdm-2, which regulates p53 expression in a negative regulatory feedback fashion. Studies by Perry *et al.*[10] and Barak *et al.*[9] have also reported that, wild-type p53 activity induced mdm-2 expression. The mdm-2 protein binds to wild-type p53, inhibiting its function as a tumour suppressor gene and when amplified or over expressed, mdm-2 has been shown to increase the tumorigenicity of cells. Furthermore, there is evidence that mdm-2 can be detected mostly in cases with wild-type p53. In addition, a DNA damage-inducible protein GADD45 is also highly expressed in both organs. Induction of GADD45 was also observed following treatment with many other types of DNA-damaging agents, including various environmental stresses, hypoxia, genotoxic agents during apoptosis. Therefore, inactivation of p53 function in response to DNA damage would results in unrepaired DNA breaks leading to aneuploidy and DNA synthesis and resulting in tetraploidy and finally cells undergoing apoptotic death[11]. Consequently, a multiplicity of cell cycle checkpoints in response to DNA damage may well involve redundant controls involving both p53-independent and -dependent pathways.

The attention was also focused on the bcl-2 family is widely accepted as regulators cell death pro-apoptotic and anti-apoptotic genes bad and bcl-2 genes, which are considered dominant regulators for apoptosis[12]. During these phenomena, the expression levels of pro-and anti-apoptotic Bcl-2 family members are altered so that the cells are prone to death. Expression levels of two pro-apoptotic members bad and bcl-x were up regulated while those of anti-apoptotic gene, bcl-2 and bcl-w were down regulated after DES-treatment. Another pro-apoptotic member of the Bcl-2 family, Bax was also up regulated. Furthermore, it has been demonstrated that steroid hormones may regulate pro-apoptotic or anti-apoptotic genes in breast and ovarian cancer cells. The transcription factor NFκb1 has been implicated in apoptosis suppression in many contexts and appears to be elevated in its activity in many types of cancer. Present study showed that NFκb1, a redox-sensitive transcription factor is also up regulated in response to DES-treatment. It has also been recently demonstrated that p53 activation induced apoptosis through Nfκb1-dependent pathway. We postulated that Nfκb1 activation might be responsible for up-regulation of bcl-x and bax, because one of the target genes of NFκb1 that is relevant to apoptosis is Bcl-2 family. These results are in agreement with previous reports that both *in vivo* and *in vitro* studies, suggests that activation of NFκb1 have strong connections between pro-and anti-apoptotic genes. We and other researchers believe that, NFκb1 might be regulating the expression of either anti-or pro-apoptotic effects depending on specific cell types and apoptotic stimuli in response to apoptotic signaling pathway. Interestingly, the expression of those genes is high in wild-type mice compared to $p53^{+/-}$ hemizygous knockout mice[13].

On the other hand, as mdm-2 is an *in vitro* substrate of caspases-3, -6 and -7, which become activated also during c-myc induced apoptosis, it is likely that mdm-2 becomes cleaved by one or several of these caspases in c-myc induced cell death. The present studies demonstrated that caspases-3, -7 and -8 were up regulated[14]. The expression of caspases-1, -2, -3, -7 wasm also up regulated in response to DES treatment. The expression of those genes slightly higher in wild-type mice compared to $p53^{+/-}$ hemizygous knockout mice. This study agreed with previous findings that the activation of caspases-3, -7 and -8 might be involved and necessary for c-myc-induced apoptosis[14-16]. In addition, activation of caspases leading to cleavage of a wide range of proteins is considered to be a crucial step involved in the induction of apoptosis. Thus, present findings are, in agreement with previous reports, strongly indicated that apoptosis and cell cycle arrest occur in isogenic wild-type parental strains compared to $p53^{+/-}$ hemizygous knockout mice. This is further supported by reports showing that p53 heterozygosity results in a significant decrease in radiation-induced apoptosis in mouse and thymocytes.

In conclusion, cDNA gene expression analysis data from our results indicate that treatment with DES can induces apoptosis in the uterus as a reproductive system and the elimination of one functional p53 allele results in an altered response to genotoxic insult. The data presented here clearly demonstrate that the haplo-insufficient phenotype of $p53^{+/-}$ knockout mice was less pronounces stimulates programmed cell death than C57BL/6J wild-type mice, suggesting that wild-type p53 might be one of the factor modulating the sensitivity to DES induces apoptosis. In addition, some of tissue specificity of genotoxic carcinogens is similar in both wild-type and $p53^{+/-}$ knockout mice, suggesting that alteration may play a role in the rapid tumour development of carcinogenicity in this model.

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