UNIVERSITI PUTRA MALAYSIA

GENE EXPRESSION AND METABONOMIC PROFILINGS IN p53+/- KNOCKOUT MICE FOLLOWING DIETHYLSTILBESTROL TREATMENT

MOHD NAZIL SALLEH

FPSK (P) 2003 2

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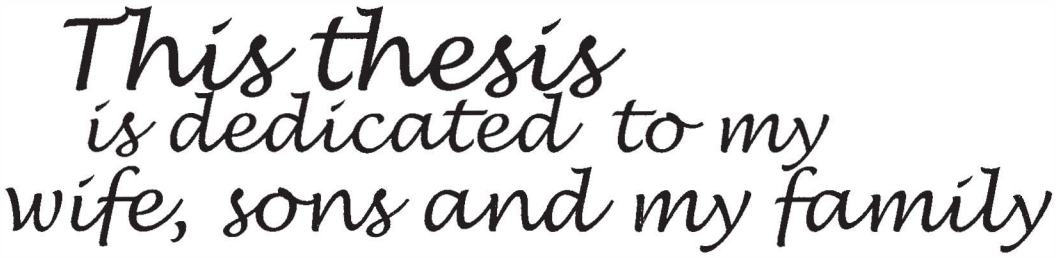
By

MOHD NAZIL SALLEH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of Requirements for the Degree of Doctor of Philosophy

September 2003


This thesis
is dedicated to my
wife, sons and my family

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

GENE EXPRESSION AND METABONOMIC PROFILING IN $p53^{+/-}$ KNOCKOUT MICE FOLLOWING DIETHYLSTILBESTROL TREATMENT

By

MOHD NAZIL SALLEH

May 2003

Chairman: Associate Professor Patimah Ismail, Ph.D.

Faculty: Medicine and Health Sciences

Clastogenic carcinogen diethylstilbestrol (DES) results in a broad of spectrum of toxic and carcinogenic effects in humans and rodents. Female C57BL/6J wild-type mice (23-25g) and female $p53^{+/-}$ hemizygous mice were treated with DES (500μmol/kg) i.p., once daily for 4 days. Control animals were treated with the trioctanoin vehicle only. All animals were sacrificed 24 hours after the last dose, and liver, kidney and uterus were harvested and frozen at –80°C. Analysis of differential expression levels of multiple genes involved in apoptosis and cell was performed using cDNA array technology. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis was carried out in order to verify the expression of these genes.

In hepatocytes, murine apoptosis arrays showed three genes: *bcl-w, caspase-3* and *E2F1* were transcriptionally down-regulated and eight genes: *bad, bax, caspase-1, caspase-7, c-myc, p21, p53 and Rb* were up-regulated. In cell-cycle arrays thirteen genes were up-regulated: *CDK6, CKK1, Cyclin C, Cyclin* D_2, *Cyclin* D_3, *Cyclin E, Cyclin* E_2, *E2F1, p16, p19, p21, p57* and *Skp1*. The greatest change was in *p21* gene expression. There was a 3-fold *versus* 10-fold increase induction for apoptosis-

associated arrays; 0.8-fold *versus* 5-fold for cell cycle-associated arrays (for $p53^{+/-}$ and wild-type mice, respectively). Similar pattern of genes expression was also found in the kidney and uterus. There are seventeen 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 change in response to DES treatment in wild-type mice compared to $p53^{+/-}$ hemizygous knockout mice. Most genes are involved in cell cycle regulation, signal transduction, apoptosis, or transcription. In comparing *p53* and *p21* gene expression in wild-type mice and $p53^{+/-}$ hemizygous knockout mice, there was a 8-fold vs. 5.2-fold; 4.4-fold vs. 1.8-fold for kidney and 2.1-fold vs. 8.3-fold; 16-fold vs 5.5-fold for uterus samples increase in induction (respectively). RT-PCR was used to confirm the biggest changes of *p21, p53, bcl-x* and *bax* mRNA genes.The novel application of high-resolution proton nuclear magnetic resonance (^{1}H NMR) analysis in this study revealed the increase in taurine, creatine and succinate acid in treated mice compared to control animals, giving further insight of hepatotoxic effects of DES as shown in histopathological studies.

The combination of cDNA arrays technology and H-NMR spectroscopy provides a direct link and good prediction of genes profile and endogenous metabolite in order to increase the sensitivity of early detection of the potential toxic effects of environmental chemical and for genes therapy.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

EKSPRESI GEN DAN METABONOMIK PROFIL KE ATAS MENCIT TERSINGKIR $p53^{+/-}$ BERIKUTAN RAWATAN DIETHYLSTILBESTROL

Oleh

MOHD NAZIL SALLEH

Mei 2003

Chairman: Profesor Madya Patimah Ismail, Ph.D.

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Kajian ke atas klastogenik karsinogen diethylstilbestrol (DES) memberi kesan yang amat meluas dalam spektrum toksin dan karsinogenik baik untuk manusia mahupun rodensia. Penggunaan mencit betina daripada baka liar (induk; C57BL/6J) dan mencit hemizigos $p53^{+/-}$ telah diberi suntikan secara intraperitoneum setiap hari selama 4 hari pada kadar dos 500 μmol/kg DES mengikut berat badan mencit. Manakala kumpulan kawalan diberi suntikan minyak zaitun. Kesemua mencit dimatikan secara dislokasi servikal selepas 24 jam selepas suntikkan terakhir. Organ hepar, ginjal dan uterus diambil dan dibekukan serta merta pada suhu -80^{0}C. Analisis pembedaan penzahiran tahap pelbagai gen yang terlibat di dalam murin sel apoptosis and kitaran sel telah dibuat menggunakan teknologi cDNA array. Analisis transkripsi tindakbalas polimerase berantai pendek (RT-PCR) telah digunakan untuk mempastikan penzahiran gen-gen tersebut.

Dari sel-sel hepar, apaptosis murin array didapati tiga gen; *bcl-w, caspase-3* dan *E2F1* telah menurun rangsangannya secara transkripsi, lapan gen; *bad, bax, caspase-1, caspase-7, c-myc, p21, p53* dan *Rb* telah meningkat rangsangannya. Manakala di dalam kitaran sel array 13 gen telah meningkat rangsangannya iaitu; *CDK6, CKK1, Cyclin C, Cyclin* D_2, *Cyclin E, Cyclin* E_2, *p16, p19, p21, p57* dan *Skp1*. Perubahan yang paling ketara ialah penzahiran gen mRNA *p21*. Masing-masing terdapat gandaan 3 melawan gandaan 10

peningkatan induksi gen yang terlibat di dalam apoptosis; 0.8 gandaan melawan gandaan 5 untuk kitaran sel bagi mencit hemizigos $p53^{+/-}$ dan mencit liar. Corak penzahiran gen yang serupa juga didapati di dalam organ ginjal dan uterus. Terdapat 16 gen yang meningkat rangsangannya iaitu; *bad, bax, bcl-x, caspase-3, caspase-7, caspase-8, c-myc, E124, GADD45, mdm2, FasL, NKκb1, p53, p21, Rb* dan *trail*, 2 gen iaitu; *bcl-2* dan *bcl-w* menurun rangsangannya dan 6 gen iaitu *caspase-1, caspase-2, DR5, E2F1, FasL* dan *iNOS* yang tidak bertindak balas terhadap perlakuaan DES ke atas mencit liar berbanding mencit hemizigos $p53^{+/-}$. Kebanyakan gen-gen tersebut terlibat di dalam kawalan kitaran sel, tranduksi isyarat, apoptosis dan transkripsi. Apabila perbandingan dibuat di antara penzahiran gen mRNA p53 dan p21 bagi mencit liar dan mencit hemizigos $p53^{+/-}$, terdapat peningkatan induksi masing-masing iaitu gandaan 8 melawan gandaan 5.2; gandaan 4.4 melawan gandaan 1.8 untuk ginjal dan gandaan 2.1 melawan gandaan 8.3; gandaan 16 melawan gandaan 5.5 bagi uterus. RT-PCR teknik telah digunakan untuk mengesahkan perubahan terbesar gen mRNA p21, p53, bclx dan bax. Pengunaan analisis terkini iaitu menggunakan proton nuklear magnetik bergelombang tinggi (^{1}H-NMR) di dalam kajian ini turut mendapati kenaikan taurina, kreatinin dan asid suksinat pada mencit mencit liar yang telah disuntik dengan DES berbanding dengan hemizigos $p53^{+/-}$. Ini menggambarkan kesan hepatotoksik oleh DES, juga telah disahihkan daripada kajian histopatologi.

Penggabungan daripada kedua-dua teknik iaitu teknologi cDNA array dan ^{1}H NMR spektroskopi memberi kesan terus menerus serta predaksi yang baik di dalam mengenalpasti perubahan gen profil dan mengetahui metabolik endogenus akibat pemberian sintetik estrogen, DES di dalam meningkatkan tahap sensitiviti dan prediksi peringkat awal penentuan barah dan bahan kimia secara semulajadi dan untuk rawatan gen terapi.

ACKNOWLEDGEMENTS

My utmost appreciation goes to Associate Professor Dr. Patimah Ismail, that without her continuous support, help, limitless patience, encouragement and advice, I won't be able to continue and complete this project. I wish to express my deepest thanks to Professor Dato' Dr. Abdul Salam Abdullah, Associate Professor Dr. Mohd. Taufiq Yap Abdullah, Dr. Paul Carmichael and Prof. John Cadwell for their guidance and support.

I acknowledge the financial support of the Universiti Putra Malaysia for conducting my research at Imperial College, London. I am grateful to all the warm-hearted people who helped me throughout the project, especially Charles, Jess, Suzie, Sandra and Faisal. I owe a special debt of thanks to Khairi, Dos, Nasir, Fezah, Hazizi, Hasiah and Latifah for their friendships that made working in the Faculty of Medicine and Health Sciences enjoyable.

I am very grateful to all my family for their continued moral support in pursuing my dreams. This work may not have materialised without the understanding, sacrificing and love, from my wife Dr. Norashikin Shamsudin. Last but not least to my boys, Alif Farhan (along) and Arif Hakimi (kimi) for the incredible joy, gift and privilege, they give to me while I'm writing my thesis. I would like to dedicate this PhD's thesis to them……amin.

I certify that an Examination Committee met on 2nd September 2003 to conduct the final examination of Mohd Nazil Salleh on his Doctoral thesis entitled "Gene Expression and Metabonomic Profilings in $p53^{+/-}$ Knockout Mice Following Diethylstilbestrol Treatment" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that candidate be awarded relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. Members of the Supervisory Committee are as follows:

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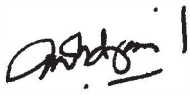
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



(MOHD. NAZIL SALLEH)

Date: 03.12.2003

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LIST OF ABBREVIATIONS

%	-	percentage
^{o}C	-	degree Celsius
g	-	gram
mg	-	milligram
mL	-	milliliter
mM	-	millimolar
mw	-	molecular weight
M	-	molar
Nm	-	nanometer
µl	-	microliter
µg	-	microgram
C	-	control
p.d	-	post-dose
NS	-	normal saline
DES	-	diethylstilbestrol
Kb	-	kilo base
Min	-	minute
hrs	-	hours
PCR	-	Polymersae Chain Reaction
NMR	-	Nuclear Magnetic resonance
RT-PCR	-	Reverse Transcriptase Polymersae Chain Reaction
BSA	-	bovine serum albumin
NADPH	-	β-nicotinamide adenine dinucleotide phosphate
EtBr	-	Ethidium bromide
H_2O_2	-	hydrogen peroxide
CDNB	-	1-chloro-2,4-dinitrobenzene
K_2HPO_4	-	potassium hydrogen phosphate
KH_2PO_4	-	potassium dihydrogen phosphate
H_3PO_3	-	orthophosphoric acid
KCl	-	potassium chloride
EDTA	-	ethylenediaminetetra acetic acid
NaN_3	-	sodium azide
NaOH	-	sodium hydroxide
DNA	-	deoxyribonucleic acid
RNA	-	ribonucleic acid
H & E	-	hematoxylin & eosin
LD_{50}	-	lethal dose 50
i.p	-	intraperitoneal injection
U	-	unit
v/v	-	volume per volume
w/v	-	weight per volume
w/w	-	weight per weight
x *g*	-	gravity

CHAPTER I

INTRODUCTION

In the twenty-first century, cancer has become one of the greatest killers of human in the world, especially in developed countries. Cancer is the second leading causes of death after heart disease. Thus, the search for the causes of cancer has been the subject of much investigation. According to Peto, (2001), cancer is a disease characterised by a loss of normal control of cell growth.

Epidemiological studies have shown that the majority of human cancers result from exposure to environmental and other chemical carcinogens including natural chemicals, radiation, viruses and hormones (Vogelstein, 1990). Furthermore, minority of cancer-prone mutations are hereditary. In these cases, a mutated gene on one of a pair of chromosomes is inherited. Several inherited diseases that are associated with cancer susceptibility have defective checkpoint control. Li-Fraumeni syndrome is a hereditary disease characterised by cancer arising in close relatives. It results from a germline mutation in the p53 gene that abrogates the G_1 checkpoint.

The p53 protein is the product of a tumour suppressor gene, which, has been the subject of intensive research efforts for the past few years. Loss of function of p53 occurs in over half of all human tumours, suggesting that inactivation of this tumour suppressor gene is an important factor in tumourigenesis. p53 function is impaired in majority of the human cancers. This

Although oestrogens in general are now known to be carcinogenic, the exact cellular mechanisms of carcinogenicity and toxicity have yet to be fully understood. Even though, more than 50% of human cancers involve the mutation of the p53 tumour suppressor gene, which is vital in many cellular processes like apoptosis and cell cycle control (Shaw, 1996).

The two-year rodent bioassay plays a central role in deciding whether a compound is carcinogenic. It has recently been suggested that six-month studies in transgenic mouse models could reduce costs and animal numbers without impairing the validity of cancer risk assessment. One of such new experimental methods is the use of a specific type of transgenic mice, the $p53^{+/-}$ knockout mouse The genetic manipulated $p53^{+/-}$ mouse is a phenotypically stable carcinogenicity model and tumours will be develop tumours during the six-month study period only in response to chemical and physical stimuli and shows a high concordance with genotoxic rodent carcinogens. Belonging to the C57BI/6J strain, these transgenic mice are heterozygous at the p53 locus, missing an intact p53 tumour suppressor gene allele. Since p53 plays a vital role in pathological pathways, both in mice and in humans, a non-functional p53 allele would make the mice model more sensitive to any genotoxic reactions on the DNA. Thus, once the functional allele is damaged by a mutagen, the entire p53-dependent pathway would be affected.

In this era of "post-genomic biology", research based upon gene expression array technology (macro/microarrays) can pose interesting problems. To give a better understanding of DES induced carcinogenesis, we need to have a

has stimulated efforts to understand the function of this gene in normal and neoplastic states. A large numbers of functions have been attributed to p53, including cell cycle checkpoints, apoptosis, angiogenesis and genetic stability (Bates and Vousden, 1999).

From 1940s to 1960s, the synthetic stilbene oestrogen, diethylstilbestrol (DES), was used to treat women with oestrogen deficiencies and to prevent miscarriages. The anti-oestrogenic properties of DES in the mammary tissues made it an effective treatment of breast cancer before 1970's (Carmichael, 1998). However, DES was banned in 1971 due to a link to various adenocarcinomas in treated women. DES was found to form adducts with DNA (Henderson and Feigelson, 2000) and thus provided a vital clue that cancers caused by DES are genetic in nature.

Epidemiological, clinical and experimental evidences indicate that steroid hormone, especially DES with oestrogenic action and, despite its lack of a steroid structure, is crucial in the induction of cancer, even though the mechanisms underlying its mitogenic effect have not been fully understood. In addition, women exposed to pharmacological levels of DES also exhibit an increased risk of breast cancer and may also increase the occurrence of endometrial adenocarcinomas (Marrselos and Tomatis, 1993). In laboratory studies, DES has been shown to have carcinogenic effect on various organs when given prenatally, neonatally or postnatally (Marselos and Tomatis, 1993). DES is classified as 'Group 1' human carcinogen by the International Agency for Research on Cancer (IARC) and results in a broad spectrum of toxic and carcinogenic effects in human and animal studies.

large body of information regarding the alteration of various genes involved in this process. To this end, we have applied gene expression through cDNA expression array (*Sehgal et al.*, 1998) which showed the expression profiles of thousands of genes in a single experiment, providing clues to the functional role of many genes, including potentially important oncogenes and tumour suppressor genes (DeRisi, *et al.*, 1996). In addition, there are no studies has been done on transcriptional gene expression using this models. Using these techniques, the analysis of the gene expression profiles of apoptosis and cell cycle genes was applied in order to see the effect of diethylstilbestrol treatment in this model. Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) also was performed in order to verify the expression of selected genes especially *p21*, *p53*, *bax*, *bcl-x* and *GAPDH* genes. Histological analysis of the liver sections was performed to look for evidence of hepatocellular damage following DES treatment. Finally, the endogenous metabolic state of DES-treated animals was also investigated by analysing urine using Proton Nuclear Magnetic Resonance (^{1}H-NMR)-based metabonomics studies.

This thesis aims to investigate the cellular mechanisms of estrogenic synthetic stilbene oestrogen, diethylstilbestrol (DES) induced development of cancer in the liver and specific-organ targets by comparing wild-type C57BL/6J isogenic parent strain and $p53^{+/-}$ knockout mice as a model.

Following strategies were employed.

1) Gene expression profilings using arrays technology involved in cell cycle control and apoptosis in several organs (liver, kidney and uterus) between female wild-type C57BL/6J isogenic parent strain and $p53^{+/-}$ knockout mice.

2) To verify selected gene expression changes from arrays analysis by using quantitative RT-PCR and densitometic analysis.

3) Endogenous metabonomic using ^{1}H-NMR was applied to compare urinary profiles following DES treatment in female wild-type mice.

4) To determine the cellular mechanisms involve in $p53^{+/-}$ hemizygous knockout mice and wild-type mice after DES administration.

CHAPTER II

LITERATURE REVIEW

2.1 Chemical Carcinogenesis

Approximately 80-90% of cancer incidence in human beings is caused by a myriad of genetic and environmental factors (Doll and Peto, 1981; Ponder, 2001). Cancer progression is promulgated by an even larger number of genes and environmental factors. These environmental factors include hormonal, diets, chemicals, biological agents and ionising radiation. Cancer remains the prominent killer in industrialized nations. Distinguishing and removing cancer cells from normal cells continue to be the key experimental design for therapy and prevention. Modern approaches to treating cancer take advantage of critical biochemical differences between cancer cells and normal cells - from radiation therapy to chemotherapy to experimental gene therapy. The Warburg hypothesis was based on the metabolic differences between cancer cells and normal cells, and proposed that increased glycolysis by transformed cells conferred a bio-energetic advantage for survival over normal counterparts under anoxic conditions. This hypothesis laid the foundation for cancer research strategies to find critical differences between transformed cellular processes and normal cellular processes.

2.1.1 Mechanisms of Chemical Carcinogenesis

The involvement of chemicals in damaging DNA is only one part of their potential role in carcinogenesis. In progression to the neoplastic state, the cells