

p53 and p21 mRNA and Protein Expression in Treated Synthetic Oestrogen in Mouse Transgenic Animal Model

¹Mohd. Nazil Salleh, ¹Henkie Isahwan Ahmad Mulyadi Lai,
¹Mohd Irfan Raimi Mustaffa, ¹Wan Khairuzzaman Wan Ramli and ²Patimah Ismail

¹Genomic Laboratory, Department of Medical Laboratory Science, Faculty of Biomedical and Health Sciences, Shah Alam City Campus, University Selangor, 40000 Shah Alam Selangor Malaysia

²Unit Molecular Biology and Cell, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 UPM Serdang Selangor, Malaysia

Abstract: Studies with Diethylstilbestrol (DES) in humans and rodents have resulted in a spectrum of toxic and carcinogenic effects. Previous findings on gene expression profiles following DES treatment showed that p53, p21 and bax was transcriptionally regulated in this model. In the present studies, we used Reverse Transcriptase *in situ* Polymerase Chain Reaction (RT *in situ* PCR) and immunohistochemistry techniques for localisation and expression of p53, p21 and bax at cellular levels. Animal were housed individually and treated with 500µmole/kg b.w of DES, (ip) once daily up to four days. Our results have shown, the expression of p53, p21 and bax mRNA were greater in wild-type compared to p53^{+/−} knockout mice. In addition, p53, p21 and bax mRNA were significantly high in DES- treated compared to control-vehicle animals. Collectively, similar patterns of expression also were seen in p53 and p21 proteins and scored according to the percentage of positive nuclear staining. Therefore, the combination of p53 and p21 were concluded to be a good prognostic marker for development of carcinogenesis.

Key words: Diethylstilbestrol • p53 • p21 • mRNA • RT *in Situ* PCR

INTRODUCTION

Diethylstilbestrol is an orally active synthetic estrogen [1] that possesses potent estrogenic activity. First synthesized in 1938, Diethylstilbestrol (DES) was prescribed to women with risk of miscarriages in pregnancies. However in 1971, DES was banned from use after clear evidence of an increase risk in developing rare cancer known as clear-cell adenocarcinomas of the vagina and cervix of daughters exposed to DES *in utero* [2-4]. At the time, it was estimated approximately two to three million people were exposed to DES worldwide [5]. Previous studies had demonstrated the carcinogenicity of DES in rodents [6, 7]. Although DES is a known carcinogen in humans and rodents, the cellular and molecular mechanism responsible for the adverse effect of DES induces cancer has not been fully elucidated, though

both genotoxic and epigenetic effects have been characterized [8]. Research using cDNA expression arrays had proved that DES had up-regulated expression of 12 genes and down-regulated 8 genes in liver of DES treated mice. Among these genes, p53 and p21 showed the greatest changes and demonstrated significant different between the wild type and p53^{+/−} transgenic mice [9].

In this research, the wild-type (Normal) p53 plays an important role in cell cycle control, genomic stability, repair and apoptosis. The p53 gene encodes a nuclear phosphoprotein that functions as a transcription factor and a 'sensor' of DNA damage [10]. The wild-type p53 is located in the nucleus and expressed at a low concentration due to its relatively short half-life of only 20 minutes. The exposure of cells to DNA damaging agents initiate an increase in p53 protein levels which caused by an increase in p53 protein half-life by post-translational

Corresponding Author: Mohd Nazil Salleh, Faculty of Biomedical and Health Sciences, University Selangor, Shah Alam City Campus, 40000 Shah Alam, Selangor, Malaysia.
Tel: (+603) – 5522 3471, Fax: (+603) – 5522 4323.

stabilization and an increase in p53 protein translation [11, 36]. Once p53 is activated it will cause cell cycle arrest [12] induce cell death through apoptosis [13]. p53 transcriptionally p21 which acts as a inhibitor of cyclin dependent kinases (CDKs) causing cell cycle arrest occurs in G1 phase, which allows the damaged DNA to repair genes [14]. If the DNA damage cannot be successfully repaired, p53 will initiate apoptosis with help of cell death genes. The p53 role as a tumour suppressor is exemplified by using p53^{-/-} knockout mice which only has one functional copy of the wild-type allele of the p53 tumour suppressor gene while a wild-type mouse has two functional p53 alleles. p53 knockout mice demonstrated a delayed initiation of cell cycle arrest or apoptosis compared to wild-type mice, indicating the presence of a p53-dependent pathways [15, 16].

In present studies, both the mRNA and protein level of p53 and p21 genes are investigated in hepatocytes of wild type and p53^{-/-} transgenic female mice following DES treatment.

MATERIALS AND METHODS

Animals: Wild type mice (C57BL/6J) and p53^{-/-} transgenic mice (C57BL/6J) (n=15) treated with 500 µmole/kg of DES intraperitoneally, once daily for 4 days. Controls were treated with trioctanoin vehicle only. Liver were obtained, formalin fixed and paraffin embedded. Samples were sectioned from liver tissue blocks at 5µm thickness and placed on glass slides. Each sample was done in triplicate. The cut sections were also stained with H&E for histology studies.

RT *in situ* PCR: The alteration of gene expression in response to DES is evaluated by using Reverse Transcriptase *in situ* Polymerase Chain Reaction (RT *in situ* PCR) which allows detection of low copy of mRNA in cellular level rapidly and sensitively. To elucidate these implications of genes for apoptosis and cell cycle control, the localisation of mRNA expression of the p53 and p21 in hepatocytes are done by combining the RT *in situ* PCR and immunodetection technique and compared between wild-type and p53^{-/-} knockout mice following DES administration. **One-step RT *in situ* PCR assay:** A modified one-step RT *in situ* PCR approach was performed using the techniques of Nuovo [17]. *In situ* synthesis and amplification of cDNA were performed in one step using the EZ recombinant Thermus thermophilus (rTth) DNA polymerase (Applied Biosystems). Each slide was loaded with 50 µl of

a RT-PCR mixture: 22.0µl DEPC-ultra pure water, 10.0 µl 1X EZ buffer, 1.5 µl dNTP for each dGTP, dATP, dTTP and dCTP (300µM), 1.5 µl for each forward primer and reverse primer (Table 1), 1.5µl 2% BSA, 0.5 µl digoxigenin-11-dUTP, 2.0 µl of 5 Units rTth DNA polymerase and 5.0 µl of 2.5 mM Mn(Oac)₂. All the RT-PCR reagents and reaction were kept on ice. The slides were covered with *in situ* Frame (Eppendorf Mastercycler) after assembly at 95°C for 3 min and placed on *in situ* Adapter in thermocycler (Eppendorf Mastercycler). The Reverse Transcription was performed on 60°C for 30 min. The PCR was performed with an initial denaturation at 94°C for 2 min followed by 25 cycles of 94°C for 45 sec, 58°C for 40sec, 72°C for 1 min. A final elongation was performed at 72°C at 7 min. It was cooled at 4 °C at 10 min and preceded to immunodetection technique. **Two-step RT *in situ* PCR:** *In situ* Frame was placed on slides before the *in situ* RT started. The slides were covered with 33 µl of mixtures containing 3 µl random hexamer and 30 µl of sterile DEPC treated ultrapure water and then incubated at 70°C heat block for 5 min. Eventually, the slides were loaded with RT reagent mixture which containing 12 µl of 5X reaction buffer, 16µl of 10mM dNTP mix, 3 µl of ribonuclease inhibitor, 3 µl sterile DEPC treated water and 3 µl of MMLV Reverse Transcriptase enzyme. After incubation at 37°C for 1 h, the reaction was terminated by incubation at 70°C for 10 min. *In situ* Frame was gently removed and slides were washed with DEPC-treated water for 3 times. Then the slides were preceded immediately to PCR step. For PCR, 50µl of PCR mixture (25 µl of DEPC treated water, 10 µl of 10X PCR buffer, 6 µl of 25 mM MgCl₂, 2 µl of 10 mM dNTP, 2 µl of 2% BSA, 1 µl of forward primer and 1 µl reverse primer, 1 µl of 1mm digoxigenin-11-dUTP and 2 µl of Taq polymerase 5U) were placed on each section. After that, the slides were sealed under *in situ* Frame and placed in the thermocycler. The PCR was performed according to standard PCR procedure. The expression of GAPDH mRNA was used as an internal control.

Immunodetection: After RT-PCR process, the *in situ* Frame was removed and the slides were flooded with PBS twice for 5 min. Prior to immunodetection of PCR products, the endogenous peroxidase in liver tissue was blocked by incubation in 6% hydrogen peroxide in absolute methanol for 30 min in dark humid chamber and followed by rinsing with PBS in Tween 20 for 2 min. The slides were treated with blocking goat serum for 15 min in humid chamber. The slides were flooded with PBS in Tween 20 for 2 min and then washed slides for 10 min with PBS in Tween 20. The digoxigenin-labeled PCR

products were detected by incubation with 30 μ l anti-digoxigenin-POD 150 U/ml diluted 1:80 in 100mM Tris-HCl, 150 mM NaCl, pH 7.5 for 30 min at room temperature in humid chamber. The slides were washed in PBS in Tween 20 and in deionised water. The sections were developed for 10-20 min at room temperature with 100 μ l DAB chromogen. The slides were kept in dark and monitored at intervals until brown color developed in dark humid chamber. Again, the slides were flooded with PBS in Tween 20 and were then rinsed in distilled water. Counterstained with Hematoxylin and covered with coverslips, the slides were viewed by light microscope and analysis of the image was carried out.

Immunohistochemistry: Sections were deparaffinized and rehydrated using standard procedure. The sections were brought to incubation for 10 min at RT with 3% hydrogen peroxide in methanol for blocking endogenous peroxidase activities. The sections were then incubated with 3% bovine serum albumin (BSA) as primary antibody diluents up to 30 min to block non specific antigen sites. Immunohistochemical staining was performed using streptavidin peroxidase procedure (Goat and Rabbit ImmunoCruz Staining System) to detect p53 and p21 protein. The negative control sections were subjected to the same procedures except by replacing the primary antibody with non-specific goat or rabbit IgG. The primary anti-p53 mouse polyclonal antibody (Santa Cruz) and anti-p21 rabbit polyclonal antibody (Santa Cruz) were used in the study.

Scoring

Immunohistochemistry: The nuclear immunoreactivity of p53 and p21 were classified into four grades with a uniform scoring system according to the number of

positive cells, i.e., negative staining (0-5%); 1+, weak staining (6-20%); 2+, moderate staining (21-50%); 3+, strong staining (>50%)

RT *in situ* PCR: All the specimens were tested for the presence of p53, p21 and GAPDH mRNA by one-step RT *in situ* PCR or by standard RT *in situ* PCR. Brown color staining in perinuclear cell represents a positive signal with DAB chromogen. It was identified by microscopic examination of the tissue sections. The intensity of reaction was variable, therefore a scoring system was used to score as follows: 0 (No signal); 1+ (Mild intense); 2+ (Moderate intense) and 3+ (Strong intense). The distribution of reactivity within the target cell population on the mRNA reaction was recorded as follows: 0 (No cells positive), 1+ (1-25% of cells positive); 2+ (26-50% cells positive); 3+ (51-75% cells positive) and 4+ (75-100% cells positive)[18].

RESULTS

The wild type and p53^{-/-} knockout control mice revealed normal architectures in the centrilobular region and periportal area in the liver parenchyma. Contrary, liver sections from the p53^{+/-} knockout and wild-type DES-treated mice hepatocytes underwent degenerative changes and inflammation in the periportal region. In addition, the treatment also induced hepatocellular necrosis in hepatocytes of p53^{+/-} knockout DES-treated mice. (Figure 1A & 1B).

In wild-type mice, the p53 mRNA was expressed greater in DES-treated compared to control mice with more than 75% of positive cells with strong intense were observed in DES-treated mice. p53^{+/-} knockout DES-treated also showed greater expression of p53 mRNA compared

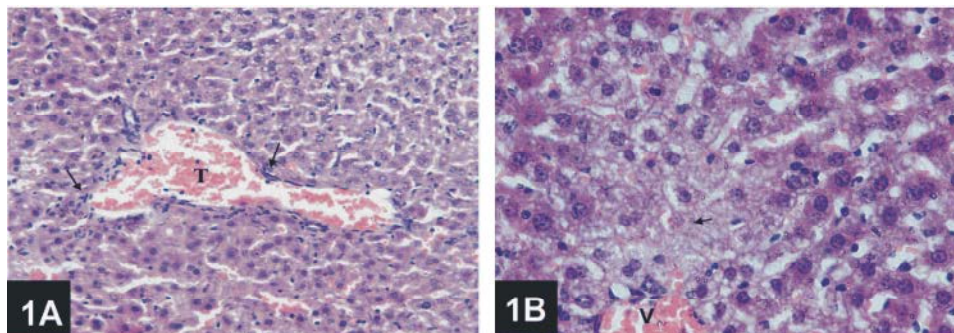


Fig. 1: Representative slides for liver histology studies using H&E stain: A) wild-type DES-treated mice showing hepatocytes underwent focal inflammation in the periportal region. (T= Portal tracts) (200x). B) p53^{+/-} knockout DES-treated mice showing hepatocytes underwent focal necrosis in the liver parenchyma. (V = Centrilobularvenule) (400x).

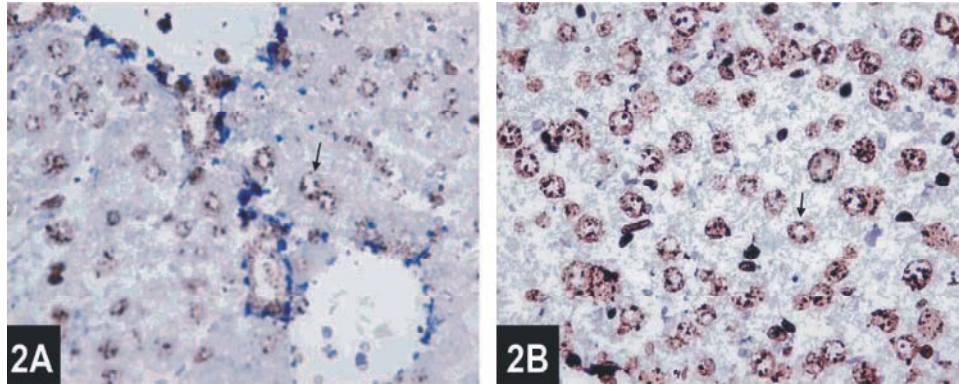


Fig. 2: RT in situ PCR detection of p53 mRNA in hepatocytes of: A) DES-treated p53^{+/-} knockout mice (400x). B) DES-treated wild-type mice (400x).

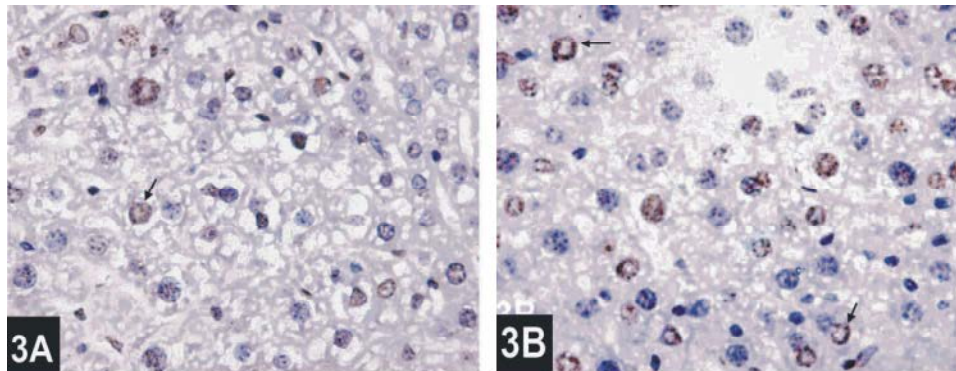


Fig. 3: RT in situ PCR detection of p21 mRNA in hepatocytes of: A) DES-treated p53^{+/-} knockout mice (400x). B) DES-treated wild-type mice (400x).

to control mice with moderate intense stain were observed in 25-50% of positive cells (Figure 2A & 2B). Both the wild type and p53^{+/-} knockout control mice only showed mild intense brown stain in 25-50% of positive cells.

Approximately 50-75% of p21 mRNA was strongly expressed in wild-type treated-mice. However, the localization of p21 mRNA expression in p53^{+/-} knockout DES-treated was lower compared to wild-type mice with a moderate brown signal evident in 25-50% of the p21-positive cells. (Figure 3A & 3B) The wild type control mice express mild staining in 25-50% of positive cells while the p53^{+/-} knockout control mice displayed low expression of p21 mRNA with mild intensity in least than 25% of cells.

Expression of nuclear p53 proteins based on intensity of immunoreactivity was higher in treated mice compared to control mice and its intensity was slightly increased in wild-type mice compared to p53^{+/-} knockout mice. For p53^{+/-} knockout DES-treated, moderate nuclei staining (2+ staining) were observed in liver parenchyma

accompanied by moderate cytoplasmic staining predominantly in periportal and centrilobular region extended into mid zonal region. Expression of p53 proteins was slightly higher in wild-types treated mice with diffuse positive nuclear staining (3+ staining). No expression of p53 nuclear protein observed in control p53^{+/-} knockout mice. Cytoplasm staining was limited to the periportal region. However, positive nuclear staining was observed in the control wild-type but only in scanty cells (1+ staining) with weak staining in cytoplasm of centrilobular and periportal region (Figure 4A-D).

Examination of p53^{+/-} knockout DES-treated mice showed the moderate (2+ staining) expression of p21 nuclear protein accompanied by moderate cytoplasm staining. The cytoplasm staining was predominantly in capsular and periportal region. Slightly higher reaction was found in the wild-type treated mice (3+ staining) but with lower cytoplasm staining. However, for control groups, there was only mild expression of nuclear p21 proteins with light cytoplasm staining observed in both p53^{+/-} transgenic and wild-type mice (Figure 5A-D).

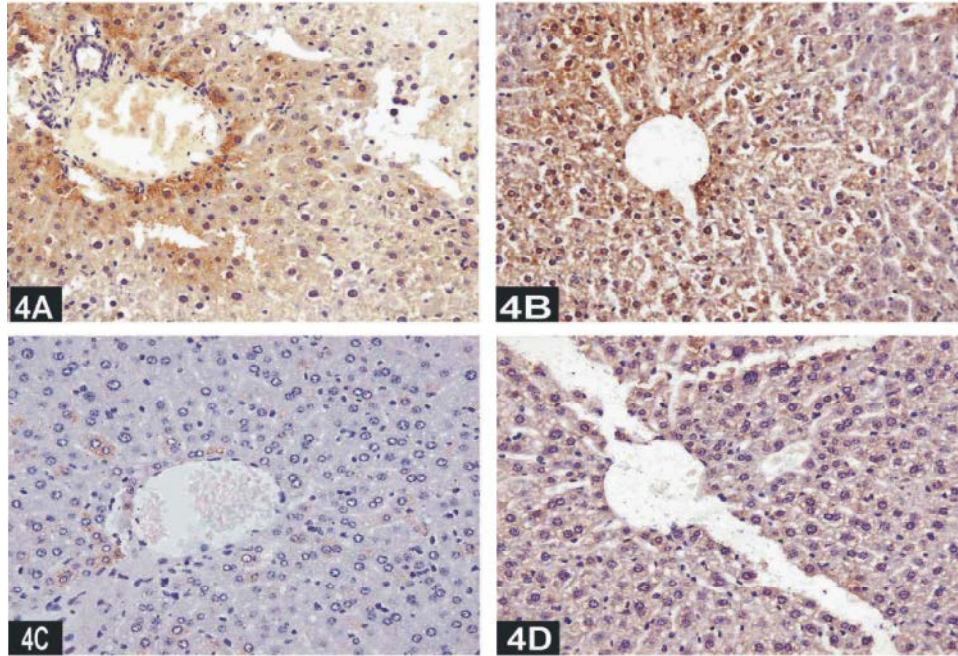


Fig. 4: Representative slides showing immunohistochemical staining for p53 protein in liver of: A) female p53^{+/-} knockout mice treated with DES, showing moderate expression of p53 nuclei proteins (200x). B) female DES-treated wild-type mice, showing diffuse p53 nuclei staining largely confined to periportal area (200x). C) female control p53^{+/-} transgenic mice showing no staining of p53 protein. Mild cytoplasm staining limited to periportal area (200x). D) female control wild-type mice, mild expression of p53 nuclear proteins predominantly in the periportal or centrilobular region (200x).

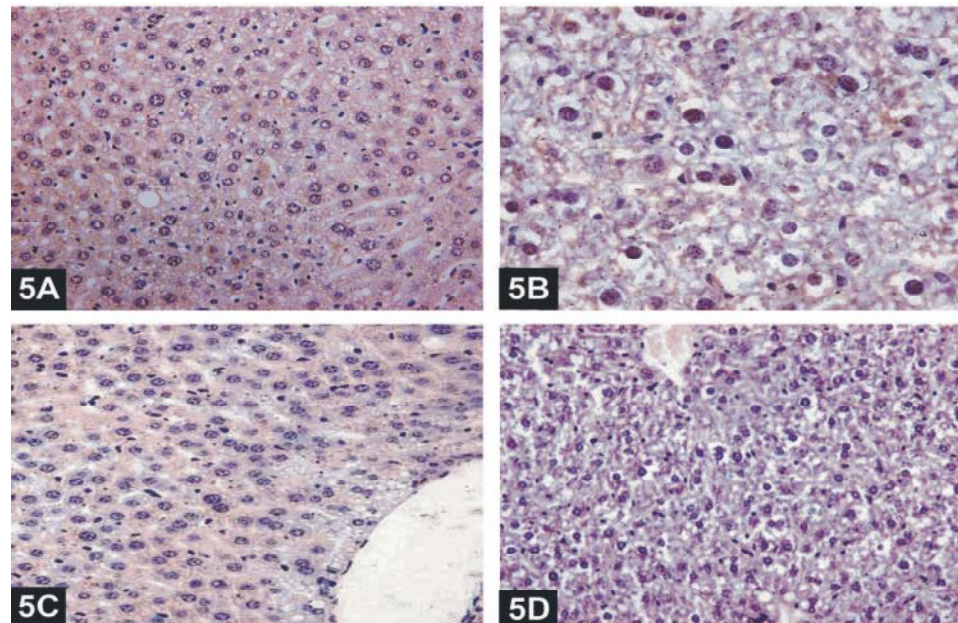


Fig. 5: Immunohistochemical staining for p21 protein in liver of: A) female DES-treated p53^{+/-} knockout mice, showing moderate p21 nuclear staining in the capsular and periportal area (400x). B) female DES-treated wild-type mice with diffuse staining of p21 proteins in over 70% of the hepatocytes (200x). C) control p53^{+/-} knockout mice with no detectable p21 staining in the liver parenchyma. (200x) and D) control wild-types mice, showing mild nuclear p21 staining (200x).

DISCUSSION

The present study offers clear evidence that localisation of p53 and p21 mRNA are expressed greater in wild-type compared to p53^{+/-} knockout mice. In addition, result also showed that mRNA of these genes are highly significant expressed in DES-treated compared to control-vehicle mice.

Previous study based on the RT-PCR analysis on hepatocytes of wild-type and p53^{+/-} knockout mice has established that p53 and p21 are induced and overexpressed by DES treatment [19]. Expression of p53 mRNA are higher than the expression of p21, suggesting that DES-induced DNA damage leads initially to p53 expression, as intense perinuclear accumulation of p53 have been observed in wild-type and p53^{+/-} knockout treated-mice in the study of Chang *et al.* [20]. The wild-type p53 proteins are expressed at low levels and in a latent state in normal cells. Overexpression of p53 may due to increased transcription of p53 mRNA, p53 mRNA stability and translation of p53 mRNA which may cause the loss of control in regulation of p53 protein. Since DES alters cell cycle kinetics, induce DNA damages and produce telomeric associations and chromosomal aberrations [21], it initiates an increase in p53 protein level as a result of the prolonged p53 protein half-life by post-translational stabilization and an increase in p53 protein translation [11]. The p53 expression precedes induction of p21 indicating that hepatocytes lead to cell cycle arrest via p53 transactivation [22, 23].

The loss of wild-type p53 tumour suppressor functions compromise the response of cell ability to genotoxic stress. p53^{+/-} knockout mice are used in the study to represent a true “loss of function” and this alteration is clearly sufficient to induce tumorigenesis [24, 25]. p53^{+/-} knockout mice model are very useful for clarifying the mechanism of organ-specific carcinogenesis in many experimental studies and clearly showed that p53 transcriptional activation is required for p53-mediated apoptosis. It explains the localisation of p53 and p21 are expressed greater in wild-type compared to p53^{+/-} knockout mice.

p21, a universal inhibitor of cyclin kinases has been identified to inhibit cell cycle progression through both G₁/S and G₂/M phases [26]. Study by Steward and Pietenpol [27] shows that p21 is induced by p53-dependent transactivation after exposure of normal cells to genotoxic agents. The elevated binds and inactivates cyclin D/Cdk4,6 and cyclin E/Cdk2 complexes resulting in cell cycle arrest. Results also show that DES mainly

induced a p53-dependent p21 expression because of the lower localisation of p21 mRNA expression was observed in p53^{+/-} knockout compared to wild-type mice [28].

Various forms of stress such as DNA-damaging drug rapidly induce a transient increase in p53 protein with showing little or no effect on p53 mRNA steady-state levels because of both enhanced p53 mRNA translation and p53 protein stabilization, which has a relatively short half-life [29]. Therefore, RT *in situ* PCR was used in present study to detect the low copy of mRNAs in cellular level with rapidly and high sensitivity [17].

Prior to immunohistochemistry study, expression of p53 and p21 proteins in the liver tissue are scored according to the positive nuclear staining. The staining was considered positive when there is brown colour found in nuclei of the hepatocytes. Staining of either p53 or p21 product in cytoplasm is not encounter as positive. This is because expression of this cytoplasmic product did not exert any changes in the growth of the parental cells. This can be support by recent findings which suggested that wild-type p53 affects the cell cycle only when localized in the nuclear cell compartment [30].

Based on staining results, p53 proteins are overexpressed in DES-treated mice compared to control mice. These findings indicate that the DES may induce genotoxic damage and has a significant impact on the expression of p53 proteins in the livers of DES-treated mice. The tumor suppressor p53 plays a central role in protection against DNA damage and other forms of physiological stress. Activation of p53 often results in cell cycle arrest, presumably to allow for DNA repair before replication or mitosis [31]. The p53^{+/-} transgenic mice which has one copy of the wild-type allele of the p53 suppressor gene and one copy of a null allele showed lower level of p53 protein expression in response to DES treatment. This suggest p53 protein play an important role in protect the cell from damage by genotoxic carcinogen. Lack of cellular protection in p53^{+/-} transgenic mice result in higher histological changes of the liver structure of p53^{+/-} transgenic mice compared to wild-type mice.

p21 protein is known to be a direct transcriptional target of p53 and is strongly induced by DNA damage in cells expressing wild-type p53. Induction of p21 expression could serve as an indicator for transactivation by p53, although p21 was also shown to be transactivated by p53-independent mechanisms [32]. Results show that DES had overexpressed nuclear p21 proteins in DES-treated mice compared to control mice. There is also slight attenuation of p21 protein expression observed in p53^{+/-} transgenic mice [33]. Likewise, cells derived from mice in

which p53 has been disrupted fail to induce p21 following UV radiation and are defective in cell cycle arrest after DNA damage [34]. The induction of p21 expression in wild-type p53-containing cells but not in p53^{+/-} containing cells by exposure to DNA-damaging agents (DES) supports the notion that p21 may be a critical effector of p53-mediated cell cycle control [35].

In conclusion, the transcriptionally of p53 and p21 at cellular levels combination will be used by many scientists as a model of early detection of prognostic in developing of carcinogenesis.

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