RESEARCH COMMUNICATION

# Defective Repair of UV-induced DNA Damage in Cultured Primary Skin Fibroblasts from Saudi Thyroid Cancer Patients

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## Abstract

**This study was conducted to examine the sensitivity of primary skin fibroblasts from Saudi thyroid cancer (TC) patients to ultraviolet (UV) irradiation. Cell survival was studied by a colony forming assay and DNA repair defects with a host cell reactivation (HCR) assay using UV-irradiated Herpes Simplex Virus (HSV). In addition, p53 gene expression was examined in the same TC cells exhibiting enhanced radiosensitivity. Skin fibroblasts from TC patients (n=4) showed significantly enhanced sensitivity to UV radiation. The average UV dose to reduce survival to 37% of the initial survival ($D_{37}$) value (in $Jm^{-2}$) for fibroblasts from TC patients was 4.6 (3.7-5.6) compared to 7.3 (6.3-8.3) for healthy individuals (n=3). UV-sensitive xeroderma pigmentosum (XP) cells, which were used as positive control, were found to be extremely sensitive with a $D_{37}$ value of 0.6 $Jm^{-2}$. In a host cell reactivation assay, UV-irradiated HSV was tested for its plaque-forming ability (PFA), by plating infected fibroblasts from TC patients (used as host cells) on African Green Monkey (Vero) kidney cells to form plaques. A significant reduction in the PFA of the UV-irradiated virus (about three fold) on TC cells compared to fibroblasts from the healthy subjects was seen, suggesting a DNA-repair deficiency in the primary fibroblasts of the TC patients. Furthermore, no significant accumulation in radiation-induced p53 expression was observed in cells from the TC patients. Our results, based on a relatively small group of subjects, indicate that Saudi TC patients primary fibroblasts (non-cancerous in nature) may be carriers of cancer-susceptible gene(s) arising from defective DNA repair/processing. These results warrant a larger study to investigate the role of UV-induced bulky DNA damage in thyroid cancer susceptibility.**

**Key Words**: DNA damage - DNA repair - ultraviolet - colony forming assay - thyroid cancer - HSV.

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## Introduction

The integrity of cellular DNA is greatly compromised due to a variety of DNA lesions produced by various endogenous and exogenous factors. UV light induces primarily cyclobutane pyrimidine dimers and pyrimidine (6-4)pyrimidone dimers along with small oxidized nucleotides such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Zhang et al., 1997; McKay et al., 1999). Most of these photolesions are repaired by two major excision pathways (Hoeijmakers and Bootsma 1994; Freidberg et al., 1995). The bulky lipophilic adducts from polynuclear aromatic hydrocarbons as well as pyrimidine dimers and 6-4 photoproducts from UV-irradiation are generally repaired by the nucleotide excision repair (NER). However, 8-OHdG, being a small-modified base, is mainly repaired by base excision repair (BER) (Dianov et al., 1998). Further, NER is comprised of two sub-pathways, one being the rapid and efficient transcription-coupled repair (TCR) while the other pathway is relatively slower global genome repair (GGR), which efficiently works for the bulky adducts and non-transcribing strand of active genes.

Cellular response to irradiation may be greatly altered by the presence of various genes responsible for the deficiencies in DNA repair and cell cycle regulation. Tumor suppressor gene (p53), the most commonly altered gene in cancer (Hollstein et al., 1991), and p53-regulated gene products have been linked with the repair of UV-induced

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DNA damage in both human and rodent cells (Smith et al., 1995; Yuan et al., 1995; Ford and Hanawalt, 1997; Huang et al., 1998). Exposure to genotoxic agents, including UV light, lead to dose-dependent accumulation of p53 through post-transcriptional mechanisms. Removal of a variety of DNA damage including the UV-induced photolesions and bulky adducts from chemical carcinogens in mammalian cells can be influenced by p53 level in the cells (Ford and Hanawalt 1997; Wani et al., 2000). Further it has been shown that removal of these bulky DNA lesions by NER occurs non-randomly across the genome and in strand-specific manner (Hanawalt, 1998). Loss or disruption of wild-type p53 function has been generally correlated with decreased GGR, however, its involvement in TCR is more controversial (Ford and Hanawalt, 1997; Smith and Fornace, 1997). Furthermore, it has been suggested that p53 participation in GGR and the recovery of mRNA synthesis following UV exposure may be through regulation of steady-state levels of p53-regulated gene products important for cellular processes (McKay et al., 1999).

Certain inherited cancer-prone disorders like UV-sensitive xeroderma pigmentosum (XP) and ionizing radiation sensitive ataxia-telangiectasia (A-T), suggest a link between cancer susceptibility and radiosensitivity (Lehman, 1982). Most of the cancer susceptibility genes identified to date result in an impairment of DNA repair and cell cycle processes that may increase genomic instability, leading to carcinogenesis (Fearon, 1997; Maser and Depinho, 2002). Earlier we have shown that defective DNA repair/processing may occur in the cultured primary skin fibroblasts of cancer patients, particularly those with carcinoma of the breast (Hannan et al., 2001).

The thyroid gland is known to be very sensitive to ionizing radiation as demonstrated by the epidemiological studies on the population exposed to high dose radiation (Sarasin et al., 1999). Earlier, we had reported that primary skin fibroblasts from thyroid cancer (TC) patients exhibited enhanced sensitivity to gamma radiation as well as a moderate radioresistant DNA synthesis (Ahmed et al., 1999). Further, it has been reported that UV radiation induced apoptosis in the rat thyroid FRTL-5 cell strain (Del Terra et al., 2001). However, not much is known about the effect of UV irradiation on the primary skin fibroblasts from TC patients. In the present study, we examined the primary skin fibroblasts of TC patients exposed to the UV irradiation for cell survival by a colony forming assay and DNA repair defects by a host cell reactivation (HCR) assay. In addition, the p53 gene expression was also studied in the same cells exhibiting enhanced radiosensitivity.

## Materials and Methods

*Cell Culture, Virus and Reagents*

Herpes simplex virus (HSV) type 1 (F strain) and African Green Monkey kidney cells (Vero) were purchased from American Type Culture Collection (Rockville, MD, USA). All the cell culture reagents and media were obtained from Sigma Chemical Company (St. Louis, MO, USA). Tissue culture flasks and the petri dishes were obtained either from Becton and Dickinson, (Franklin Lakes, NJ, USA) or NUNC (Denmark).

Vero cells were propagated in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 IU) and streptomycin (100 μg/ml). The virus stock was prepared by infecting Vero cells with HSV-1 and after 48 h incubation, cells were lysed by two cycles of freezing and thawing. Lysed cell suspension was centrifuged at 1500 g for 5 min at 4°C and the supernatant was aliquoted and kept frozen at –80°C. All the immunochemicals were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA).

Four Saudi patients (1 male and 3 females; aged 36-75 years) with confirmed papillary carcinoma (differentiated thyroid carcinomas) and 3 normal healthy Saudi subjects (1 male and 2 females; aged 26-67 years) were recruited into the study after obtaining informed consent. Approval from the Research Advisory Council of King Faisal Specialist Hospital and Research Center was obtained before commencing this study. Fibroblast cell strains were developed from gluteal skin biopsies from the TC patients and grown to confluence using Ham's F-12 medium with 15% fetal bovine serum, penicillin (100 IU) and streptomycin (100 μg/ml). These cells from the TC patients also happened to be gamma-radiation sensitive (Ahmed et al., 1999).
Skin fibroblast strain exhibiting the classical XP syndrome was used as a positive control and was developed from the skin biopsy of a Saudi XP patient belonging to the XP-A group in a complementation analysis (Hannan et al., 2002).

*Cell Survival Analysis*

A colony forming assay (CFA) was used to determine the cell survival curves as described previously (Hannan et al., 2001). Briefly, the fibroblasts (passages 10-15) grown to confluence were exposed to UV (254 nm) irradiation (0-12 $Jm^{-2}$) from a germicidal UV lamp at the rate of 1.1 $J/m^2/s$. The UV dosimetry was performed using an ultraviolet meter (Spectronics Corporation, NY, USA). UV-sensitive XP cells were used as positive control. Following an overnight incubation at 37°C in a humidified atmosphere using 5% CO2: 95% air, cells were trypsinized, washed and resuspended in fresh medium. Aliquots from appropriate dilutions of the cell suspensions were assayed for colony-forming ability in the dishes containing primary human fibroblasts (60,000 cells) inactivated with gamma rays (50 Gy) as feeder cells. After 3 weeks of incubation with a weekly change of medium, cells were washed with normal saline, stained with crystal violet, and the colonies with ≥50 cells were counted. Survival curves for each cell strain were constructed by determining plating efficiencies (25-30%) and calculating the percent colony formation after each dose of UV irradiation compared to the respective unirradiated control. All cell survival curves were analyzed by the method of Tarone et al. (1983) as described in detail in our earlier publication (Hannan et al., 2001).

*Host Cell Reactivation (HCR)*

This procedure, which is also known as infectious center assay, was performed to detect DNA repair deficiency in the cultured fibroblasts (Abrahams et al., 1984; 1998). The technique was described in detail in our previous publication (Hannan et al., 2001). Briefly, monolayers of cultured fibroblasts from healthy subjects and TC patients growing in 35 mm tissue culture dishes were infected with UV irradiated (50-150 $Jm^{-2}$) and non-irradiated HSV-1 for a time period of 90 minutes at 37°C. The UV-sensitive XP cell from Saudi XP patient was used as a positive control. Infected cultures were re-incubated for 90 minutes after the addition of 2 ml of complete medium, which is referred to as liquid holding condition (Abraham et al., 1984). The medium was aspirated and monolayers were washed with ice-cold phosphate buffered saline (PBS) (pH 7.4) and trypsinized in a mixture of trypsin (0.25%) and EDTA (0.02%). Cells were mixed with Vero cells in a ratio of 1:2000 and 60 mm dishes were seeded in triplicate at a density of $2.5x10^6$ ($2x10^5$ cells/ml) per dish in EMEM containing 10% fetal bovine serum. After 5 h incubation, the medium was removed and agar overlay (Siddiqui and Al-Ahdal, 1993) was added to these dishes and incubated for 3-4 days. The multiplicity of HSV infection was adjusted in such a way that the trypsinized cells when mixed with the permissive cell line (Vero) results in a countable number (80 $\pm$ 20) of plaques. It was calculated to be 10 virus particles per cell. The plaques were counted from the dishes containing irradiated or un-irradiated virus by adding 0.003% neutral red solution. The plaque counts from the dishes containing 30-80 plaques were used in statistical analysis and percentage survival curves were drawn using this data.

*p53 Analysis*

For p53 expression, monolayer fibroblast cultures from healthy subjects and TC patients were exposed to UV (254 nm) at a dose of 12 $Jm^{-2}$ and, after 4 h of incubation, medium was removed and the monolayers were washed in PBS. Cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.4 / 250 mM NaCl / 1.5 mM MgCl2 / 1% Triton-X-100 / 0.25% sodium deoxycholate and protease inhibitor cocktail) and after centrifugation, the supernatant containing 30 µg of protein (determined by Bio-Rad protein assay reagent) was mixed with monoclonal antibody against p53 for a period of 8-12 h. The Ag:Ab complex was removed by protein-A Sepharose (Pharmacia, Uppsala, Sweden), eluted in SDS-sampling buffer and run on 10% acrylamide gel. After fixing the gel in 10% trichloroacetic acid, polypeptide bands were stained with coomassie blue.

The p53 expression was also confirmed by immunoblotting procedure. The lysates were run in SDS-PAGE, transblotted onto PVDF membrane (Amersham Biosciences, UK) and probed with monoclonal antibody against p53. Blots were incubated with anti-mouse IgG-labeled with horseradish peroxidase. After an extensive washing, the chemiluminiscent substrate was added and the signal was captured on Kodak Biomax films. The autoradiograms were scanned using Biorad GS-800 calibrated densitometer and analyzed by QuantityOne software. Equal protein loading was confirmed by β-actin as a standard.

*Statistical Analysis*

The data for CFA and HCR represents the mean $\pm$ SD from two independent experiments. The p values were calculated by student t test and $p<0.05$ considered significant when compared to the controls.

## Results and Discussion

Figure 1 depicts typical survival curves resulting from UV exposures of cultured skin fibroblasts from the TC patients. It shows significantly ($p<0.05$) enhanced sensitivity to UV irradiation (up to 12 $Jm^{-2}$) in a dose-dependent manner compared to those from (normal) healthy individuals. The $D_{37}$ (UV dose resulting in 37% survival) values (in $Jm^{-2}$) for TC skin fibroblasts (n=4) was 4.6 (3.7-5.6) compared to those from (normal) healthy individuals (n=3) showing $D_{37}$ values of 7.3 (6.3-8.3). On the other hand, the well-known UV-sensitive XP cells, used as positive controls, were found to be very sensitive with a $D_{37}$ value of 0.6 $Jm^{-2}$. The host cell reactivation assay detected the impaired DNA-repair ability in fibroblasts from TC patients when infected with UV-irradiated herpes simplex virus. There was a significant reduction ($p<0.05$) in virus recovery (number of plaques) in fibroblasts from TC patients when compared to the normal subjects (figure 2). This reduction in the viral reactivation in radiosensitive TC patient's fibroblasts indicated a possible repair deficiency in these cells for the UV irradiation-induced DNA damage in the virus. Earlier we reported similar

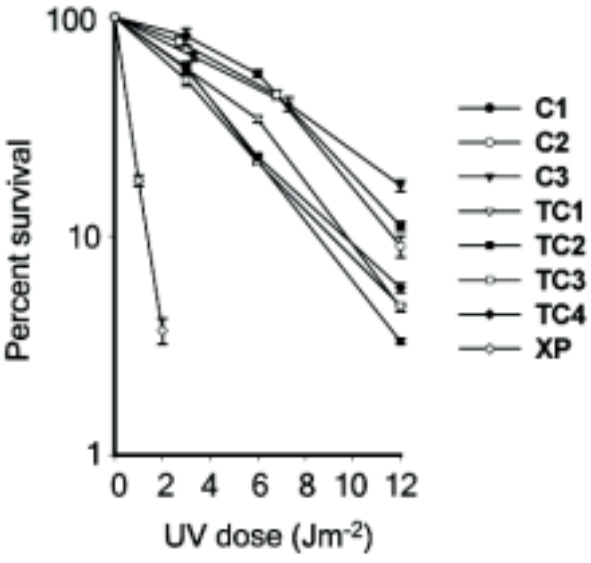


**Figure 1. Fibroblast Cell Survival Curves, After Low dose UV Irradiation (up to 12 $Jm^{-2}$), for Normal Healthy Subjects (C) and Thyroid Cancer Patients (TC).** Each data point represents mean $\pm$ SD from two independent experiments.

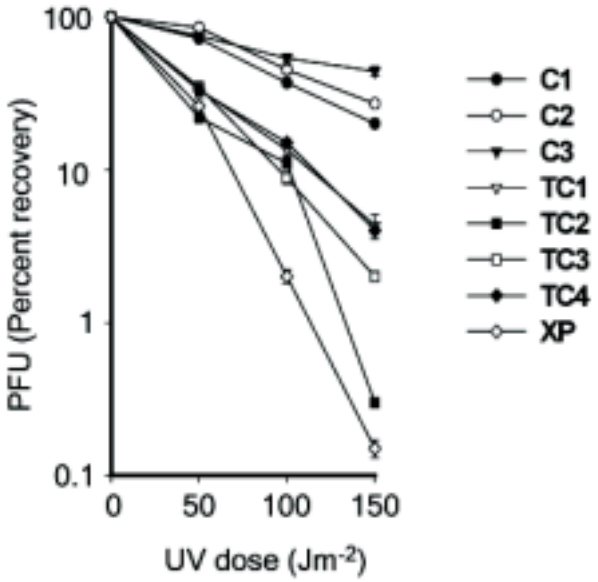


**Figure 2. Host Cell Reactivation (HCR) Assay Showing the Plaque-forming Virus (HSV-1) After Irradiation with UV (50-150 $Jm^{-2}$) Following Adsorption into Fibroblasts from Normal Healthy Subjects (C) and Thyroid Cancer Patients (TC).** Each data point represents mean ± SD from two independent experiments. PFU represents the plaque-forming unit.


**Figure 3. Expression of p53, by Immunoprecipitation and Western Blotting, in Primary Skin Fibroblasts from Normal Healthy Subjects (C) and Thyroid Cancer Patients (TC) with and without UV Irradiation (12 $Jm^{-2}$). Letter 'R' Represents UV Irradiated.** Equal amount (30 μg) of protein, confirmed by β-actin as standard, was loaded in each lane.

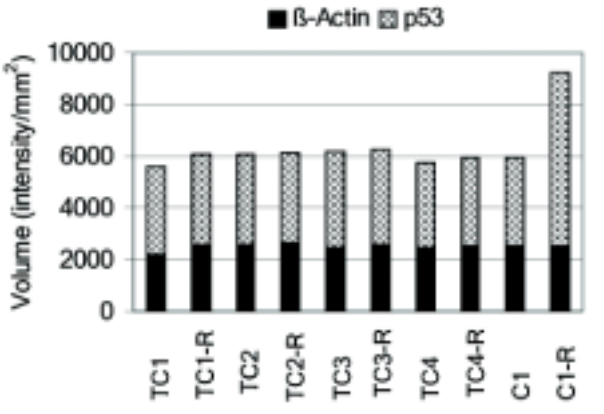


**Figure 4. Levels of β-actin and p53 in Primary Skin Fibroblasts from TC Patients and Normal Healthy Subjects with and without UV Irradiation (12 $Jm^{-2}$).** The autoradiograms were scanned using Biorad GS-800 calibrated densitometer and analyzed by QuantityOne software.

findings in the primary skin fibroblasts from breast cancer patients, after gamma irradiation (Hannan et al., 2001). Similar observations were also made when the fibroblasts from TC patients were used in the HCR assay, where HSV-1 was exposed to ionizing radiation at doses of 0, 250, 500 and 1000 Gy (data not shown). It is interesting to note that the primary skin fibroblasts from Saudi TC patients are in fact defective in repair of both UV and ionizing radiation derived DNA damage. Although, A-T cells are known to have enhanced sensitivity to the killing effects of ionizing radiation and defective DNA repair, we recently reported that the skin fibroblasts from Saudi A-T patients are also unexpectedly sensitive to UV irradiation with moderate radioresistant DNA synthesis and a reduced recovery of UV-damaged virus in the HCR assay (Hannan et al., 2002). Moreover, we also showed that these UV-sensitive A-T cells were compromised for the ATM gene and found to be somewhat deficient in the excision of cyclobutane pyrimidine dimers in non-replicative conditions.

p53 has been shown to play a role in removal of a variety of DNA damage, including UV-induced photolesions and bulky adducts arising from chemical carcinogens (Ford and Hanawalt, 1997; Wani et al., 2000). Further, p53 conferred, at least in part, its antineoplastic effect by enhancing the DNA repair, promoting cell cycle arrest and/or facilitating apoptosis (Levine, 1997; McKay et al., 1999). UV exposure generally led to dose-dependent accumulation of p53 in cells by post-transcriptional mechanisms (Hall et al., 1993; Lu and Lane, 1993; Ko and Prives, 1996). In the present study, we noticed no significant accumulation of p53 in the UV-irradiated primary skin fibroblasts from TC patients (Figure 3), suggesting that these primary fibroblasts may be compromised of p53 accumulation. On the other hand, the normal fibroblasts from healthy control subjects showed an increased accumulation (~2-fold) of p53 following UV irradiation (Figures 3 and 4). Furthermore, UV exposure remarkably enhanced p53 level in both normal and A-T cells indicating that these cells are not compromised for UV-mediated p53 induction (Hannan et al., 2002). From our preliminary results, significantly decreased DNA repair with no accumulation of p53 in the primary skin fibroblasts from TC patients seems to indicate that these cells may be defective in repair of UV-induced DNA damage.

The possibility that cancer patients may carry the defective genes/functions compromising both DNA repair and post-irradiation DNA processing seems to be applicable for these TC patients, which is also true for the Saudi A-T patients. Since most of the cancer susceptibility genes discovered to date have been linked to the DNA repair and/or cell cycle checkpoint defects (Fearon, 1997; Maser and Depinho, 2002), the enhanced radiosensitivity in the TC patients primary fibroblasts resulting from the DNA damage and/or repair defects could be responsible for genomic instability, which in turn led to the susceptibility towards the malignant diseases. Further, our findings on the unexpected sensitivity of primary fibroblasts from Saudi A-T and TC patients to both UV and ionizing radiations

warrants a detailed study to investigate the underlying cellular mechanism(s) for their relative susceptibilities to the respective malignancies.

## Conclusions

Although based on a relatively small number of subjects, our results from this study led us to conclude that Saudi TC patient's normal body cells may be carrier of cancer—susceptible genes with defective repair and/or cell cycle regulation. Further studies are required to study the DNA damage profile as well as the expression of various DNA repair and cell cycle controlling genes in the body cells from TC patients, which may help in identifying the cancer-prone individuals and the defective genes underlying their susceptibility to malignancies. These studies could further help identify etiologic agents that could induce TC.

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