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Allele-Specific Wild-Type *TP53* Expression in the Unaffected Carrier Parent of Children with Li-Fraumeni Syndrome

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Abstract

Li-Fraumeni Syndrome (LFS) is an autosomal dominant disorder where an oncogenic *TP53* germline mutation is passed from parent to child. p53 is a key tumor suppressor regulating cell cycle arrest in response to DNA damage. Paradoxically, some mutant *TP53* carriers remain unaffected, while their children develop cancer within the first few years of life.

To address this paradox, response to UV stress was compared in dermal fibroblasts (dFb) from an affected LFS patient vs. their unaffected carrier parent. UV induction of *CDKN1A/p21*, a regulatory target of p53, in LFS patient dFb was significantly reduced compared to the unaffected parent. UV exposure also induced significantly greater p53[Ser15]-phosphorylation in LFS patient dFb, a reported property of some mutant p53 variants.

Taken together, these results suggested that the unaffected parent dFb may express an increased proportion of wild-type vs. mutant p53. Indeed, a significantly increased ratio of wild-type to mutant *TP53* allele-specific expression in the unaffected parent dFb was confirmed by RT-PCR-RFLP and RNA-seq analysis.

Hence, allele-specific expression of wild-type *TP53* may allow an unaffected parent to mount a response to genotoxic stress more characteristic of homozygous wild-type *TP53* individuals than their affected offspring, providing protection from the oncogenesis associated with LFS.

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Conflict of interest

The authors declare no potential conflicts of interest.

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Keywords

p53; Li-Fraumeni Syndrome; allele-specific expression; tumor suppression; UV stress

Introduction

The tumor suppressor, p53, has long been recognized as a major transcriptional regulator in the cellular response to environmental, genotoxic stress and programmed cell death or apoptosis. It is believed that over 50% of all cases of human cancer can be attributed to its acquired 'loss of function', with missense mutations of the *TP53* gene resulting in the inability to repair DNA damage, regulate cell division, or effectively eliminate cancer cells through programmed cell death [1]. Since p53 is so intimately involved in these activities, it is not surprising that acquired mutations have a profound effect on human disease and oncogenesis. Although mutations do arise spontaneously throughout the gene, in the majority of cancers the *TP53* mutations occur in the DNA binding domain of the gene, exons 4–8 [2]. Mutations in exons 5–8 of p53 comprise over 90% of all somatic or acquired mutations in tumors characterized thus far. Moreover, it is clear that the spontaneous occurrence of *TP53* mutations in tumors plays a critical role in cancer progression [2].

Although the most commonly observed *TP53* mutations are those arising spontaneously in tumor cells, there is also a rare, autosomal dominant disorder, Li-Fraumeni Syndrome (LFS), where the *TP53* mutation is present in the germ line, which can then be passed on from parent to child [3]. This disorder is associated with an inherited predisposition for the early onset of cancer in multiple family members throughout multiple generations. Carriers of this germ line mutation have a high cancer incidence rate, with 68% of males and 93% of females developing some form of cancer in their lifetimes. Compared to the general population, the average age for cancer onset is also much earlier in LFS patients. Whereas only 2% of cancer cases occur before age 30 and 11% before age 50 in the general population, those figures rise to 56% and 100% for LFS patients.

In contrast to studies of tumors with acquired p53 mutations, there is limited data on the prevalence of cancer in LFS family members who carry the same mutant *TP53* allele but remain unaffected by cancer [4,5]. Accordingly, the selective cellular and genomic regulatory factors that contribute to the absence of cancer in LFS carriers have also been largely ignored in this disease. And yet, why some individuals with *TP53* mutations go on to develop cancer and others do not is a question whose answer may hold profound impact on the future direction of cancer detection, targeted therapy, and ultimately patient survival [6, 7]. It is our intent to identify these differences between members of LFS families and characterize the elements which either protect carriers or promote oncogenesis in others.

For these reasons, we determined that LFS family studies with both affected and unaffected members would help to illuminate mutation-specific mechanisms, and gain a more complete understanding of possible intervention points to prevent oncogenesis. This strategy also takes advantage of the family members' otherwise comparatively similar genetic backgrounds. Several years ago, we began studying a highly informative family who has been deeply affected by a *TP53* mutation [8]. Despite carrying a *de novo*, constitutional

TP53 mutation, the father has never developed a malignancy. However, an additional informative event in his medical history was a diagnosis of aplastic anemia in his third decade of life, which was successfully treated with a fully matched sibling, allogeneic stem cell transplant. He remains tumor-free in his fourth decade of life. He was unaware of his *TP53* mutation until he was screened after his two children developed choroid plexus carcinomas before the age of 4. At that time, he was found to carry a *de novo* Ser241Tyr C>A mutation in exon 7 of *TP53*, and had passed this mutation on to his two children. The mother is negative for any *TP53* mutation.

Previous efforts to examine the impact of p53 mutations on downstream regulatory pathways have been performed in cell lines derived from tumors, murine knock-out or knock-in models, or comparison of fibroblasts or lymphocytes from patients with LFS [9]. The data derived from these experiments has been very useful for elucidating the impact of mutant p53 on cell cycle arrest and DNA repair. Therefore, we have established primary dermal fibroblast (dFb) cultures using tissue samples from both the affected LFS daughter and unaffected father from this family. The goal of this study was to compare the *in vitro* response of their dFb to UV stress [10, 11, 12]. These previous and the present studies all focus on response to UVC irradiation. While UVC radiation is not typically present in the terrestrial environment, experimental evidence suggests that UV radiation dose has a much greater impact on p53-mediated responses than its particular wavelength [13].

It was hypothesized that the unaffected father may mount a response to genotoxic stress more characteristic of homozygous wild-type *TP53* individuals than that of his affected heterozygous daughter. Comparing the genotoxic response profiles of these heterozygous *TP53* family members offered a rare opportunity to gain insight into the potential “protective” vs. oncogenic genomic factors involved in tumorigenesis. Understanding the mechanism of this protection may uncover critically important targets to develop new therapeutic agents for cancer and marrow failure syndromes [6, 7].

Materials and methods

Culture, irradiation, and harvest of human dermal fibroblasts

Proliferating cultures of human dFb were established from fresh LFS family tissue samples, as described [14]. Control dFb were derived from unrelated foreskin samples. Cultures were maintained in Dulbecco's modified Eagle's medium (D-MEM) containing GlutaMAX (GIBCO-ThermoFisher, Grand Island NY USA), supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins CO USA), non-essential amino acids (Irvine Scientific, Santa Ana CA USA), and Primocin antimicrobial reagent (InvivoGen, San Diego CA USA). Experiments were performed with uniform dFb cultures of equivalent passage, during their sixth through eighth passage in culture. All experimental replicates were biological from separate cultures in six-well plates.

For UV irradiation, dFb six-well plate cultures were immediately challenged by exposure to UVC (254 nm) radiation ($1\text{--}4\text{ mJ/cm}^2 = 10\text{--}40\text{ J/m}^2$) in a GS UV Chamber (BioRad, Hercules CA USA) after D-MEM aspiration, directly followed by D-MEM replenishment.

Total cellular RNA was isolated from confluent dFb cultures $\pm$ UV irradiation using the RNeasy Mini Kit (QIAGEN, Hilden DEU) and quantified using the RNA 6000 Nano Kit with a BioAnalyzer 2100 (Agilent, Santa Clara CA USA).

Cellular proteins from confluent dFb cultures $\pm$ UV irradiation were extracted in PathScan® Lysis Buffer (Cell Signaling Technology, Danvers MA USA), supplemented with additional phosphatase and protease inhibitors (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.5 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 30 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM β -glycerophosphate, 4 mM Na₃VO₄, 1 μ M okadaic acid, 3 μ g/ml leupeptin, 1 mM PMSF, 5 μ g/mL pepstatin A, 10 μ g/mL aprotinin). Cell extract protein content was determined using the modified Lowry DC Protein Assay (Bio-Rad).

Reverse-transcribed (RT)-qPCR mRNA expression

RT-qPCR (two-step) was performed using the iScript Advanced cDNA Synthesis Kit (BioRad) with 25 ng RNA/sample, followed by the SsoAdvanced Universal SYBR Green Supermix (BioRad) on a CFX Connect Real-Time PCR System (BioRad). Validated qPCR primers specific for human gene transcripts of *CDKN1A* (p21; qHsaCID0014498), along with *ACTB* (β -actin; qHsaCED0036269) and *B2M* (β_2 -microglobulin; qHsaCID0015347) reference standards, were utilized according to the manufacturer's specifications (BioRad). Reference gene-normalized relative gene expression was calculated based on C_q values by the CFX Manager 3.1 operational software (BioRad).

Immunoblot analysis

Protein extracts (30 μ g/lane) were electrophoresed on denaturing, SDS-polyacrylamide gels (10%, 1:37.5 bis:acrylamide, Bio-Rad), along with Precision Plus Kaleidoscope Protein molecular weight standards (Bio-Rad). Proteins were electroblotted onto low-fluorescence PVDF membranes (Bio-Rad,) using a MiniTrans-Blot Electrophoretic Transfer Cell (Bio-Rad), according to the manufacturer's recommendations. The electroblotted membranes were incubated with 1% casein in TBS (Bio-Rad) for 60 min at room temperature, followed by either 1:2000 anti-p21 (Cell Signaling Technology; #2946), 1:1000 anti-phospho-p53[S15] (Cell Signaling Technology; #9284), 1:500 anti-p53 (Santa Cruz Biotechnology, Dallas TX USA; sc-6243) or 1:5000 anti- β -actin (Santa Cruz Biotechnology; sc-47778) overnight at 4°C, and then with the corresponding horseradish peroxidase-conjugated goat anti-rabbit (Cell Signaling Technology; #7074) or – mouse (Santa Cruz Biotechnology; sc-2055) IgG for 2 h at room temperature. Membranes were washed with antibody binding buffer (0.05% casein in TBST) for 15 min at room temperature between incubations and with TBST (Santa Cruz Biotechnology) for 10 min x 3 before developing with Pierce ECL Plus chemifluorescent substrate (ThermoFisher, Rockford IL USA). Chemifluorescent signals were detected with a Typhoon FLA 9000 Laser Scanner using a 473 nm band pass filter (GE Healthcare, Pittsburgh PA USA). Optical densities of the fluorescent signals were then quantified using ImageQuant TL (GE Healthcare). Mean values were calculated from replicate immunoblots using signal sum normalization [15].

Antibody array analysis

Protein extracts (30 µg/well) were incubated on glass PathScan® Stress/Apoptosis Signaling Antibody Array slides, according to the manufacturer's recommendations (Cell Signaling Technology). Following subsequent incubation with the horseradish peroxidase-conjugate provided by the manufacturer, the glass array slides were developed with the Pierce ECL Plus chemifluorescent substrate (ThermoFisher). Chemifluorescent signals were detected with the Typhoon FLA 9000 Laser Scanner using a 473 nm band pass filter (GE Healthcare). Optical densities of the fluorescent signals were quantified using ImageQuant TL (GE Healthcare).

RT-PCR-RFLP analysis for *TP53* allele-specific expression

RNA samples (100 ng each) were reverse-transcribed using a First-Strand cDNA Synthesis Kit (GE Healthcare). A 94 bp region of exons 6–7, carrying the inherited Ser241Tyr C>A *TP53* mutation, was amplified using custom, intron-spanning primers; 5'-GGTGCCCTATGAGCCGCC-3' and 5'-CCGGTTCATGCCGCCcT-3' (IDT, San Diego CA USA). A mismatched substitution of cytosine for adenosine, represented as a lower case 'c', generated a PCR product with a derived, single *SbfI* restriction site [16] cleaved in the wild-type allele only. In the *SbfI* recognition sequence, 5'-C*CTGCAGG-3', C* represents the location of the inherited C>A *TP53* mutation, which is not cleaved when mutated, while the lower case 'g' indicates the mismatched primer-derived base pair required for *SbfI* recognition.

PCR amplification with FastStart Taq DNA Polymerase (Roche, Mannheim DEU) was carried out by incubating reactions for 5 min at 94°C, followed by 30 sec at 94°C, 30 sec at 60°C, and 1 min at 72°C for 40 cycles, and then for 7 min at 72°C, which was determined to be within the linear phase of exponential amplification (data not shown). The 94 bp PCR amplicons were then digested with *SbfI* (New England BioLabs, Ipswich MA USA) and the RFLP products quantified using the DNA 1000 Kit with a BioAnalyzer 2100 (Agilent). Expression of the wild-type vs. mutant *TP53* alleles in the heterozygous subjects was calculated by determining the molar ratio of the 75 bp *SbfI* RFLP product from the wild-type *TP53* allele vs. the uncleaved 94 bp product from the mutant allele for each sample.

RNA-seq for *TP53* allele-specific expression

RNA subjected to RNA-seq had an RNA Integrity Number (RIN) of at least 9.5, as determined by the RNA 6000 Nano Kit on a BioAnalyzer 2100 (Agilent). RNA samples (300 ng each) were ribo-depleted using the Ribo-Zero Gold rRNA Removal Kit (Epicentre, Madison WI USA). Sequencing libraries were then prepared from the ribo-depleted RNA using a NEBNext® Ultra Directional RNA Library Prep Kit for Illumina, according to the manufacturer's specifications (New England BioLabs). Libraries were loaded onto a HiSeq2000 (Illumina, San Diego CA USA) and sequenced for 100 bases, with a 7 base indexing read. Transcript sequences were aligned to the Hg19 human genome reference sequence using TopHat v2.0.13 [17] with Bowtie2 [18], and annotated using Partek Genomics Suite 6.6 (Partek, St. Louis MO USA). Finally, SAMtools/BCFtools [19] was used to generate variant reports for quantitating the *TP53* allele-specific expression ratios in the heterozygous subjects.

Statistics

StatMate2 (GraphPad Software, La Jolla CA) was used to assist in selecting appropriate sample replication. Data were presented as the mean $\pm$ standard-error-of-the-mean (SEM) for two or more samples. Probability of significant differences ($p < 0.05$) between two data sets was calculated using Student's *t*-test (two-sided) on InStat for Windows (GraphPad Software).

Results

***CDKN1A*/p21 mRNA up-regulation by UV exposure was not impaired in unaffected LFS fibroblasts**

In order to study response to genotoxic stress by dFb from the affected LFS patient compared to the unaffected carrier father or an unrelated control subject, their isolated dFb were exposed to UVC radiation, a well-characterized inducing agent of *TP53*/p53 [10] and *CDKN1A*/p21 [20], which is a key cell cycle regulatory target of p53. The UVC stress responses of both p53 [11] and p21 [20] have also been found to be markedly dose-dependent. Since cellular UV radiation responses are often dose-dependent [13] and it has been further suggested that lower doses induce a more p21-dependent DNA damage response [11, 21, 22], *CDKN1A*/p21 mRNA expression by these dFb was tested following UVC irradiation at fluences of 1, 2, and 4 mJ/cm² (10, 20, and 40 J/m², respectively).

Induction of *CDKN1A*/p21 mRNA expression was significantly greater in response to 1 vs. either 2 or 4 mJ/cm² UV for dFb from all three subjects, 24h after irradiation (Fig. 1A). Since 1 mJ/cm² UV irradiation clearly elicited the most robust induction of *CDKN1A*/p21, reduced *CDKN1A*/p21 mRNA expression by the affected LFS dFb was further analyzed from 0–24h after 1 mJ/cm² UV irradiation. *CDKN1A*/p21 mRNA expression by the affected LFS dFb was significantly less than that of the unrelated dFb at all time points and of the unaffected dFb at 24h after irradiation (Fig. 1B). This reduced induction of *CDKN1A*/p21 mRNA expression could bring about an impaired p21-dependent cell cycle arrest in response to UV stress [20, 21].

p21 protein up-regulation by UV exposure was not impaired in unaffected LFS fibroblasts

In order to further characterize the decreased expression of *CDKN1A*/p21 by the affected LFS dFb in response to UV stress, p21 protein induction was compared in dFb from the affected LFS patient vs. the unaffected father and the unrelated control subject in response to 1 mJ/cm² UV irradiation using immunoblot analysis. p21 protein expression by the affected LFS dFb was significantly less than that of both the unaffected and unrelated dFb at all time points 2–24h after irradiation, with maximum induction 8h after irradiation (Fig. 2). In fact, p21 expression was actually reduced by UV irradiation in the affected dFb. Consequently, the affected LFS dFb may suffer from a reduced capacity for p21-dependent cell cycle arrest in response to UV stress [20, 21].

Stress/apoptosis signal transduction in response to UV exposure was altered for affected LFS fibroblasts

The central role of p53 regulating cellular senescence and apoptosis in response to genotoxic stress has been well-established, including its influence on p21 activity [21]. To initiate a broader investigation of alterations in this response by affected LFS dFb to UV stress, antibody arrays were used to compare protein cleavage, phosphorylation, or induction for 17 key cellular stress and apoptosis signal modulators [23] following 1 mJ/cm² UV irradiation. The response was assessed 2h after UV irradiation, since signal protein modifications are often rapid and transient [12, 24]. No significant differences were detected for 16 of these signal modulators in dFb protein extracts from the affected LFS patient vs. the unaffected father 2h after UV exposure (Fig. 3). However, UV irradiation induced significantly greater phosphorylation of p53 itself at Ser15 in dFb protein extracts from the affected LFS patient (Fig. 3).

Phospho-p53[S15] induction was up-regulated by UV exposure in affected LFS fibroblasts

To further characterize the significantly increased phospho-p53[S15] in the affected LFS dFb following UV stress, phospho-p53[S15] induction was compared in dFb from the affected LFS patient vs. both the unaffected father and the unrelated control subject in response to 1 mJ/cm² UV irradiation using immunoblot analysis. This comparison also found significantly increased phospho-p53[S15] when normalized to total p53 in dFb protein extracts from the affected LFS patient compared to both the unaffected father and the unrelated control subject, 0–24h after UV exposure (Fig. 4A & 4B). Since altered patterns of phosphorylation have been reported for mutant compared to wild-type p53 [25], particularly involving Ser15 [26, 27], the differential response of p53 to phosphorylation in dFb from the affected LFS patient (Fig. 4) suggested that there may be a different proportion of wild-type and mutant p53 expressed in the heterozygous dFb from the affected LFS patient vs. the unaffected father.

Allele-specific wild-type *TP53* expression by heterozygous unaffected father in response to UV stress

To determine if there was a bias toward expression of either *TP53* allele in heterozygous dFb from the affected LFS patient or the unaffected father, a custom reverse-transcribed-PCR-restriction fragment length polymorphism (RT-PCR-RFLP) assay [28] was designed to detect the Ser241Tyr C>A *TP53* mutation in this LFS family. The RT-PCR-RFLP reaction product yields from this assay were used to calculate the wild-type vs. mutant allele-specific expression ratios for each heterozygous subject before and after UV irradiation. The ratio of wild-type to mutant *TP53* allele-specific expression was found to be significantly less in dFb from the affected LFS patient vs. the unaffected father 0–24h after UV exposure (Fig. 5A). RNA-seq was utilized for additional confirmation of *TP53* allele-specific expression in dFb from these heterozygous subjects before and 24h after UV irradiation. This analysis also found a significantly decreased ratio of wild-type to mutant *TP53* allele-specific expression in dFb from the affected LFS patient, 0 and 24h after UV exposure (Fig. 5B). Furthermore, dFb from the unaffected father showed a significant shift toward expression of the wild-type

allele 24h after UV exposure, whereas the allele-specific expression ratio was unchanged by UV exposure in dFb from the affected LFS patient (Fig. 5B).

The consequent allele-specific expression of mutant p53 by dFb from the affected LFS patient may contribute to the impaired up-regulation of *CDKN1A*/p21 by UV stress observed in comparison to dFb from the unaffected heterozygous father or the unrelated control subject (Figs. 1 & 2).

Discussion

This study was designed to test the hypothesis that an unaffected *TP53* carrier father may mount a response to genotoxic stress that is more characteristic of homozygous wild-type *TP53* individuals than that of his affected heterozygous daughter. Our approach was to compare the response of dFb isolated from an affected LFS patient vs. from the unaffected carrier father to genotoxic stress. Sadly, the affected heterozygous *TP53* daughter succumbed to LFS-related brain cancer at age 11, as had her younger brother at age 3 [8]. However, their heterozygous father, carrying the identical Ser241Tyr C>A *TP53* mutation, remains unaffected.

To test this hypothesis, the first portion of our study compared the induction of *CDKN1A*/p21, a key cell cycle regulatory target of p53 [20], as well as an array of other cellular stress and apoptosis signal modulators [23], by UV stress in dFb from the affected LFS patient vs. the unaffected father and an unrelated control subject. The second portion then investigated whether there may be an alteration in *TP53* allele-specific expression between dFb from the affected LFS patient and the unaffected father that could contribute to a differential genotoxic stress response.

Following UV irradiation, induction of *CDKN1A*/p21 mRNA (Fig. 1B) and protein (Fig. 2) by dFb from the LFS patient was significantly less than that of dFb from the unaffected father or the unrelated control subject, which displayed comparable responses to UV stress. In fact, p21 protein accumulation was actually reduced by UV irradiation in the affected dFb (Fig. 2). Similarly decreased induction of *CDKN1A*/p21 mRNA and protein in response to an equivalent UVC dose has been previously reported for dFb from affected LFS patients with different p53 mutations compared to homozygous wild-type controls [21]. However, that study did not compare the UV response of *CDKN1A*/p21 in unaffected, heterozygous *TP53* dFb. Note that the decrease in p21 protein accumulation observed by 24h after irradiation for all dFb in the present study (Fig. 2) may be due to its previously reported ubiquitin-dependent degradation in response to low dose UVC [29]. This decreased p21 protein accumulation was eventually detectable by both the previously reported and present studies despite ongoing UV induction of *CDKN1A*/p21 mRNA expression in both (Fig. 1b) [29]. Notwithstanding its ensuing apparent degradation, there is considerable evidence indicating that p53-induced p21 is a key mediator of cell cycle arrest to facilitate DNA repair in response UV exposure [20, 21]. Thus, the reduced induction of p21 by affected LFS dFb in response to UV irradiation, as reported previously [21] and herein (Fig. 2), is likely to predispose affected dFb to undue propagation of UV-induced DNA damage.

More importantly, the comparable UV-induced expression of *CDKN1A*/p21 mRNA (Fig. 1B) and protein (Fig. 2) by dFb from the heterozygous *TP53* unaffected father and unrelated control subject was significantly greater than that of the affected patient, suggesting that the impaired response expected from mutant p53 [21] may be suppressed in dFb from the heterozygous father.

A broader comparison of cellular stress and apoptosis signal transduction [23] was undertaken to determine if there were any additional differences between dFb from the affected LFS patient vs. the unaffected father in response to UV exposure that may be p53-regulated. Interestingly, the only significant UV-induced difference found concerned p53 itself, which was much more highly phosphorylated at Ser15 in dFb from the affected LFS patient (Fig. 3). Again, UV-induced p53[S15] phosphorylation was found to be comparable in dFb from the heterozygous *TP53* unaffected father and the unrelated control subject, but both were significantly less than that of the affected patient (Fig. 4A). Increased phosphorylation has been reported for mutant compared to wild-type p53 [25], particularly involving Ser15 [26, 27]. Therefore, the increased UV-induced phospho-p53[S15] in dFb from the affected LFS patient (Fig. 4) suggested that there may be more phosphorylation-susceptible mutant p53 expressed in the heterozygous dFb from the affected LFS patient vs. the unaffected father.

Taken together, the potential suppression of a mutant p53 regulatory contribution to UV-induced *CDKN1A*/p21 expression (Figs. 1B & 2) along with a UV-induced phospho-p53[S15] response more similar to that of homozygous wild-type dFb (Fig. 4) suggested that the heterozygous dFb from the unaffected father may express an increased proportion of wild-type vs. mutant p53 than the heterozygous dFb from the affected LFS patient. Comparison of the *TP53* allele-specific expression ratios in the heterozygous dFb from the affected LFS patient vs. the unaffected father found that this was indeed the case (Fig. 5). Not only was the ratio of wild-type to mutant *TP53* allele-specific expression significantly greater in dFb from the unaffected father 0–24h after UV exposure but his dFb showed an additional significant shift toward even greater expression of the wild-type allele 24h after UV exposure, while the expression ratio was unchanged by UV exposure in dFb from the affected LFS patient (Fig. 5).

The p53 protein is tetrameric, and its mutants often result in a dominant-negative phenotype [30]. Hence, it may be reasonable to suspect that expression of any mutant *TP53* would be harmful, even at the lower levels observed for the unaffected father (Fig. 5). However, many *TP53* mutations do not exert a strictly dominant-negative effect [30], and the activity of heterotetrameric p53 has also been found to be very complex and mutation-dependent [31]. With respect to the present study, the activity and tumorigenic mechanism of p53 carrying the Ser241Tyr mutation have yet to be determined. Thus, it is possible that this particular mutation results in a tumorigenic gain of function [32], for example, manifested at a higher mutant allele expression level than that of the unaffected father [33]. Indeed, the significantly increased phospho-p53[S15] observed in dFb from the affected LFS patient (Fig. 4) may signify such a gain of function [34]. To begin addressing these oncogenic possibilities, experiments are underway to compare p53 activity in dFb from the affected LFS patient vs. her unaffected father, both quantitatively and qualitatively.

Above all, this alteration in *TP53* allele-specific expression ratios suggests that *TP53* may be subject to genomic imprinting [35] in this LFS family. Although a role for genomic imprinting of *TP53* in human oncogenesis has not yet been established, it has been implicated in mice [36], as well as for other tumor suppressors in humans [37]. Furthermore, considerable evidence for the epigenetic regulation of *TP53* expression has also been reported [38,39], though not yet connected to parental imprinting in humans. Thus, silencing of the mutant *TP53* allele in dFb from the unaffected father may account for its significantly reduced expression in response to UV stress (Fig. 5), as well as his ongoing protection against the oncogenic onset of LFS [8].

Conversely, reversal of this mutant *TP53* silencing in the LFS daughter may then account for the impaired induction of *CDKN1A*/p21 (Figs. 1B & 2) and increased phospho-p53[S15] (Fig. 4) observed in response to UV irradiation of her dFb. Finally, comparatively increased expression of the mutant *TP53* allele in the affected daughter could have, thus, contributed to the loss of effective p53 tumor suppression and subsequent oncogenic progression associated with LFS [8]. Elucidation of an epigenetic mechanism regulating *TP53* allele-specific expression in dFb from the affected LFS patient vs. the unaffected father will be invaluable for establishing its molecular basis [38], along with the potential for future therapeutic intervention [6, 7, 40].

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Highlights

- A heterozygous mutant *TP53* carrier parent of children with Li-Fraumeni syndrome (LFS) remains unaffected in their fourth decade of life.
- UV induction of *CDKN1A*/p21 mRNA and protein, a regulatory target of p53, in LFS patient dermal fibroblasts (dFb) was significantly reduced compared to dFb from the unaffected parent.
- UV irradiation induced significantly greater phosphorylation of p53 at Ser15 in dFb from the affected LFS patient, suggesting there may be a different proportion of wild-type and mutant p53 in heterozygous dFb from the affected LFS patient vs. the unaffected parent.
- The ratio of wild-type to mutant *TP53* allele-specific expression was found to be significantly greater in dFb from the unaffected parent vs. the affected LFS patient.
- Allele-specific expression of wild-type p53 in the unaffected parent may contribute to their increased up-regulation of *CDKN1A*/p21 in response to UV stress and protection from the oncogenesis associated with LFS.

Figure 1A

Figure 1B

Figure 1.

CDKN1A/p21 mRNA expression by affected LFS compared to unaffected or unrelated control dFb in response to UV stress. Total dFb RNA was analyzed by RT-qPCR $\pm$ UV irradiation, as indicated. Reference gene-normalized relative *CDKN1A/p21* mRNA expression was calculated based on C_q values for each sample time point. (A) 24h after 1–4 mJ/cm² UV irradiation (n=4; ‡ $p < 0.05$ vs. corresponding 2 or 4 mJ/cm² irradiated dFb, * $p < 0.05$ vs. control dFb, † $p < 0.05$ vs. unaffected dFb). (B) 0–24h after 1 mJ/cm² UV irradiation (n=6; * $p < 0.05$ vs. control dFb, † $p < 0.05$ vs. unaffected dFb).

Figure 2A

Fig. 2B

Figure 2.

p21 protein induction by affected LFS compared to unaffected or unrelated control dFb in response to UV stress. Protein extracts of cultured dFb were subjected to immunoblot analysis following UV irradiation, as indicated. Fluorescent signals corresponding to p21 (21 kD) were normalized to those of the corresponding β -actin (43 kD) reference standard. (A) Results presented in comparison to uninduced, reference-normalized p21 for each dFb

source (n=4; * $p<0.05$ vs. control dFb, $^{\dagger} p<0.05$ vs. unaffected dFb). (B) Results from representative immunoblot.

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Figure 3.

Stress/apoptosis signal profiling of affected LFS compared to unaffected dFb with antibody arrays in response to UV exposure. Protein extracts of cultured dFb were subjected to antibody array analysis following UV irradiation, as indicated. Fluorescent signals of thirteen phosphorylated stress and four apoptosis target modulators were normalized to those of α -tubulin reference standard. The results shown represent signal protein target abundances 2h after 1 mJ/cm² UV irradiation (n=4). Significantly increased phospho-p53[S15] was detected in protein extracts from the affected LFS compared to unaffected dFb. [†] $p < 0.05$ vs. unaffected dFb.

Figure 4A

Fig. 4B

Figure 4.

Phospho-p53[S15] induction by affected LFS compared to unaffected or unrelated control dFb in response to UV stress. Protein extracts of cultured dFb were subjected to immunoblot analysis following UV irradiation, as indicated. (A) Fluorescent signals corresponding to phospho-p53[S15] (53 kD) were normalized to those of total p53 protein (53 kD) following

UV irradiation for each dFb source (n=4; * $p<0.05$ vs. control dFb, $^{\dagger}p<0.05$ vs. unaffected dFb). (B) Results from representative immunoblot.

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a. RT-PCR-RFLP **b. RNA-seq** **Figure 5.**

TP53 allele-specific expression (ASE) by heterozygous affected LFS compared to unaffected dFb in response to UV stress. (A) RT-PCR-RFLP assay customized to detect the heterozygous Ser241Tyr C>A *TP53* mutation in this LFS family was used to calculate the wild-type vs. mutant ASE ratios 0–24h after 1 mJ/cm² UV irradiation (n=6). (B) RNA-seq

analysis to confirm wild-type vs. mutant *TP53* ASE ratios, 0 and 24h after 1 mJ/cm² UV irradiation (n=2). [†] $p < 0.05$ vs. unaffected dFb. [‡] $p < 0.05$ vs. uninduced dFb.

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