The p53 tumor suppressor gene of the marsupial Monodelphis domestica: cloning of exons 4–11 and mutations in exons 5–8 in ultraviolet radiation-induced corneal sarcomas

Donna F.Kusewitt1,2, Tamara E.Sherburn, Katarzyna B.Miska1, Gregory B.Tafoya, James M.Gale and Robert D.Miller1

Department of Cell Biology and Physiology, Room 149, Basic Medical Sciences Building, Health Sciences Center, University of New Mexico, Albuquerque, NM 87131 and 1Department of Biology, Castetter Hall, University of New Mexico, Albuquerque, NM 87131, USA

2To whom correspondence should be addressed
Email: dkusewitt@salud.unm.edu

Inactivating p53 mutations are found in many ultraviolet radiation (UVR)-induced skin tumors. We examined 12 UVR-induced corneal tumors of the marsupial Monodelphis domestica for mutations in exons 5–8 of p53 and compared their mutational spectrum with that of UVR-induced skin tumors of other species. First we cloned and characterized a cDNA extending from the middle of exon 4 through exon 11 of the Monodelphis p53 gene. Based on the sequence information obtained, primers were designed to amplify introns 4–9 of the gene; intron primers to amplify individually exons 5–8 were subsequently developed. ‘Cold’ single strand conformational polymorphism analysis followed by reamplification of DNA with altered mobility and cycle sequencing revealed single p53 mutations in four of 12 tumors (33%), including one mutation in exon 5, two identical mutations in exon 7 and one mutation in exon 8. All mutations were at dipyrimidine sites and occurred on the non-transcribed strand. Three of the four were hallmark UVR-induced C→T alterations. Three of the mutations were found at sites corresponding to human codons 248 and 273, which are mutational hotspots in human and murine UVR-induced squamous cell carcinomas. Our findings suggest that UVR-induced corneal sarcomas in Monodelphis will be valuable in studying mechanisms of p53 mutation in UVR-induced tumors.

Introduction

The p53 tumor suppressor gene functions as the ‘guardian of the genome’ by promoting DNA repair in cells with minimal genomic damage and initiating apoptosis in cells with DNA damage in excess of that which can be accurately repaired (1,2). The importance of normal p53 protein function in the prevention of tumor development is particularly apparent when ultraviolet radiation (UVR)-induced skin tumors are considered (3–5). Up to 100% of UVR-induced skin tumors in mice contain mutationally inactivated p53 alleles (6–10). p53-knockout mice have a significantly shorter latent period for the development of UVR-induced squamous cell carcinoma than wild-type mice (11). Transgenic mice expressing wild-type p53 have been identified in the majority of human non-melanoma skin cancers (13–22). The mutations appear to arise at an early stage of tumor development: sun-damaged skin and tumor precursor lesions such as actinic keratosis and Bowen’s disease also harbor mutant p53 alleles (3,16,22). p53 mutations in UVR-induced skin tumors are concentrated in exons 5–10 of the gene (23,24).

Monodelphis domestica is a small South American opossum that is particularly suitable for studies of UVR carcinogenesis (25). Monodelphis possesses a photolyase that catalyzes the repair of UVR-induced pyrimidine dimers (25–27). The enzyme binds specifically to pyrimidine dimers and uses energy absorbed from visible light to monomerize the dimers, a process termed ‘photoreactivation’. A variety of DNA lesions are induced by UVR in the skin and eyes of this animal; subsequent exposure to visible light can specifically remove pyrimidine dimers. This permits attribution of adverse effects of UVR to a specific DNA lesion: UVR effects abrogated by photoreactivation are generally considered to be due to DNA damage, specifically to the formation of pyrimidine dimers (25). For example, when Monodelphis are chronically exposed to low doses of UVR and photoreactivation of pyrimidine dimers is prevented by housing the animals under red or yellow lights that cannot drive the process, the animals develop a variety of skin and corneal tumors (28–32). Photoreactivation treatment after each UVR exposure reduces the incidence and delays the onset of these tumors, indicating that UVR-induced pyrimidine dimers play an important role in tumor development (30,31).

Virtually all Monodelphis chronically exposed to low doses of UVR develop corneal sarcomas (30,32). The development of frank tumors is preceded by preneoplastic fibroplasia and neovascularization. Fully developed tumors are locally invasive, but do not metastasize. Previous studies showed that the tumors express the H-ras and K-ras oncogenes, however, activating ras mutations were identified in only two of 12 invasive corneal sarcomas examined (33,34). Both of these mutations occurred at dipyrimidine sites, suggesting that UVR-induced DNA lesions were the initiating events. In the present study we determined the incidence and location of mutations in exons 5–8 of the p53 gene in these same 12 UVR-induced corneal sarcomas. Our goal was to determine if p53 mutation was a frequent event in corneal tumorigenesis in Monodelphis and if p53 mutations in the tumors occurred at dipyrimidine sites. It was of particular interest to compare p53 mutations in these UVR-induced mesenchymal tumors with those in UVR-induced epithelial skin tumors of other species.

Materials and methods

Tumor induction, collection and processing

Animal experimentation was carried out in accordance with all applicable state and national animal welfare requirements, using protocols approved by the Institutional Animal Care and Use Committee. Shaved Monodelphis received either 250 or 500 J/m² UVR three times weekly for periods of up to 104 weeks. A dose of 500 J/m² to the shaved skin of Monodelphis is
approximately one minimal erythema dose (35). Opossums were UV irradiated at an approximate dose rate of 4.0 W/m², as determined with a spectroradiometer (model 742; Optronic Laboratories, Orlando, FL), with a bank of unfiltered FS40 fluorescent sunlamps (Westinghouse, Bloomfield, NJ) that emitted most of their output between 280 and 380 nm. Tissue was frozen immediately in liquid nitrogen and stored at −70°C. DNA was isolated from this tissue using previously described techniques (36). For several of the eye tumors, a small portion was used to establish cell lines in vitro, as previously reported (36).

Cloning p53 cDNA

We were unable to detect Monodelphis p53 mRNA sequences on northern blots of mRNA isolated from a variety of normal tissues and cell lines using a human p53 cDNA probe kindly provided by Dr. B. Vogelstein (37) and standard techniques (34), thus we concluded that screening existing Monodelphis cDNA libraries with human cDNA was unlikely to prove successful. However, we were able to use radiolabeled human p53 cDNA as probe to identify by standard techniques (33) a 370 bp p53 fragment in a Monodelphis liver genomic library cloned into ZAP II phage (Stratagene, La Jolla, CA). The fragment proved to contain sequences highly homologous to conserved region IV of the human p53 gene and hybridized to mRNA of an appropriate size for p53 (2.5 kb) on northern blots of mRNA isolated from cultured Monodelphis corneal tumor cell lines.

Using intron sequence information, we were able to screen ~100,000 colonies of a Monodelphis cDNA library. Construction of this library and techniques for screening have been described (33,34). The library was made from mRNA isolated from a cell line derived from one of the primary corneal sarcomas examined in this study. Two positive clones, each containing an insert ~900 bp in length, were identified. Nucleotide sequences were determined in both directions for both clones, using the Sequenase v.2 kit (Amersham Life Science, Arlington Heights, IL) as recommended by the manufacturer and vector primers purchased from Stratagene or internal primers (Figure 1) synthesized by Scientific Resources (Albuquerque, NM). For each clone, the sequences obtained from the two strands were consistent and the two clones had identical sequences. An additional cloned cDNA p53 sequence was subsequently identified that yielded a short stretch of additional 5′ sequence.

Characterization of introns 4–8

Using cDNA sequence information, the primers shown in Table I were designed for PCR amplification of introns 4–8. Primers for amplification and sequencing in this and subsequent steps were purchased from Southwest Scientific Resources, from Genosys (The Woodlands, TX) or from Integrated DNA Technologies (Corailva, IA). We were able to amplify introns 5, 6 and 8 from genomic DNA using AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) and standard PCR protocols. To obtain intron 7 sequences, we initially used a 2.5 kb fragment, using the upstream primer for intron 6 amplification, the downstream primer for intron 8 amplification, AmpliTaq DNA polymerase and standard PCR conditions. Intron 4 sequences were PCR amplified using Hot Tub DNA polymerase (Amersham Life Science) and a stepdown technique (38,39). Amplified fragments were cleaned on glass beads (GeneClean; Bio 101, Vista, CA) or DEAE-cellulose (40) and cloned into pCR2.1 using the TA cloning kit (Invitrogen, Carlsbad, CA) or into pPCR-Script Amp SK (+) using the PCR-Script Amp cloning kit (Stratagene) as directed. For introns 5–8 sequencing of cloned material was performed with the Sequenase v.2 kit; for intron 4, cycle sequencing of the cloned fragment was performed as described below.

Amplification and sequencing of exons 5–8

Using intron sequence information, we developed primers to amplify individually exons 5–8 from genomic DNA (Table I). Each 100 μl reaction mixture contained 20–25 pmol of each primer, 1–2 μg of tumor DNA, 1.5 mM MgCl₂, 200 μM each dNTP and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer). An initial 5 min denaturing step at 94°C was followed by 30–40 amplification cycles (melting at 94°C for 1 min, annealing at 52–59°C for 1 min and extension at 72°C for 1 min) and a final 10 min extension step at 72°C. For direct sequencing, amplified material was cleaned with the QIAquick kit (Qiagen, Santa Clarita, CA). Sequencing was performed using the ABI Prism or the BigDye terminator cycle sequencing kit (Perkin Elmer) and a model 377 automated sequencer (Perkin Elmer). In all cases, amplification primers were used as sequencing primers. The nucleotide sequence of exons 5–8 individually amplified from liver DNA and sequenced directly was in complete concordance with the previously determined cDNA sequence. Sequences obtained individually from liver DNA from 12 invasive corneal sarcomas as described above. PCR-amplified fragments were analyzed by ‘cold’ single-strand conformational polymorphism (SSCP), using the Thermoflow apparatus supplied by Novex (San Diego, CA) (41). Briefly, 2–5% of the PCR reaction was denatured for 5 min at 95°C in a mixture containing 20 mM methyl mercury hydroxide and 1/10 loading dye supplied by Novex. Samples were rapidly chilled on ice, then loaded on a 20% polyacrylamide gel and electrophoresed in 1.25X TBE at 250 V for 2–3 h. Amplified material from each exon was tested at 10, 15 and 20°C. For exons 5 and 7, a temperature of 20°C proved best for detecting a band shift, while for exon 8, a temperature of 10°C was optimal. Gels were stained with the Silver Xpress kit (Novex) according to the directions of the manufacturer.

When a fragment with altered mobility was detected, the occurrence of the band shift was confirmed by a second PCR amplification from genomic DNA and an SSCP gel stained with ethidium bromide. For each fragment showing altered mobility, a small plug of the band was punched out of the gel using a pipet tip. This fragment was used for a reamplification reaction under the original PCR conditions for a total of 18–25 cycles. For comparison, an unaltered band from another tumor was treated similarly. Reamplified material was directly sequenced as previously described using the two amplification primers individually as sequencing primers. Sequences obtained using the upstream and downstream primers were always in complete agreement.

Results

We characterized cDNA sequences that extended from within exon 4 through the final exon of the gene, exon 11 (Figure 1). Exon boundaries were assigned based on their location in other species (42). The portion of Monodelphis p53 cDNA characterized contained the highly conserved regions II–V (43). Based on the sequence we obtained, Monodelphis p53 appeared to be ~71% identical to human p53 at the nucleotide level and 73% identical at the amino acid level overall (43,44). Evolutionarily conserved regions II–V were more similar to the human gene, showing 85% nucleotide identity and 95% amino acid identity. The extreme conservation of these regions highlights their essential function in DNA binding (45). Other conserved sequence features of the protein included few charged residues and two hydrophobic regions (conserved regions IV and V) in the internal amino acid sequence and a highly charged and hydrophilic C-terminus (43).

Using intron primers allowed us to amplify and examine all nucleotides in exons 5–8. As shown in Figure 2, band shifts were detected for exon 5 in one tumor, for exon 7 in two tumors and for exon 8 in one tumor; no DNA fragments with altered mobility were seen for exon 6 at any temperature. In all cases, a second amplification from genomic DNA and subsequent SSCP analysis confirmed the occurrence of a band shift. This demonstrated that sequence alterations were not a PCR artefact. The two bands of altered mobility seen for exon 5 in a single tumor yielded the same mutant sequence when analyzed. Thus, no tumor gave evidence of two different mutations.

Altered nucleotides were identified in all apparently mutant fragments (Table II), whereas apparently normal fragments had p53 germline sequence. The mutation in exon 5 was located just 3′ of conserved region II and changed the coding triplet CAG to the termination codon TAG. Both tumors with mutant exon 7 sequences had the same mutation, a CGG→TGG codon alteration in conserved region IV, resulting in an Arg→Trp change. The mutation in exon 8 was a CGT→TGT change that caused substitution of a cysteine for an arginine: the mutation was located in conserved region V. When compared with human p53, the alteration in exon 5 was at amino acid 144, the change in exon 7 was at amino acid 248 and the mutation in exon 8 was at amino acid 273.

Discussion

In the present study, inactivating p53 mutations were identified in four of 12 (33%) UVR-induced corneal sarcomas of...
Monodelphis domestica p53 tumor suppressor gene

Fig. 1. Nucleotide and predicted amino acid sequence of cDNA derived from the p53 gene of Monodelphis. Sites of internal sequencing primers are shown in lower case letters and the direction of DNA synthesis is indicated above by an arrow. Exon boundaries are based on those of other species. Evolutionarily conserved regions II–V are indicated by bold italics. Amino acids altered by mutation in the present study are shown in bold. The amino acid sequence of the human protein and the amino acid number of the human protein are shown below the Monodelphis sequence; hyphens indicate amino acid identity and gaps have been introduced as necessary to maximize homology.

Monodelphis. In man, p53 mutations have been reported to occur in >90% of sunlight-induced squamous cell carcinomas (17) and in up to 56% of basal cell carcinomas (18). The frequency of p53 mutations in UVR-induced squamous cell carcinomas in hairless mice has been determined to approach 100% (6–10). In comparison, the frequency of p53 mutations in UVR-induced corneal sarcomas of Monodelphis was somewhat less than in UVR-induced epithelial skin tumors of other species. In sarcomas in man, the percentage of tumors with p53 mutations has been reported to vary, depending on the tumor type (46–50). For sporadic soft tissue sarcomas, a p53 mutation frequency of 20–30% is usually found, while post-radiation sarcomas have a very high frequency of p53 mutations (88%) (46–50). p53 inactivation in some human sarcomas has
Exon 8

Table I. Primers used for PCR amplification of individual introns and exons of the p53 gene of Monodelphis

<table>
<thead>
<tr>
<th>Product</th>
<th>Upstream primer sequence</th>
<th>Downstream primer sequence</th>
<th>T_a (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron 4</td>
<td>5'-CTCAGTTTCTTACATGCTGGA-3'</td>
<td>5'-GGACACGTCTTGCACAGCTGC-3'</td>
<td>90-60</td>
</tr>
<tr>
<td>Intron 5</td>
<td>5'-TGGATGGTTAAAGCGGCGTTC-3'</td>
<td>5'-TTCCACCTGCTTCTTTTAGGCC-3'</td>
<td>56</td>
</tr>
<tr>
<td>Intron 6</td>
<td>5'-CTATCTATGTCACCACCAAC-3'</td>
<td>5'-GGGAAAATTGAGCTTCTTC-3'</td>
<td>56</td>
</tr>
<tr>
<td>Intron 7</td>
<td>5'-TCTACCAAGAGGGAGGTTTCA-3'</td>
<td>5'-TGTTGGGAGCTATTTCTGGA-3'</td>
<td>56</td>
</tr>
<tr>
<td>Intron 8</td>
<td>5'-GCAATCATATGGGTTTCTC-3'</td>
<td>5'-TTCCACAAGAAGGGAGGTCCCA-3'</td>
<td>52</td>
</tr>
</tbody>
</table>

*Annealing temperature.

Stepdown technique.

**Fig. 2.** SSCP analysis of p53 mutations in exons 5–8 for 12 UVR-induced corneal sarcomas of Monodelphis. Arrows indicate fragments with altered mobility that were re-amplified and sequenced. All of the indicated fragments contained point mutations.

**Table II.** p53 mutations identified in UVR-induced primary corneal sarcomas of Monodelphis

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>5</td>
<td>144</td>
<td>cCAG→cTAG</td>
<td>Gln→Ter</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>248</td>
<td>cCGG→cTGG</td>
<td>Arg→Trp</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>248</td>
<td>cCGG→cTGG</td>
<td>Arg→Trp</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>273</td>
<td>tCTG→tTGT</td>
<td>Arg→Cys</td>
</tr>
</tbody>
</table>

*Equivalent position in human p53 gene

also been shown to result from gross rearrangements of the gene, resulting in loss of gene expression, or from amplification and overexpression of the MDM-2 protein, which functionally inactivates the p53 gene product (51,52). The frequency of p53 mutations we observed in the corneal sarcomas of Monodelphis was similar to the frequency of p53 mutations in sporadic soft tissue sarcomas of man. We did not specifically investigate the possibility of large deletions in the gene or rearrangements of the gene. However, we were able to amplify exons 5–8 from at least one p53 allele in every tumor; this indicates that no tumor was homozygous for the loss of any of these exons. We have not determined if the mdm-2 gene is amplified or overexpressed in the tumors; preliminary studies indicate that the mdm-2 gene of Monodelphis can be detected on Southern blots using a human mdm-2 cDNA probe.

UVR-induced skin tumors may contain more than one p53 allele. In one study, >60% of tumor cell lines derived from UVR-induced skin tumors of mice had multiple p53 mutations (8). Multiple p53 mutations were detected in 45–88% of human non-melanoma skin tumors (14,18). Multiplicity of p53 mutations is believed to reflect secondary events during clonal evolution of tumors (14) and indicates the mutagenic potency of UVR (5). In our study, no Monodelphis tumor appeared to harbor more than one p53 mutation and no tumor with a p53 mutation also had a K-ras or H-ras mutation (33,34).

Our techniques did not detect all p53 mutations present in tumors. First, because the DNA analyzed was isolated from primary tumors, germline DNA sequences from admixed non-tumor cells diluted mutant alleles to a variable extent. Second, even under the best conditions, SSCP does not detect all sequence alterations (53). The ‘cold’ SSCP technique we used is reported to detect p53 mutations with sensitivity equal to direct sequencing of amplified exons and somewhat better sensitivity than radioactive SSCP (41). However, the sensitivity of some radioactive SSCP techniques appears to exceed that of ‘cold’ SSCP (14). In our hands, ‘cold’ SSCP was more effective when silver staining rather than ethidium bromide staining was used to detect band shifts.

Most of the p53 mutations in sunlight-induced skin tumors in man and UVR-induced skin tumors in hairless mice are missense base substitutions, primarily C→T and CC→TT transitions at dipyrimidine sites (3,6–10,13–21). These are hallmark UVR mutations; they occur where UVR-induced cyclobutane pyrimidine dimers or pyrimidine-pyrimidone (6–4) photoproducts are formed as the direct result of the interaction of UVR with DNA (20). Likewise, all mutations we identified in corneal tumors in the present study were point mutations at dipyrimidine sites and three of the four mutations were C→T alterations. All mutations we detected appeared to arise on the non-coding strand of the DNA. This is consistent with the strong non-coding strand bias reported for p53 mutations in UVR-induced murine skin tumors (6–9,50). This strand bias is believed to result from the preferential repair of photoproducts located on the coding strand of the DNA (54).

Hotspots for UVR-induced mutations are located at codons 245, 247/248, 273, 278, 285/286 and 294 in man (18,23) and at codons 267 and 272 (equivalent to codons 273 and 278 of man) in the hairless mouse (6,7). In our studies, similar sites were altered. Both mutations in exon 7 were at the site equivalent to codon 248 of man and the mutation in exon 8...
was at a site corresponding to codon 273 of man. The frequency of mutations at these UVR hotspots does not appear to be correlated with the frequency of photoproduct formation at the sites. Although UVR photoproducts occur at high frequency in codons 278 and 286 of the human p53 gene, they form rarely in codons 245 and 248 (55). Instead, hotspots in human p53 may represent sites at which DNA repair is unusually slow (56). It has been reported that dipyrimidines containing 5-methylcytosine are more susceptible to pyrimidine dimer formation than other dipyrimidines (57), while pyrimidine-pyrimidone (6–4) photoproducts cannot form at such sites (20). Cytosine can be methylated when it occurs at a CpG site; CpG sites in the human p53 gene appear to be universally methylated (57). Interestingly, of the four mutations we identified, three involved cytosines at CpG sites. These mutations seem likely to have occurred at cylobutane pyrimidine dimers.

Although the number of mutations we examined was small, our study of p53 mutations in UVR-induced cornal sarcomas of Monodelphis suggested that p53 mutations in these tumors occurred at sites of DNA photoproduct formation and were the direct result of UVR interacting with DNA. The Monodelphis sarcomas had a mutational spectrum very similar to that seen in UVR-induced epithelial skin tumors of other species, despite their origin from mesenchymal rather than epidermal cells. These studies suggest that these Monodelphis tumors and cell lines derived from the tumors may prove valuable in studying the role of DNA damage and repair in UVR-induced p53 mutation.

Acknowledgements

We would like to thank Dr R.Mittlstaedt, Dr R.Hefflich and Dr J.James-Gaylor of the National Center for Toxicological Research (Jefferson, AR) for their generous assistance. These studies were funded by National Institutes of Health grant 1 R01 CA57368.

References


Monodelphis domestica p53 tumor suppressor gene

967

Downloaded from https://academic.oup.com/carcin/article-abstract/20/6/963/2733486 by guest on 19 December 2018


Received September 22, 1998; revised January 19, 1999; accepted February 5, 1999.