Investigations on a clinically and functionally unusual and novel germline $p53$ mutation

The tumour suppressor gene $p53$ was discovered in 1979 because of its ability to bind to large T antigen (Lane and Crawford, 1979). $p53$ is a nuclear phosphoprotein that plays a central role in the cellular response to DNA damage by inducing either G1 arrest or apoptosis. It functions mainly through its ability to transactivate or repress target genes. Inactivation of wild-type $p53$ by mutation or interaction with cellular or viral proteins has been found to occur in over 50% of human cancers (Levine, 1997). Li-Fraumeni Syndrome (LFS), which is a rare dominantly inherited cancer predisposition syndrome, is associated with germline $p53$ mutations (Malkin et al, 1990). Individuals with LFS are at increased risk of developing a large spectrum of cancers, often with a very early onset and multiple primary tumours are common. Primary choroid plexus tumours are rare and usually occur in early childhood. However, several families with LFS have been described, in which there are individuals with choroid plexus tumours, some- times in young adults (Garber et al, 1990). There have been four reports of germline $p53$ mutations in families with LFS and childhood choroid plexus tumours (Frebourg et al, 1995; Jolly et al, 1994; Sedlacek et al, 1998; Vital et al, 1998). In this report we describe an adult patient with a novel germline 7 base pair insertion in the $p53$ gene who presented with an osteosarcoma of the femur at the age of 22 years. This tumour was treated successfully by surgery and chemotherapy. At 29 years, she presented with a choroid plexus tumour which was shown on histology to be an atypical choroid plexus papilloma. There was no unusual family history of cancer (Figure 1) and both parents were alive and well in their 50s. Unexpectedly in routine screening this mutation appeared functional in two assays of $p53$ function (FASAY and apoptotic assay). The FASAY (Flaman et al, 1995) is a yeast based assay that looks at the transactivational ability of $p53$. This assay has been shown to reliably identify both germline (Lomax et al, 1997) and sporadic mutations (Duddy et al, 2000). The apoptotic assay (Camplejohn et al, 1995, 2000), can detect germline $p53$ mutations by measuring a reduction in the radiation-induced apoptotic response of peripheral blood lymphocytes (PBL) compared with that seen in cells from normal individuals. Due to the unexpected results in these two assays, further functional studies were carried out on this mutation, including an investigation of its ability to induce apoptosis in mammalian cells, to transrepress a target gene and to inhibit colony growth in transfected Saos-2 cells. However, surprisingly, data from irradiated peripheral blood lymphocytes and transfected Saos-2 cells, suggested that this truncated, mutant protein retains significant ability to induce apoptosis.

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consensus sequence and there is no transactivation of the Ade2 gene. In this case the yeast cells accumulate a red coloured intermediate of the adenine biosynthesis pathway.

### Apoptotic assay

This assay was carried out as described by Camplejohn et al (1995). Briefly, PBL were separated from whole blood and cultured for 3 days, at which point half of the PBL were exposed to 4 Gy of radiation. Control and irradiated cells were cultured for a further 24 h, when they were fixed in 70% ethanol. Prior to analysis the samples were acid denatured in 0.1 M HCl (BDH) and stained with propidium iodide (Sigma). The amount of apoptosis was measured by the size of the sub-G1 peak by FACS (FACSCalibur, Becton Dickinson).

### Western blotting

For Western blotting, Saos-2 cells were transfected as described and were lysed using NP40 lysis buffer (BDH) after 24 h. The protein concentration was measured using the Bio-Rad DC assay (Bio-Rad Laboratories) and 15 μg of protein denatured in the presence of SDS was loaded onto a 12% SDS–PAGE gel. Proteins were transferred onto a nitrocellulose membrane (Amersham Life Science) and were detected using DO-1 antibody (Santa Cruz Biotechnology). The secondary antibody used was a rabbit anti mouse HRP (DAKO) and this was in turn detected using the ECL Western blotting detection reagent (Amersham Pharmacia Biotechnology).

### Induction of apoptosis in Saos-2 cells

Cells were transfected as described using the calcium phosphate precipitation kit (Promega). Cells were washed after 16 h and cultured for a further 72 h. The Saos-2 cells were then fixed in 70% ethanol and were stained with propidium iodide (Sigma) and with anti-p53 monoclonal antibody DO-1 (Santa Cruz Biotechnology) for FACS analysis. Apoptosis was determined for both the p53-positive and p53-negative sub-populations by the sub-G1 peak size and all samples were compared to the wild-type control.

### Transrepression

5 × 10⁵ Saos-2 cells were transfected in 60 mm dishes with 1 μg pC53-SN3 (containing wild-type or mutant p53) and 3 μg SV40 β-gal. Cells were washed after 16 h and then cultured for 24 h and washed in PBS. 1X reporter lysis buffer (Promega) was used to lyse the cells. We added 20 μl lysate to 150 μl chloroformolred β-D-galactopyranoside (Boehringer Mannheim) in a 96-well plate, and this was incubated at 37°C. Measurements of absorbance were recorded using a plate reader at 570 nm every 15 min.

### Suppression of colony growth

1 × 10⁶ Saos-2 cells were transfected in 60 mm dishes with 7 μg pC53-SN3 (containing wild-type or mutant p53). Cells were washed after 16 h and were cultured for 48 h. G418 (Calbiochem) at 400 μg ml⁻¹ was then added to the media on each dish, and cells were cultured for 4 weeks. Cells were washed in PBS, fixed by adding 70% ethanol, and stained using 0.1% methylene blue. The number of colonies on each plate was counted.

### RESULTS

#### Genomic sequencing of DNA

Genomic sequencing of DNA from PBL was carried out in both directions in two independent laboratories from exon 5 through
to exon 9. A frameshift mutation, the result of a 7 base pair insertion, was discovered in exon 5 of the p53 gene, after the first base of codon 161 (Figure 2). The alteration in frame would produce amino acid substitutions beginning with alanine to glycine at position 161 and a stop codon at position 182 in the mutated protein.

The insertion, GCCATGGG, was found to be a direct repeat of the 7 base pair sequence immediately upstream in the p53 gene.

One of the proband’s parents elected to have predictive testing and was found not to have the mutation. The other parent declined testing.

**FASAY results**

The FASAY is a yeast based functional assay, designed to investigate the ability of p53 to transactivate a target gene by binding to a consensus sequence from the ribosomal gene cluster. Wild-type p53 will result in white colonies in the FASAY and mutant p53 will give red colonies. mRNA from the patient’s PBL was subjected to RT–PCR and the resulting cDNA was tested in the FASAY. The results show that only 3% red colonies were produced in this experiment, indicating a wild-type result (any value under 10% is considered wild-type). Wild-type p53 gave 2% red colonies, a non-functional mutant (344P) gave a result of 100% red colonies, and a partially functional mutant (337C) resulted in 98% pink colonies; all these results were as expected (Figure 3). The 7 base pair insertion mutation was then synthesised by site-directed mutagenesis, which was found not to have the mutation. The other parent

**Induction of apoptosis**

Apoptosis in Saos-2 cells can be induced by the expression of wild-type p53. Saos-2 cells were transfected with the mammalian expression plasmid pC53-SN3, which contained the 7 base pair insertion mutation of p53. Controls used included wild-type p53, 337C p53 (a semi-functional mutant) and 344P p53. The results showed that the 7 base pair insertion mutation retains 65% of the ability to induce apoptosis compared with wild-type (Figure 5). The 344P mutation only retained about 20% of the activity of wild-type p53 and the 337C mutant retained about 55%. Statistical analysis using both parametric (paired t-test) and non-parametric tests (Fisher’s exact test) on these data showed that the 7 base pair insertion mutant was significantly different from both wild-type p53 and the 344P mutant, (P < 0.02). The 7 base pair mutation was not significantly different from the 337C mutant.

**Transrepression**

This method was carried out on the mutants described previously and involved co-transfection of the mammalian expression plasmid pC53-SN3 with a reporter construct consisting of an SV40 promoter upstream of the β-galactosidase gene. Wild-type p53 will repress the SV40 promoter and therefore will prevent the expression of β-galactosidase. Addition of a β-galactosidase substrate should produce no colour change. The results obtained in this assay, showed that the 7 base pair insertion mutant and the 344P mutant could not effectively repress the SV40 promoter (Figure 6). The 337C mutant retained about 50% of the ability of wild-type to repress the SV40 promoter.

**Suppression of growth**

Wild-type p53 can inhibit the growth of Saos-2 colonies and so a colony forming assay was carried out to determine the ability of the 7 base pair insertion to inhibit growth. Saos-2 cells were again transfected with the pC53-SN3 expression plasmid, in order to observe which of the mutants could suppress the growth of colonies. The results showed that the 7 base pair insertion and the 344P mutants could not suppress the growth of colonies, compared to wild-type p53 (Figure 7).

**DISCUSSION**

The 7 base pair insertion is a novel mutation and is not recorded on the p53 database. The mutation is suspected to be de novo.
to the lack of family cancer history and the lack of mutation in the parent tested. The insertion being so large is unusual, as most reported insertions are between one and three base pairs. Cooper and Krawczak (1993) looked at 20 short insertions, nine of which were single base insertions. Two mechanisms for these insertions were proposed; they could be caused either by slipped mispair mediated by direct repeats (trinucleotide expansions) or mediated by inverted repeats (palindrome). The repeats stabilise a hairpin loop structure. The present reported insertion is a duplication of the previous 7 base pairs and occurs at a palindrome sequence of 8 base pairs.

An association between choroid plexus tumours and LFS has been suggested by others (Garber et al., 1990; Yuasa et al., 1993). Choroid plexus tumours are rare and are more common in childhood (Dohrmann and Collias, 1975). There have been three previous reports of adult onset choroid plexus tumours in families with multiple malignancies. Two of these were choroid plexus carcinomas, presenting at ages 16 and 27 (Li et al., 1988) and

**Figure 3** FASAY results. The FASAY gave a normal wild-type result with only 3% red colonies when mRNA was extracted from the patients PHA-stimulated leukocytes. In contrast the 7 base pair mutation synthesised by site directed mutagenesis gave 100% red colonies. The 344P mutation, which is an inactive mutant, also gave 100% red colonies and wild-type p53 gave only 2% red colonies.

**Figure 4** Western blot. The 7 base pair mutation was cloned into the mammalian expression plasmid pC53-SN3, which was transfected into Saos-2 cells. A Western blot was carried out using lysates from these cells, cells transfected with WT p53 and PHA stimulated lymphocytes from the patient. The blot showed WT p53 with the expected size of 53 kDa (lane 1) and the p53 from the patient’s lymphocytes also at size 53 kDa (lane 2). The cells transfected with the manufactured mutation showed a protein on the blot at about size 27 kDa (lane 3).

**Figure 5** Induction of apoptosis. The pC53-SN3 plasmid containing the p53 insert of interest was transfected into Saos-2 cells, which were washed after 16 h and reincubated for 72 h. The results show that the 7 base pair mutation retained about 65% of the apoptotic ability of WT p53. 344P, the non-functional mutant only retained about 20% apoptotic ability. Statistical analysis showed that the 7 base pair mutation was significantly different from both 344P and WT, with P < 0.02.
one, a choroid plexus papilloma, at age 29 (Faber, 1934). There have been only four previous reports of mutations in p53 found in families with choroid plexus tumours. Jolly et al., 1994; Frebourg et al., 1995; Sedlack et al., 1998; Vital et al., 1998). However, none of the previously reported mutations were insertions and the patients were all children.

The results from the first two functional assays carried out were normal (FASAY and Apoptotic assay). Further investigations were therefore carried out in order to characterise this mutation. The FASAY using the site-directed mutagenesis 7 base pair insertion cDNA gave an abnormal result (100% red colonies). This result most likely indicated that the mutant allele is expressed at an undetectable level in the original FASAY carried out using mRNA from PHA stimulated PBL from the patient. However, the normal apoptotic response of the patient’s cells was surprising as the presence of a non-expressed mutant allele would be expected to result in an abnormal apoptotic response. It is possible that stimulation by PHA of the lymphocytes favoured expression of the wild-type allele hence the wild-type result in the original FASAY. The lymphocytes used in the apoptotic assay are not treated with PHA and it is possible that both alleles were expressed at the very low levels seen normally in unstimulated PBL. This result would then be consistent with the ability of the 7 base pair insertion to induce apoptosis in Saos-2 cells. Western blotting of the patient’s PHA stimulated lymphocytes gave a single band present at the expected size of 53 kDa. The transfected Saos-2 cells showed a band at about 27 kDa, which was the size expected for a truncated protein with a stop codon at position 182. No truncated protein was seen in PBL from the patient and sequencing of the mRNA showed no mutation. The induction of apoptosis results showed that the 7 base pair insertion mutation retained 65% of the ability of wild-type to induce apoptosis in Saos-2 cells. However, the mechanism by which a 182 amino acid truncated p53 protein can partially induce apoptosis is not known. The studies on transactivation and transrepression in this report show the 7 base pair insertion mutation is not functional. The suppression of colony growth assay also showed the 7 base pair insertion mutation to be clearly inactive. However, in 1995 Haupt and colleagues reported a truncated protein, containing only the first 214 amino-terminal residues of murine p53, which was found to retain its ability to induce apoptosis in HeLa cells, but was transactivationally non-functional. These results are consistent with the findings described here for the 7 base pair insertion mutation. Wild-type p53-induced apoptosis may involve the processes of transactivation or transrepression of target genes. However, an intriguing possibility has arisen following reports that p53 can play a pro-apoptotic role by binding directly to the mitochondrial membrane and interacting with protein members of the bcl-2 family (Marchenko et al., 2000). It was suggested by these latter authors that such binding might indeed be the mechanism by which the truncated protein described by Haupt et al. (1995) induces apoptosis and the same could be true for the 7 bp insertion mutation.

In summary, the 7 base pair insertion mutation is a novel and unusual mutation. The clinical details for the patient were unusual, with the occurrence of a choroid plexus tumour at 29 years. The p53 mutation found in this individual is clearly functionally abnormal in terms of transactivation and transrepression of target genes and in suppression of colony growth. However, the normal results obtained for apoptosis induction in lymphocytes and the partial ability of the mutant to induce apoptosis in Saos-2 cells implies that a p53 protein with only half of the DNA binding domain present and no oligomerisation domain can retain significant ability to induce apoptosis. In terms of clinical significance it may be advisable to consider the possibility of germline p53 mutations in adults presenting with choroid plexus tumours since it may influence decisions regarding treatment and imaging.

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Functional studies on a novel germline p53 mutation

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