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Original Paper

A New Germline *TP53* Gene Mutation in a Family With Li–Fraumeni Syndrome

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This report describes an unusual clinical presentation of Li–Fraumeni syndrome. Family history revealed a mild aggregation of adult cancers in one generation, and an unusual clustering of brain tumours of early childhood in the following generation. In order to evaluate the genetic basis for cancer predisposition in this family, molecular genetic analysis for the occurrence of germline *TP53* tumour suppressor gene mutations was performed on 12 siblings of two generations. Indirect mutation analysis was performed by the single-strand conformation polymorphism (SSCP) technique. Alterations were characterised by automated direct fluorescence sequencing analysis. Tumour material was also examined for p53 protein accumulation by immunohistochemistry. Initially, a *TP53* gene germline missense mutation was detected in an 11-year-old kindred with acute myeloid leukaemia (AML) following intensive treatment of a brain tumour. In peripheral blood and bone marrow samples of this proband, a reduction to hemizygosity occurred. During AML treatment, detection of LOH of 17p was used as a marker for clonality and treatment control. The mutation was found to be inherited from the proband's mother, who was diagnosed with breast cancer at the age of 48 years. Further, three siblings were carriers, and two are apparently healthy at the age of 21 and 23 years. Knowledge of germline mutations may allow accurate DNA-based carrier diagnosis which is of important clinical significance for treatment strategy and control. Furthermore, the occurrence of unaffected carriers in this family raises questions about appropriate methods of cancer surveillance and counselling for these people.

Key words: Li–Fraumeni syndrome, familial cancer, childhood cancer, p53, tumour suppressor gene, brain tumour, leukaemia
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INTRODUCTION

ACQUIRED ALTERATIONS in the *TP53* tumour suppressor gene and its encoded protein represent the most common genetic changes in malignant human neoplasms [1]. The gene, located on the short arm of chromosome 17, encodes a 53 kDa nuclear phosphoprotein that binds specific DNA sequences and appears to be a transcription factor that may regulate the expression of other genes in either a positive or a negative manner [2, 3]. p53 protein is involved in the control of the cell cycle, DNA repair and synthesis, cell differentiation and programmed cell death. Mutant forms can act as dominant oncogenes, whereas wild-type *TP53* has the characteristics of a recessive tumour suppressor gene [4–8]. Somatic mutations in the *TP53* gene have been found in half of almost all types of cancer arising from a wide spectrum of tissue. Eighty per cent are missense mutations causing an amino acid to be substituted for another one, usually altering protein conformation and nuclear protein accumulation [9, 10]. These mutations are often associated with the complete loss of the other *TP53* allele, which is consistent with the two-hit model of inactivation of tumour suppressor genes first described by Knudson [11].

Inherited *TP53* gene mutations were identified in Li–Fraumeni syndrome (LFS), an autosomal dominant familial cancer syndrome [12–16]. Members of a Li–Fraumeni cancer family

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are at high risk of a wide spectrum of malignant tumours including breast cancer, soft tissue and bone sarcomas, and other diverse malignant neoplasms, particularly brain tumours and leukaemia. Furthermore, a high frequency of second primary tumours is observed in patients who survive their first cancer. The definition of Li–Fraumeni requires an individual to have a sarcoma diagnosed before the age of 45 years, a first-degree relative with cancer before the age of 45 years, and another first- or second-degree relative with either sarcoma diagnosed at any age or any cancer diagnosed under the age of 45 years [17]. Furthermore, germline *TP53* mutations have been reported in children with second malignant neoplasms and in patients with a strong familial history of cancer, but lacking the criteria for diagnosis of LFS [18–24].

These observations indicate that germline *TP53* mutations confer increased susceptibility to cancer, and the detection of these genetic alterations may allow the identification of patients and families at high risk.

We report the clinical implication of a new germline *TP53* gene mutation associated with LFS, identified in an 11-year-old child with acute myeloid leukaemia (AML), following aggressive treatment for a brain tumour.

PATIENTS AND METHODS

Family history

The cancer family pedigree of the proband (marked by an arrow) is given in Figure 1. A high incidence of cancer occurred in the family of the mother, whereas in the family of the father (data not shown) no unusual cancer history was found.

Malignant tumours occurred in two siblings of the mother, who has seven brothers and sisters altogether. One sister died at the age of 43 years of a soft tissue sarcoma of the upper extremities. The patient's mother was diagnosed to suffer from breast cancer when she was 48 years old. With anti-oestrogen therapy, she is still in remission. One sister was diagnosed with an endometrial carcinoma at 48 years and treated by surgery. She is still in remission and apparently healthy at the age of 73 years.

In the third generation, two children died of brain tumours at 5 and 13 years of age, respectively.

The patient presented was diagnosed for a primitive neuroectodermal tumour of the brain (WHO grade IV) when she was 7 years old. Four years after surgery, high-dose chemotherapy and radiotherapy treatment, she developed a myelodysplastic syndrome (MDS) with a rapid progression to an acute myeloid leukaemia (AML). During AML induction chemotherapy, she caught pneumonia and finally died of septic shock.

The other siblings of this generation, including two brothers and one sister of the patient were apparently healthy.

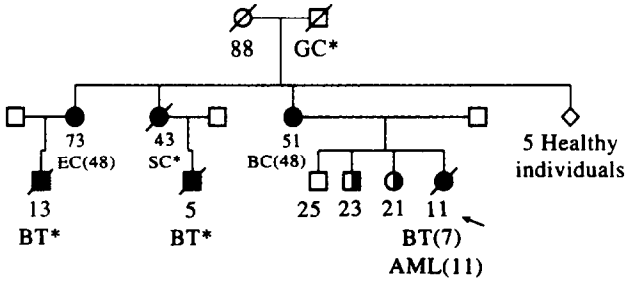

Figure 1. Pedigree of the family. The patient presented here is marked by an arrow. ● or ■, affected by neoplasm and presence of *TP53* germline mutation in codon 213 in white blood cells; ◨ or ◑, presence of *TP53* germline mutation in codon 213 in white blood cells, but not affected by neoplasm; numbers below represent current age or age at death (slash through symbol). *No material available for molecular genetic analysis. BT, brain tumour; EC, endometrial carcinoma; SC, soft tissue sarcoma; BC, breast cancer; AML, acute myeloid leukaemia.

Immunohistochemistry

Four micrometre sections of paraffin-embedded tumour material were mounted on poly-L-lysine coated glass slides and air-dried overnight at room temperature. Dewaxed sections were immersed in sodium citrate buffer (0.01 M Na-citrate monohydrate, pH 6.0) in plastic coplin jars and incubated in a labour autoclave (Göessner, GLA 40-2) at 120°C for 10 min. Following autoclaving, the slides were allowed to cool down to room temperature and rinsed in 0.1 M PBS (phosphate-buffered saline, pH 7.4). A polyclonal anti-p53 antibody (CM1, Medac) was applied at a dilution of 1:20000 and incubated at 4°C overnight. Immunohistochemical detection was performed by means of single phosphatase/anti-alkaline phosphatase (APAAP) technique [25]. A negative control for the immunostaining was carried out by replacing the primary antibody. As a positive control, sections were used from a previously studied gastric cancer that had a missense mutation of *TP53* in codon 248 and expressed p53 protein in most neoplastic cells.

DNA extraction

DNA was extracted from white blood and bone marrow cells and paraffin-embedded tissue by Proteinase K digestion and phenol/chloroform extraction [26]. DNA was precipitated in ethanol and resuspended in sterile water and stored at 4°C. Formalin-fixed and paraffin-embedded material were cut in 10 μm specimens. Excess paraffin was removed before Proteinase K digestion (0.1%) was carried out overnight at 55°C. After digestion, residual paraffin could be removed from the top of the digestion mixture with a pipette. DNA was subsequently extracted by standard phenol/chloroform methods as described above.

Single-strand conformation polymorphism (SSCP)

For analysis of genomic DNA by SSCP, *TP53* exons 4–8 were amplified in five different polymerase chain reaction (PCR) reactions. Oligonucleotide primers used for the different amplification regions are listed in Table 1. Briefly, each PCR reaction of a final volume of 20 μl contained: 40 ng of genomic DNA, sense and anti-sense primers at a final concentration of 0.2 pmol/μl, 2.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, 20 mM Tris–HCl pH 8.4, 50 mM KCl, 0.5 units Ampli-Taq polymerase (Perkin–Elmer Cetus). Samples were subjected to 35–40 cycles of denaturing (92°C for 30s), annealing (see Table 1 for individual annealing temperature for 30 s) and extension (72°C for 60 s).

For SSCP analysis, 1 μl of the amplification mixture was added to 1.5 μl 95% deionised formamide heated to 94°C for 2 min and kept on ice until loading. Samples were applied to a 10% native ultrathin polyacrylamide gel baked on GelBond Pag™ (FMC). Electrophoresis was carried out in a discontinuous buffer system with 35 mM sulphate–borate (pH 9.0) as the leading ion and 141 mM Tris–borate (pH 9.0) as the trailing ion [27]. Gels were run on a horizontal electrophoresis system (Multiphor, Pharmacia) at 15°C and 10 mA for 1.5 h. Bands were visualised by silver staining.

Table 1. PCR primers used for SSCP and sequencing of p53 exon 6

Exon	Primers	Annealing	Direction
5	5′-TTCCTGCAGTACTCCCCTGCC-3′	58°C	Sense
	5′-GCAAATTTCCTTCCACTCGG-3′		Antisense
6	5′-ACGACAGGGCTGGTTGCCCA-3′	60°C	Sense
	5′-CTCCCAGAGACCCCAGTTGC-3′		Antisense
7	5′-GTTGGCTCTGACTGTACCAC-3′	58°C	Sense
	5′-GGAAGAAATCGGTAAGAGGTGG-3′		Antisense
8	5′-ATTTCCTTACTGCCTCTTGC-3′	55°C	Sense
	5′-CCACCGCTTCTTGTCCTGCT-3′		Antisense
S_1	5′-ACGACAGGGCTGGTTGCCCA-3′	58°C	Sense
S_2	5′bio-CAAATAAGCAGCAGGAGAAAGC-3′		Antisense
S_3	5′-ACGACAGGGCTGGTTGCCCA-3′		Sense
S_4	5′-CTCCCAGAGACCCCAGTTGC-3′		Antisense

Detection of LOH of chromosome 17p

PCR amplification of the VNTR region YNZ.22 was carried out with primers (Table 2) flanking the YNZ.22 locus [28, 29]. The PCR reaction of a final volume of 20 µl contained: 40 ng DNA, 10 pmol of each primer and 0.5 units Taq-polymerase. Buffer conditions were the same as described above. Subsequent to initial denaturation, PCR was carried out with 40 cycles of denaturation (94°C, 1 min), annealing (63°C, 1 min) and extension (72°C, 5 min). Two microlitres of the reaction mixture were applied without further purification to a native 12% PAGE prepared for the Multiphor System. Gels were run under the same buffer conditions as described above at 15°C and 10 mA and subsequently silver stained.

Direct sequencing of PCR products

Sequence analysis of the *TP53* gene exon 6 was performed by solid phase sequencing of single-stranded PCR products. PCR fragments were obtained by amplification with the primers S_1 and S_2 (Table 1). For magneted bead purification, S_2 was biotinylated. PCR was carried out with the following modification: 100 µl final PCR volume containing 100 ng of genomic DNA, sense and anti-sense primers at a final concentration of 0.2 pmol/µl. Amplification followed 40 cycles of denaturing (94°C, 0.5 min), annealing (58°C, 1 min) and extension (72°C, 1.5 min). PCR products were purified through a Centricon 100 microconcentrator (Amicon), and resuspended in 40 µl distilled water. Immobilisation of the PCR product and subsequent preparation of single-stranded DNA fragments were performed with streptavidin coated beads, according to the manufacturer's protocols (Dynabeads M-280, Dynal). DNA sequences of the single-stranded PCR products were determined by dideoxy sequencing using S_3 and S_4 as sequencing primers, fluorescein-15-dATP as an internal label and T7 polymerase (AutoRead, Pharmacia) [27]. Gel electrophoresis, data collection and analysis were performed on an automated laser fluorescence sequencer (A.L.F. Pharmacia).

RESULTS

Surgical pathology and cytology

Histopathological examination of the brain tumour from the patient (marked by an arrow in Figure 1) presented in this report (primary diagnosis in 1990) revealed a highly anaplastic neoplasm with marked mitotic activity and focal necrosis (Figure 2a). Immunohistochemistry showed strong nuclear staining of p53 protein in the tumour cells (Figure 2b). In normal skin tissue obtained by autopsy, no p53 protein accumulation was detected (data not shown).

Molecular biology

Initially, DNA from bone marrow and peripheral blood samples obtained at diagnosis of MDS were screened for *TP53* gene mutations by non-isotopic SSCP analysis of exons 5–8. The SSCP analysis of the *TP53* gene exon 6 showed an additional band and the loss of one wild-type band indicating reduction to hemizygosity (Figure 3a). The same pattern was observed in SSCP analysis of the brain tumour material. In contrast, in normal skin tissue, both wild-type bands were found in combination with the additional band, suggesting the presence of a germline mutation in *TP53* gene exon 6. SSCP analysis for the *TP53* gene exon 6 of the direct siblings from the patient detected the same additional band in the white blood cells from the mother, one sister and one brother, indicating that the mutation was inherited from the mother. SSCP analysis of *TP53* exon 6 of DNA obtained from the father and the other brother revealed only wild-type bands (Figure 3b). Screening of a further six family members of the

Table 2. Amplification primers used for LOH analysis of YNZ22 locus

Primers	Annealing	Direction
5′-AAACTGCAGAGAGAAAGGTCGAAGAGTGAAGTG-3′	63°C	Sense
5′-AAAGGATCCCCCACATCCGCTCCCCAAGTT-3′		Antisense

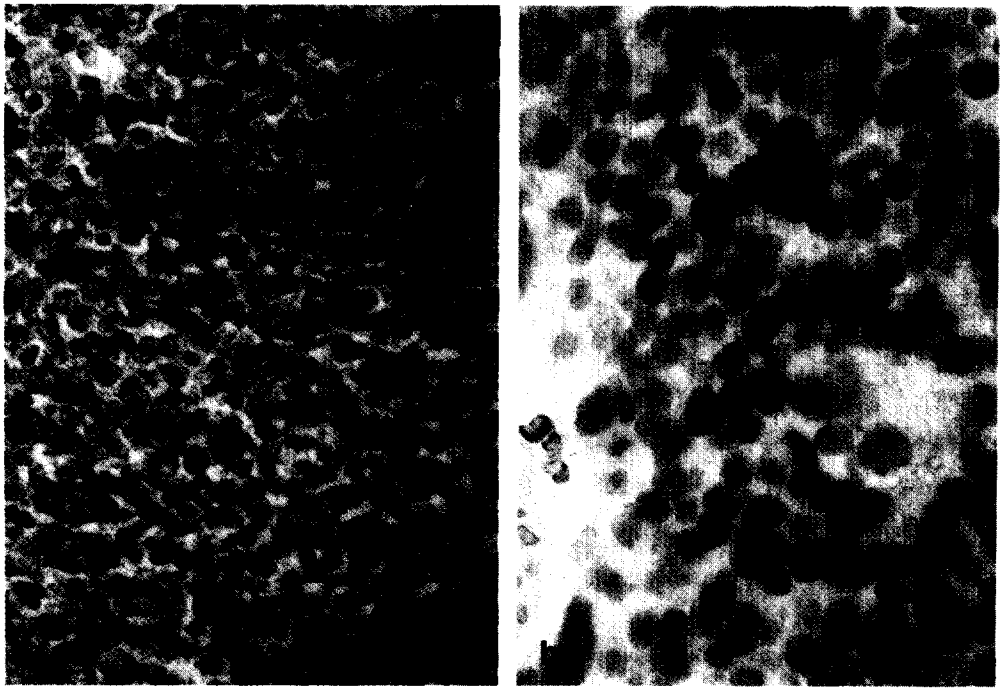

Figure 2. (a) Highly anaplastic neoplasm of the brain with strong mitotic activity and focal necrosis (haematoxylin staining × 250). (b) Immunohistochemical examination of the brain tumour revealing a heterogenous strong nuclear accumulation of p53 protein (× 400).

second generation revealed an altered migration pattern in the 73-year-old sister of the proband's mother, who was diagnosed with an endometrial carcinoma at the age of 48 years and treated by surgery. Her son, who developed a brain tumour, died at age 13 years. Material for DNA analysis was not available. Five other kindreds, who were found apparently healthy, did not exhibit mobility shifts in the SSCP analysis. No material was available from one sibling, who died of a sarcoma at the age of 43 years, nor from her son, who died of a brain tumour at age 5 years.

Direct sequence analysis identified a missense germline mutation in codon 213 of the *TP53* gene exon 6 in the constitutional DNA from all individuals with altered SSCP pattern. This mutation was characterised as a heterozygous mutation with CGA to CCA transversion leading to an amino acid exchange arginine to proline (Figure 4). The germline mutation was not present in white blood cells from all family members without altered SSCP pattern. As shown in the pedigree (Figure 1), the mutation was detectable in the second generation in two of six kindred.

To confirm LOH in the brain tumour cells and leukaemic cells of the proband, PCR amplification of the VNTR locus YNZ22 on chromosome 17p was performed. Amplification results are listed in Table 3. Reduction to hemizygosity was detected in the brain tumour and in the cells obtained from bone marrow and peripheral blood samples during MDS and AML since the paternal allele no. 5 could not be identified. In normal tissue of the patient, both alleles were present. The maternal allele no. 6 was found to be inherited in all offspring with proven occurrence of the mutation, suggesting that this allele might be linked to the *TP53* gene mutation in exon 6. In one offspring, YNZ22 allele no. 6 was inherited, but the mutation was not detected.

During AML induction therapy, peripheral blood samples were analysed by SSCP twice a week in order to detect recurrence of wild-type *TP53* allele and to monitor the success of the therapy. However, in all samples obtained, a persistent reduction to hemizygosity was detected. Furthermore, bone marrow tissue obtained during autopsy was shown to be hemizygous as well.

DISCUSSION

The Li–Fraumeni syndrome (LFS) is an autosomal dominant cancer syndrome, initially recognised through clinical observations at the bedside, followed by epidemiology studies, and molecular biology investigations to search for associated genetic alterations [12]. Germline mutations of the *TP53* gene have been found to be associated with this cancer syndrome, which is consistent with the first hit in Knudson's two-hit mutational model of hereditary cancer [13, 14]. Even though all the cells of the individuals have a *TP53* mutation, they have a propensity to develop only certain types of cancer. Soft-tissue sarcomas usually develop in the first 5 years of life, brain tumours and acute leukaemias throughout childhood and young adulthood, and breast cancer is the most common neoplasm of the adult [30–32]. The family cancer syndrome reported here best fits the definition of the Li–Fraumeni syndrome. However, the familial clustering of brain tumours in one generation is unusual. Whereas, in the second generation, a mild aggregation of adulthood cancers was found, the predominant type of cancers in the third generation were brain tumours occurring in early childhood. Two of the carriers of the germline *TP53* mutation were free of cancer and apparently healthy at the age of 21 and 23 years, respectively. Although brain tumours show a high incidence in LFS, the predominant occurrence of brain tumours in one generation is remarkable, but may reflect the variability of the clinical definition of LFS. So far, tissue-specific properties of distinct *TP53* gene mutations have not been described. A *TP53* germline mutation in a familial brain tumour syndrome has been

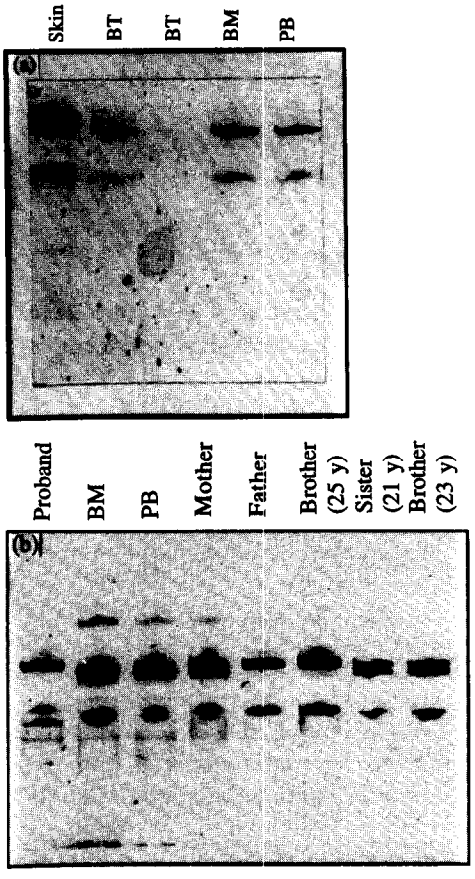

Figure 3. (a) SSCP analysis of normal skin tissue, bone marrow (BM) and peripheral blood (PB) samples obtained at diagnosis of MDS, and DNA retrospectively retrieved from formalin-fixed and paraffin-embedded brain tumour (BT) material. Whereas SSCP analysis of normal skin tissue from the patient revealed two upper bands, in bone marrow, peripheral blood and brain tumour material, only one upper band was detected, indicating loss of heterozygosity. (b) SSCP examination of the family revealed two upper bands in the constitutional DNA from the mother, the 21-year-old sister and the 23-year-old brother. In white blood cells from the father and the other brother wild-type bands as shown for the normal control band were detected. Loss of the upper wild-type band was still detected in the bone marrow and peripheral blood samples from the patient at diagnosis of AML.

recently found and was characterised as a deletion of codon 236 in exon 7 [24]. Furthermore, *TP53* mutations detected in brain tumours or in the germline of kindreds with brain tumours have not been shown to cluster at a certain site of the gene [33–35]. Interestingly, codon 213 has been described as a neutral polymorphic site with a silent mutation in the third position of the codon [36, 37]. However, codon 213 represents a rare localisation for mutations leading to protein alterations. So far, a C:G to T:A transition leading to a premature stop-codon has been detected in a Japanese family with a mild aggregation of adulthood cancer with a high prevalence of stomach cancer, and in Japanese patients with sporadic gastric cancer as a germline and somatic mutation, respectively [38, 39]. The same type of mutation has been detected in some Burkitt's Lymphoma patients [40]. The *TP53* germline mutation reported in the family presented here has not previously been described, neither in LFS nor in sporadic malignancies. This mutation most likely displays the genetic variability of *TP53* alterations found in LFS. Furthermore, the clinical and genetic findings support the concept that other factors, both genetic and environmental, may influence the onset and type of cancer in patients who are carriers of the same *TP53* germline mutation. Although the mutation is localised outside the conserved region of the gene, it may alter DNA binding properties because codon 213 lies within the region involved in binding to heat shock proteins [41]. As shown by immunohistochemistry, this mutation resulted in an accumulation of p53 protein in the tumour cells but not in normal tissue. Examination of YNZ22 locus revealed LOH of 17p in the brain tumour-derived DNA, and in the DNA of the bone marrow and peripheral blood cells obtained during MDS and progression to AML, as already suggested by SSCP and sequence analysis. DNA of normal tissue from the patient and all constitutional DNA from the kindreds carrying the mutation were heterozygous for the mutation as well as for the YNZ22 locus. Interestingly, in one sibling (25 years), the YNZ22 allele no. 6 from the mother was inherited. This allele was identified as transmitted with the *TP53* mutation and was detected in all offsprings who were carriers. However, in this sibling the mutation was neither detected in SSCP nor in sequence analysis of his constitutional DNA. This is most likely caused by crossing-over during meiosis since the *TP53* gene is located more than 40 centimorgan centromeric to the YNZ22 locus [42]. This finding demonstrates that linkage analysis using extragenic markers may lead to false positive identification of carriers. For this reason, indirect screening assays, such as the detection of SSCPs and the PCR analysis of intragenic p53 polymorphisms, provide more reliable results in the identification of carriers within a family [16, 29].

The occurrence of LOH in brain tumour cells and in the bone marrow and blood cells obtained during MDS and AML is consistent with the concept of tumour suppressor gene function. Although *TP53* alterations are infrequently detected in primary MDS and AML patients, it has been frequently identified in myeloid cell leukaemia cell lines [43–48]. Furthermore, individuals with LFS have an increased incidence of leukaemias. A high incidence of *TP53* inactivation in human myeloid leukaemic cell lines suggests that inactivation of normal p53 function plays an important role in the establishment of those lines. Whereas normal myeloid progenitor cells continuously undergo apoptosis in the absence of appropriate differentiation and proliferation signals, cells with *TP53* inactivation might have a selective growth advantage by decreasing their rate of apoptotic cell death. In the patient presented here, MDS with progression to AML was detected as secondary neoplasia 4 years after successful treatment of a brain tumour. The increasing evidence that secondary MDS and leukaemia result from the treatment of several primary malignancies generates tentative conclusions that many of the alkylating agents are leukaemogens [49]. Recently, epipodophyllotoxin and, to a lesser extent, cisplatin have been reported as secondary causes for leukaemias [50, 51]. The role of the *TP53* gene has been defined as the "genomic guardian" that monitors the integrity of the genome [4]. Cells with mutant *TP53* are only partially blocked against genotoxins and, therefore, acquire selective growth advantage and accumulate additional genetic alterations. Furthermore, it has been demonstrated that loss of wild-type *TP53* leads to gene amplification and enhances the possibility of genomic rearrangements, and the absence of wild-type *TP53* is associated with a significant increase in cellular resistance to irradiation and chemotherapy [52–54]. Thus, the occurrence of the AML is most likely the result of

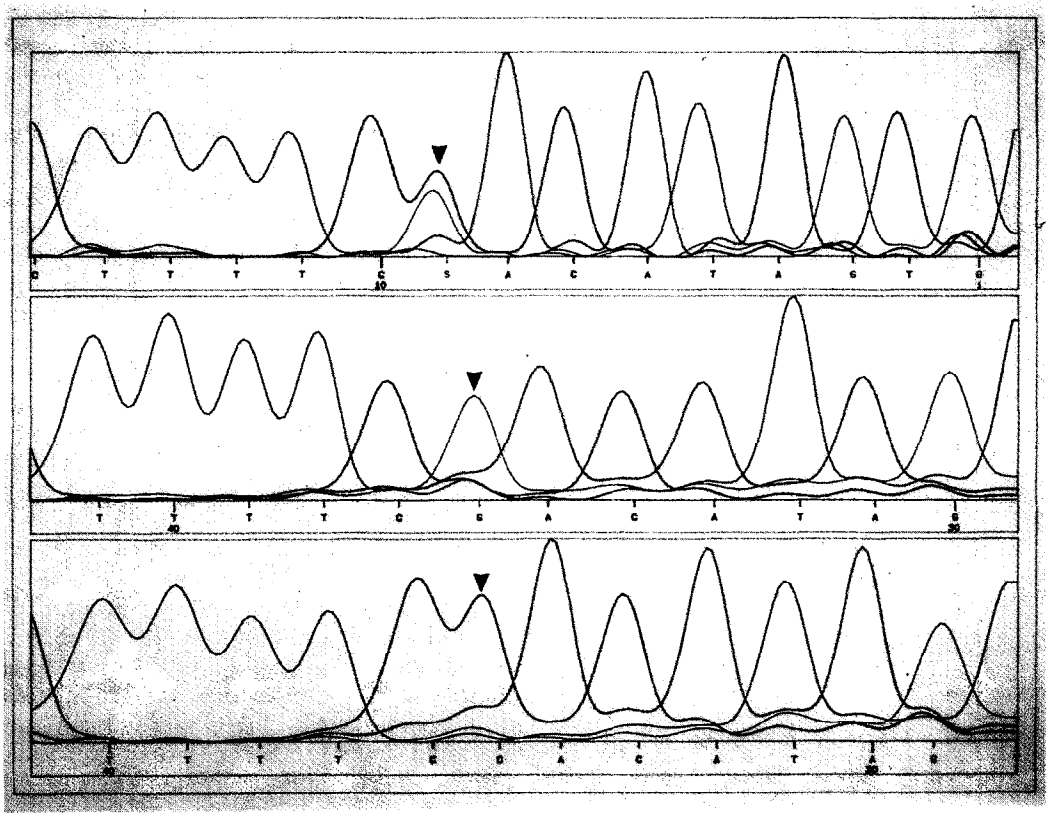

Figure 4. DNA sequencing analysis of the *TP53* exon 6. A heterozygous mutation (G to C) was identified in codon 213 in the constitutional DNA from the mother (upper lane). A wild-type sequence was found in DNA obtained from the white blood cells of the father (middle) and a loss of wild-type sequence was detected in the patient's DNA of a bone marrow sample obtained at diagnosis of MDS (lower lane). Base positions are marked by arrows.

Table 3. VNTR analysis for YNZ22 locus on chromosome 17p

	Alleles
Proband bone marrow	6/—*
Proband normal tissue	6/5
Mother	3/6
Father	5/9
Sibling 21 years	6/9
Sibling 23 years	6/9
Sibling 25 years	6/9

*LOH.
Alleles were numbered according to size [28].

the combination of the germline *TP53* mutation and treatment for the brain tumour with radiation and high dose alkylating agents applied.

As shown by SSCP and sequencing analysis, the complete reduction to hemizygosity, which was detected in bone marrow and peripheral blood samples at primary diagnosis of MDS and during AML treatment, leads to the supposition that malignant transformation has occurred in an early precursor cell. Furthermore, a persistence of clonal cell expansion was demonstrated since, during AML induction therapy, no detectable recurrence of the wild-type *TP53* allele was found, indicating a relative therapy resistance. This is consistent with findings that somatic *TP53* mutations are detected in patients with advanced variants of primary MDS and AML associated with poor response to treatment and short survival [55, 56].

In summary, the cancer pedigree of the family described in this report underlines that LFS comprises a wide variability of clinical and genetical features. Since the current understanding of LFS and its association with germline *TP53* mutations is still incomplete, additional studies—such as linkage analysis to identify genetic heterogeneity, age-specific penetrance of the mutant genes and the role of environmental carcinogens—are needed to elucidate the complete genetic background of this cancer syndrome. However, the clinical implications of germline *TP53* mutations are evident. Detection of germline *TP53* alterations may influence therapeutic intervention and lead to tailored, risk-adapted treatment regimens. Identification of carriers in predisposed families will need effective genetic counselling, but will provide the potential to develop prevention strategies and finally may lead to the diagnosis of tumours at an earlier stage and presumably reduce mortality.

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