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ORIGINAL ARTICLE

An identical, complex *TP53* mutation arising independently in two unrelated families with diverse cancer profiles: the complexity of interpreting cancer risk in carriers

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Most inherited *TP53* mutations have been identified in individuals with a family cancer predisposition syndrome, in which the activity of p53 mutants is severely reduced. However, germline p53 mutants in children with 'sporadic' adrenocortical or choroid plexus tumors exhibit a wide range of functional activity. Here, we demonstrate the occurrence of a complex germline *TP53* mutation in two unrelated families with different cancer phenotypes, neither fulfilling the classic criteria for Li-Fraumeni syndrome. The *TP53* mutation consists of a duplication of 7 bp in exon 4, resulting in a frame shift and premature stop signal. Haplotype analysis indicated that the mutation arose independently in the two families. Analysis of the DNA secondary structure predicts the *TP53* mutation occurred within a hairpin loop. Additional germline complex mutations occurring within the same region of exon 4 have been identified in the IARC database. Our findings suggest that certain *TP53* regions are prone to intrinsic genetic alterations, possibly through defects in DNA replication or repair. Further, carriers of the same *TP53* mutation can have diverse cancer profiles, illustrating the complexity of genetic counseling and risk prediction.

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INTRODUCTION

Inherited mutations in the *TP53* tumor suppressor gene are associated with susceptibility to cancer.[1] Most of these mutations have been identified in individuals from families with Li-Fraumeni syndrome (LFS), Li-Fraumeni-like syndrome or *TP53*-associated malignancies such as pediatric adrenocortical tumors (ACTs)[2,3] and choroid plexus carcinoma.[4,5] In familial cancer syndromes, functional studies have shown severe reduction in the activity of p53 mutants.[6] Conversely, the p53 mutants show varying degrees of functional activity in children with ACT or choroid plexus carcinoma, and some have wild-type (WT) activity.[2] In carriers of the latter p53 mutations, the spectrum and the onset of malignancies differ from those in carriers belonging to LFS families.[7,8] Varley *et al.*[9] and Birch *et al.*[10] suggested the existence of inherited low-penetrance *TP53* mutations that are associated with ACT but not with a specific familial cancer susceptibility syndrome. The tumorigenic effect of such low-penetrance mutants may depend on cooperating age-and tissue-specific genetic changes. Therefore, detailed and active life-long genotype/phenotype studies in the families of individuals carrying low-penetrance mutations may reveal genetic abnormalities and pathways associated with tumorigenesis. Here, we report a child with ACT who carried a complex, inherited *TP53* mutation identical to a mutation previously reported in a young woman with breast cancer from a family that did not fit the strict LFS criteria.[11] Detailed haplotype analysis of these families confirmed that the mutation arose independently in each.

RESULTS

Case report

We studied two apparently unrelated families that shared no common surnames or known relationships. The first family was that of a patient entered into the International Pediatric Adrenocortical Tumor Registry (IPACTR) (http://www.stjude.org/ipactr). A previously healthy 2-year-old girl in Honduras had a 4-week history of hirsutism, acne, pubarche and strong adult body odor. The patient weighed 12.5 kg (50th percentile) and was 82 cm tall (25th percentile). Her blood pressure was 90/60 mm Hg. She had a verrucous clitoris and Tanner stage III pubarche. A mass was palpable in her left hemi-abdomen. The family history of cancer did not fit the criteria for LFS. Her father had surgical resection of a lung mass (unknown histology) at the age of 2 years, her paternal grandmother had uterus (cervix) cancer, a great grandmother had lung cancer at age 67, and the paternal great grandfather had skin cancer (melanoma). Laboratory tests revealed adrenocorticotropic hormone <5 pg/ml (normal range <46 pg/ml); morning cortisol $=12.9$ μg/dl (normal range 5–25 μg/dl); dehydroepian-

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drosterone sulfate = 652 μg/dl (normal range 35–430 μg/dl) and 17α-hydroxyprogesterone = 1370 ng/dl (normal range 3–51 ng/dl). Abdominal ultrasonography revealed a left adrenal mass that was surgically removed. Grossly, it measured 4.4 cm and weighed 15 g. Histopathological findings revealed nuclear grade 3, clear cell, abnormal mitosis <25%, diffuse architecture and no capsular or venous invasion or necrosis. This family is referred to as HON001. Clinical and genetic studies of the second family, which resided in Mexico (MEX001), were recently reported.[11] The index case was a 23-year-old woman with breast carcinoma (lobular carcinoma plus infiltrating ductal carcinoma). Her father, carrier of this mutation, had leiomyosarcoma at age 67 years, and her half-sister died of bronchioloalveolar carcinoma at age 25 years. The patient had a healthy brother in his 20s who carried the mutation. The pedigrees of both families are shown in Figure 1.

Sequence analysis of *TP53*

TP53 sequence analysis of genomic DNA from family HON001 revealed a duplication of 7 bp (GTTTCCG) (c.329_330 ins7) affecting codons 108–110 in exon 4, which generated a frame shift and a premature stop signal at position 150. The mutation was heterozygous in the germline of the ACT patient and her father (Figure 2a). The mother was WT for *TP53* sequence. The ACT underwent loss of heterozygosity (LOH) tissue with deletion of the WT allele. The same 7 bp duplication in exon 4 was observed in the germline of the Mexican family, including the father with leiomyosarcoma (67 years), the daughter with early-onset breast cancer (25 years) and additional family members who were healthy (8). As shown in Figure 2b, the leiomyosarcoma remained heterozygous for the mutation (Figure 2b, panel II), whereas the breast tumor underwent LOH and selected the mutant allele (Figure 2b, panel III).

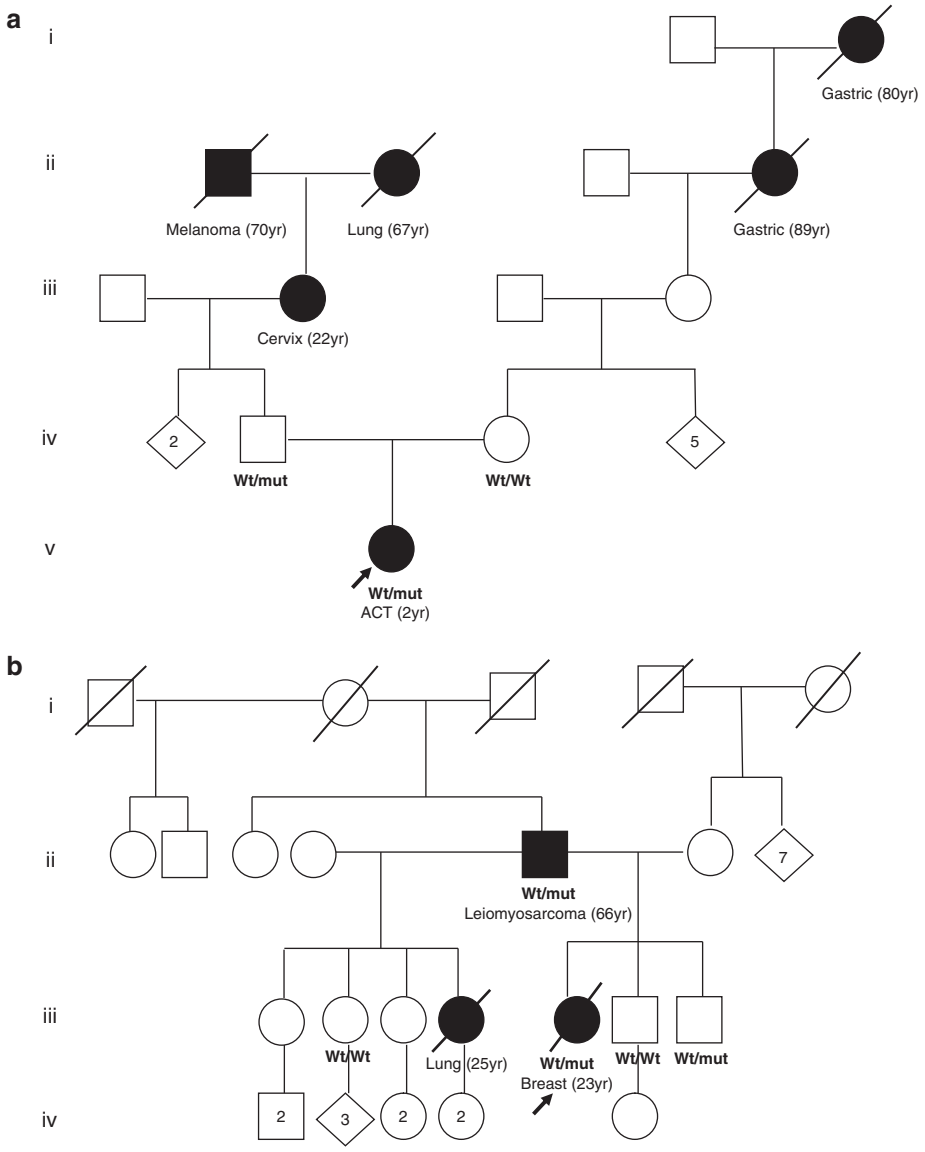

Figure 1. Genealogy of the two families' cancer history. (**a**) The Honduran family. The pediatric patient with ACT is a carrier of the duplication of 7 bp (GTTTCCG) (c.329_330 dupl GGTTTCC) affecting codons 108–110 in exon 4 of the *TP53* gene. Her father is also a carrier of this mutation. (**b**) The Mexican family. The patient with breast cancer, her father with sarcoma and a healthy brother are carriers of the same mutation observed in the Honduran family. The circles represent females, whereas the squares represent males. Open symbols indicate no presence of tumor and filled symbols represent those with tumor; crossed symbols indicate deceased individuals. Arrows indicate proband. TP53 status is identified above those cases that biological material was accessed.

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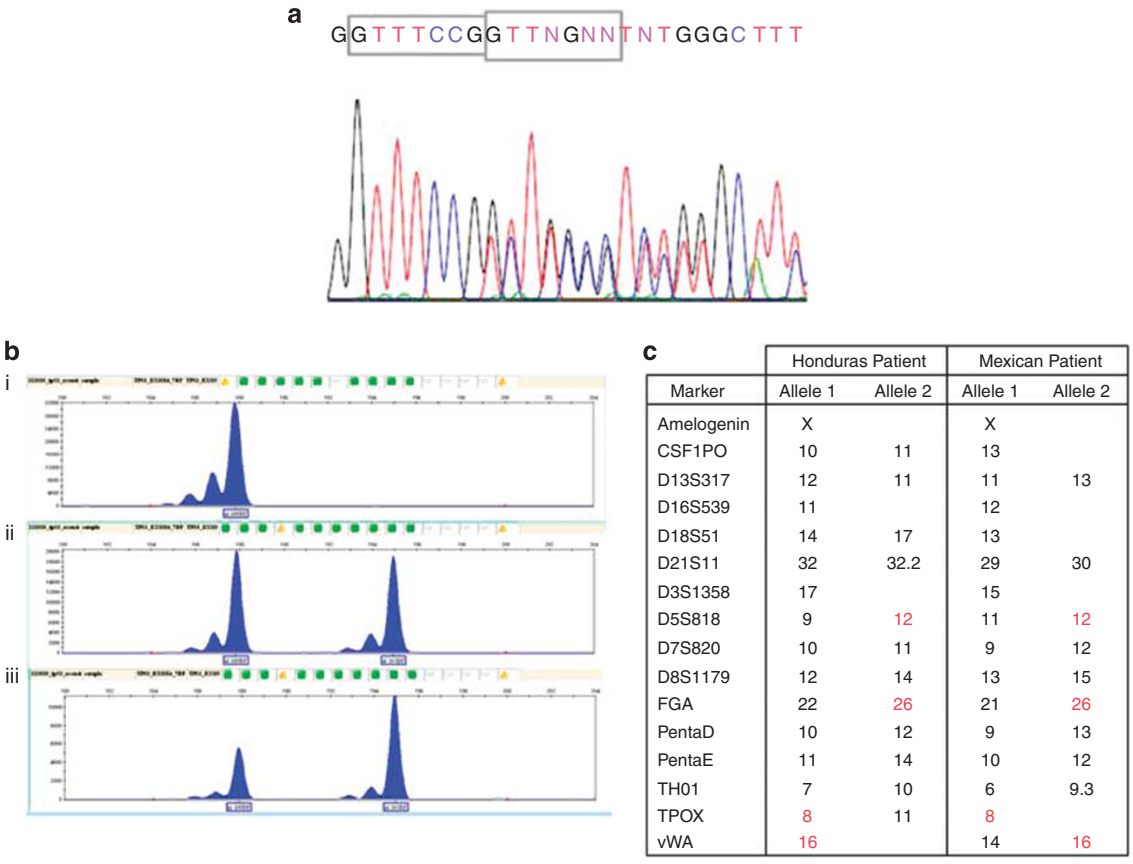

Marker	Honduras Patient Allele 1	Honduras Patient Allele 2	Mexican Patient Allele 1	Mexican Patient Allele 2
Amelogenin	X		X	
CSF1PO	10	11	13	
D13S317	12	11	11	13
D16S539	11		12	
D18S51	14	17	13	
D21S11	32	32.2	29	30
D3S1358	17		15	
D5S818	9	12	11	12
D7S820	10	11	9	12
D8S1179	12	14	13	15
FGA	22	26	21	26
PentaD	10	12	9	13
PentaE	11	14	10	12
TH01	7	10	6	9.3
TPOX	8	11	8	
vWA	16		14	16

Figure 2. Sequence analyses of *TP53*. (**a**) Chromatogram showing a 7-bp duplication, in heterozygous state, in the *TP53* gene. (**b**) Loss of heterozygosity (LOH) analysis. Upper panel (I): the WT allele in a homozygous state; center panel (II): the WT and mutant alleles in a heterozygous state; lower panel (III): an allelic imbalance indicating LOH. (**c**) Genetic profiling of the index case in each family, using 15 single tandem repeat markers. This analysis shows only alleles reported to be most common in the overall populations of their respective countries. The patients share only four alleles, red ones (the most common in both populations).

Haplotype analysis

We analyzed the structure of the *TP53* locus to precisely identify the haplotype carrying the complex *TP53* mutation. The HON001 ACT patient was heterozygous for the VNTRp53 marker (127/132) and homozygous for the p53(CA)$_n$ (122/122), allele G (rs1642785) in intron 2, arginine in codon 72 (rs1042522) and C and T for single-nucleotide polymorphisms rs12947788 and rs12951053 in intron 7. Therefore, the patient harbored the carrier haplotype represented by allele 122 for p53(CA)$_n$ and 127 for VNTRp53; this haplotype was also observed in the ACT DNA and in the germline of her father's DNA (Figure 3a).

The MEX001 breast tumor patient was homozygous for all markers, with allele 122 for p53(CA)$_n$, allele 132 for VNTRp53, allele C for single-nucleotide polymorphism rs1642785 in intron 2 and allele C (proline) at codon 72 (rs1042522). Single-nucleotide polymorphisms rs12947788 and rs12951053 at intron 7 were T and G, respectively. The carrier haplotype was also observed in this patient's father germline DNA. The paternal half-sister (39 years) and two brothers (22 and 20 years) of the patient were also analyzed. Only the younger brother, who is healthy and cancer free, inherited the carrier haplotype (Figure 3b).

These data demonstrate a different haplotype for the inherited 7 bp duplication *TP53* mutation occurring in the Honduras and Mexican families. Corroborating these findings, the two index cases (HON001 ACT and MEX001 breast cancer) were profiled by using a set of 15 single tandem repeat markers and only four alleles, all of which were commonly found in the populations of the patients' respective countries, were matched suggesting no close ancestry (Figure 2c).

Copy-number variation (CNV) analysis

Nine genomic DNA samples were tested for CNV by multiplex ligation-dependent probe amplification (MLPA). All probes for the *TP53* gene revealed extensive CNV in the ACT DNA and no CNV in the leiomyosarcoma and breast tumor tissue (Figure 4).

Analysis of DNA secondary structure of WT *TP53* exon 4

The DNA secondary structure of WT *TP53* exon 4 was predicted using the Mfold program.[12] Because of the limitations of the mathematical model and uncertainties in the thermodynamic parameters used in these methods, Mfold predicted multiple suboptimal structures with similar free energy values. The program predicted a secondary structure between codons 107 and 110, suggesting that the duplication event occurred within a hairpin structure (Figure 5).

p53 Immunostaining

The pediatric ACT showed strong to weak cytoplasmic staining for the p53 protein in almost all tumor cells. By contrast, p53 immunostaining was negative in the leiomyosarcoma and breast tumor tissue (Figure 6).

DISCUSSION

Here we report an identical, inherited, complex germline mutation in the *TP53* gene arising independently in two unrelated individuals from families with different cancer profiles. Because of the uniqueness of the mutation and the shared Hispanic

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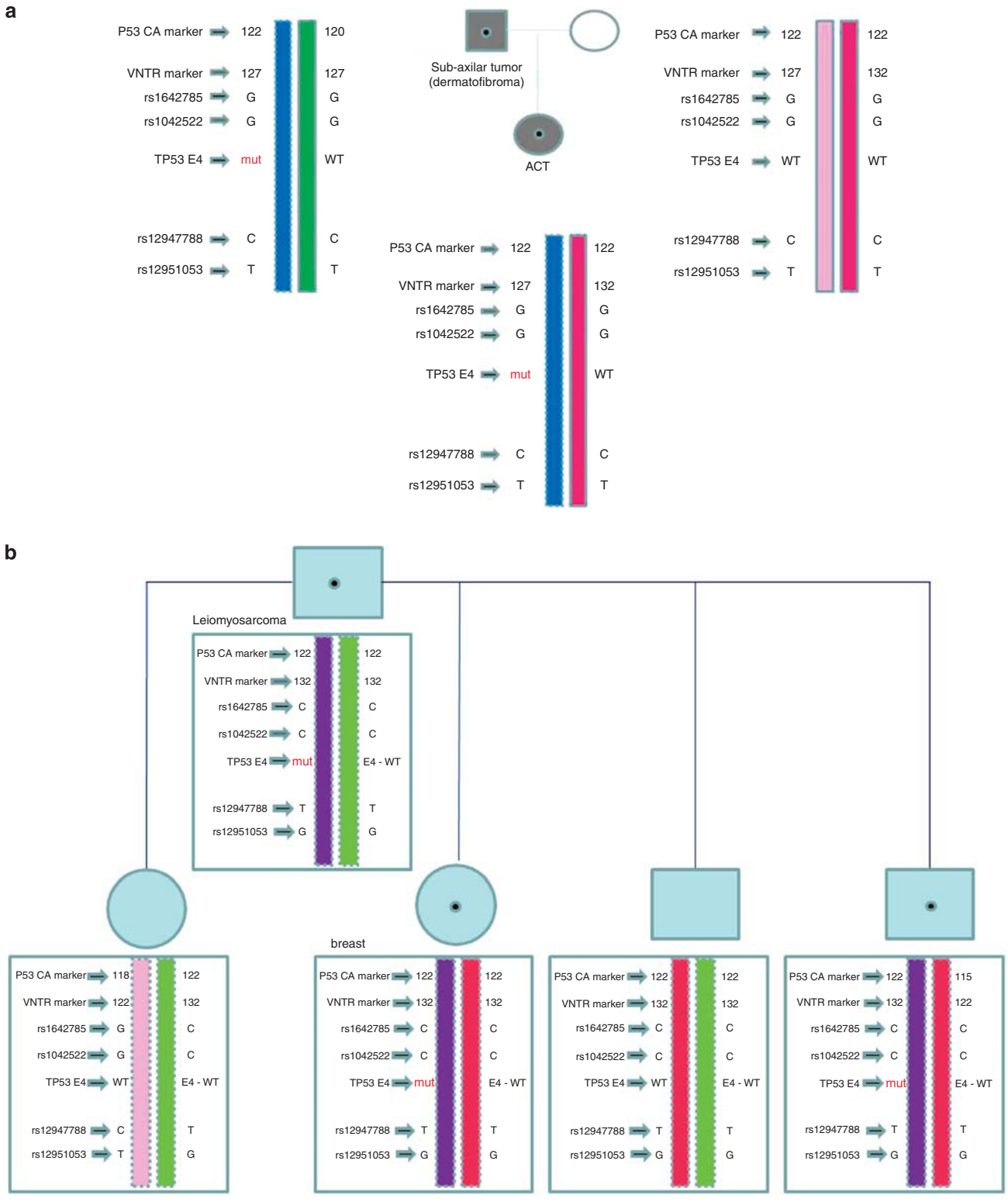

Figure 3. Detailed haplotype analysis for the *TP53* locus. (**a**) The haplotype in the Honduran family. (**b**) The haplotype in the Mexican family.

heritage, we considered the possibility of a founder effect. In population genetics, founder events occur when a new population is established from a few individuals descended from a large ancestral population.[13] The founder effect has been reported only once for a *TP53* mutation (Arg337His).[14] Our detailed *TP53* analyses in both families definitively showed different haplotypes in each family, ruling out the possibility of a common founder. Corroborating evidence was provided by genetic profiling of the two index cases with 15 single tandem repeat markers, clearly showing that they shared only alleles commonly identified in the populations of their respective countries.[15,16] We observed no shared rare alleles that would increase the likelihood of common ancestry.

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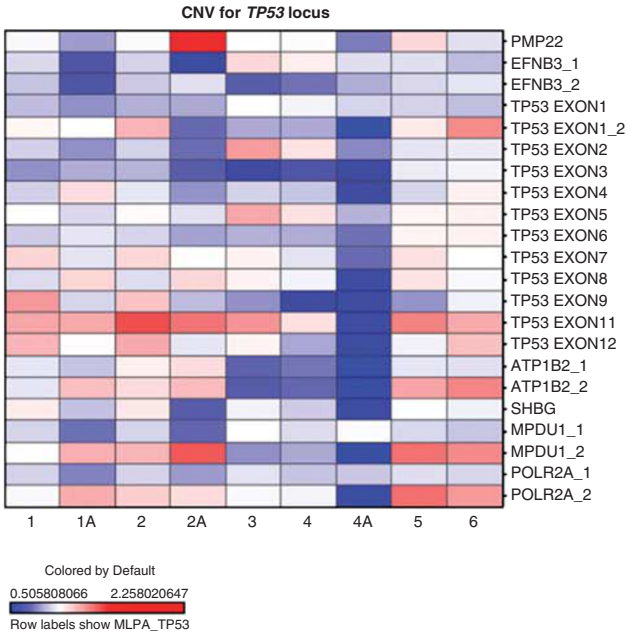

Figure 4. Heat map derived from MLPA analysis of the *TP53* locus and nearby genes in nine samples. No significant CNV was observed between germline (1) and breast tumor tissue (1A) or between germline (2) and sarcoma tissue (2A). However, extensive CNV was observed between germline (4) and tumor tissue (4A) from the patient with ACT. Healthy individuals from both families were included in this analysis (3,5,6). Two (3 and 5) were carriers of the mutation, and their germline samples showed no significant CNV. Sample 6, from the mother of the patient with ACT (4), had the WT *TP53* sequence. Red indicates duplications (>1.3 ratio to normal controls). Blue indicates deletions (<0.7 ratio to normal controls).

In the late 1960s, Li and Fraumeni observed a high concentration of cancers in four families, in which siblings or cousins had childhood sarcoma; a high frequency of soft-tissue sarcoma and breast carcinoma was noted in the ancestral line of one parent in each family. Acute leukemia, brain tumor and carcinoma of the lung, pancreas and skin frequently occurred in first- and second-degree relatives, and adrenocortical carcinomas were often diagnosed in children.[17] This nonrandom aggregation of cancer is termed Li-Fraumeni syndrome. Classic LFS criteria include (1) diagnosis of sarcoma before age 45 years; (2) at least one first-degree relative with a cancer of any kind before age 45; and (3) A

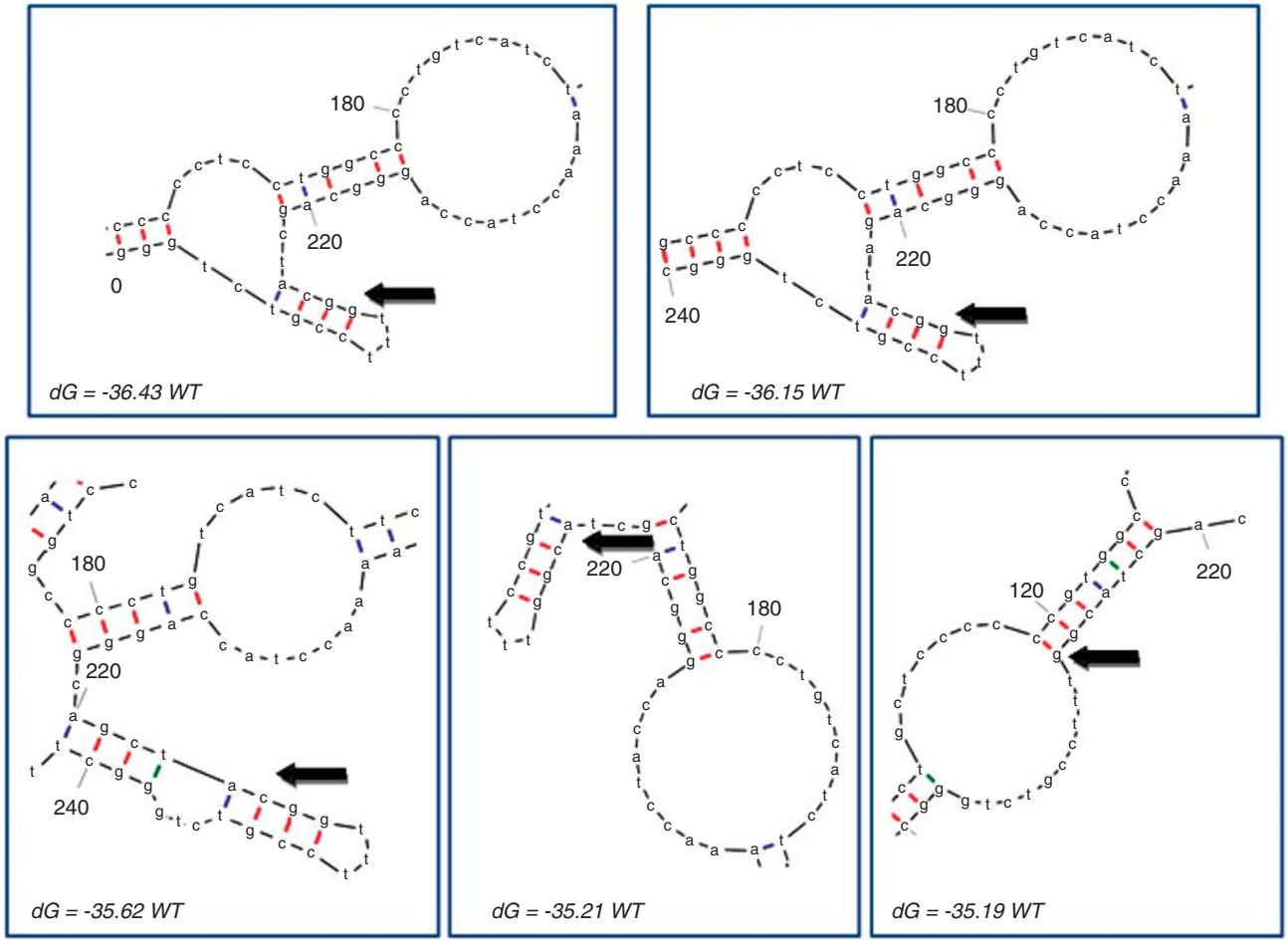

Figure 5. Predicted secondary structures for WT exon 4 TP53 DNA fragment. The positions of cleavage sites between exons 107–110 are indicated by arrows. The calculate free energies for each structure at 1 M NaCl and 37 °C are shown.

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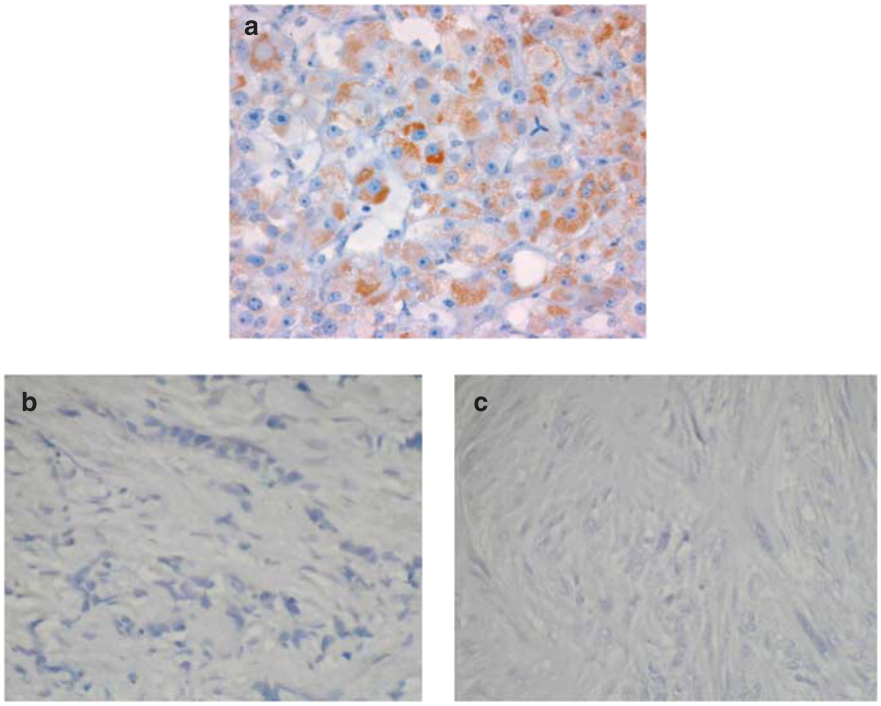

Figure 6. Immunohistochemical staining pattern for p53 protein. (**a**) Strong to weak cytoplasmic staining is seen in almost all ACT cells. Staining was negative in the breast tumor (**b**) and sarcoma (**c**) specimens. Appropriate positive and negative controls were implied.

third family member (a first- or second-degree relative) with cancer under age 45 or with sarcoma at any age.[18] Although the family histories of our cases did not fit these criteria, the presence of ACT in one family and early-onset breast cancer in the other are an acceptable indication for *TP53* mutation testing.[19] This less stringent recommendation should identify *TP53* mutations that may be associated with an increased cancer predisposition even in the absence of a defined familial pattern.

LOH at the *TP53* locus was observed in both the ACT and breast tumor, but not in the leiomyosarcoma (Figure 2). CNV supports LOH findings in ACT and leiomyosarcoma (Figure 4). However, the loss of the p53 allele in the breast tumor was not accounted for by the MLPA suggesting a neutral copy number, possibly due to gene conversion. Consistent with the loss of the nuclear localization signals in the C-terminus of p53, the truncated mutant p53 protein was expressed at detectable levels within the cytoplasm of the ACT (Figure 5). Although the other tumors (breast and leiomyosarcoma) harbored the same mutation, the samples were negative for p53 staining, indicating that immunohistochemistry alone is not always informative for assessing *TP53* status.[20]

The *TP53* mutation observed in our Honduran pediatric ACT and Mexican breast cancer patients comprises a seven nucleotide duplication, affecting codons 108–110, resulting in a frame shift and premature stop codon at position 150. The observation that this complex mutation exists as different *TP53* haplotypes in these two families, demonstrates that this mutation arose independently and suggests that this region of exon 4 is susceptible to genetic alterations. Consistent with this possibility, the Mfold program predicted a potential hairpin loop encompassing codons 107–110, which corresponds to the 7 bp duplication in exon 4. The identification of multiple complex mutations in breast carcinomas, ovarian cancer, rhabdomyosarcomas and other adrenocortical carcinomas further suggests that this region is prone to genetic alterations (Figure 7).[10,21–24]

The heterogeneity of cancer phenotype in our two study families can be partially attributed to constitutional and acquired genetic changes. Our findings suggest that this complex *TP53* mutation could be a driver of oncogenesis. The ability to differentiate between drivers and passengers will be crucial to better understanding the process of tumorigenesis and defining age- and tissue-specific factors. Registries for specific tumors associated with *TP53* mutations can provide invaluable clinical and biological correlates, allowing studies of the effects of environment, lifestyle and modifier genes on the penetrance and spectrum of tumor types associated with seemingly identical genetic lesions.

MATERIALS AND METHODS

The patient's parents provided informed consent for her participation in the IPACTR (http://www.stjude.org/ipactr), whose research protocol was approved by the St Jude Children's Research Hospital Institutional Review Board. Mexican family members provided signed informed consent with approval from the local ethics and scientific committees (Comite de Bioetica, Instituto Nacional de Cancerología, with: Reg. no. COFEPRIS 103300538X0301, Office for Human Research Protections IORG0006100). We also obtained St Jude Institutional Review Board approval to use genetic material from this Mexican family.

Genotyping of *TP53*

DNA was isolated from peripheral blood lymphocytes and tumor tissue by standard procedures. *TP53* exons 2 through 11, including each exon's flanking intron sequence, were amplified by PCR and sequenced on a high-throughput 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Two highly informative polymorphic markers, VNTRp53 and p53$(CA)_n$, were also amplified by PCR. VNTRp53 is a pentanucleotide repeat $(AAAAT)_n$ within intron 1 of the human *TP53* gene,[25] whereas p53$(CA)_n$ is a dinucleotide repeat polymorphism located 30 kb upstream of the 5′ start site of the gene.[26] The forward oligonucleotide primers for these two markers were labeled with fluorescent dye (FAM). Genomic DNA was amplified in a 15-μl reaction mixture consisting of 9 μl of True Allele PCR Premix (Applied Biosystems), 1 μl of primer mix (5 μM each), 4 μl of water and 1 μl of genomic DNA (50 ng). The PCR reaction mixture was denatured

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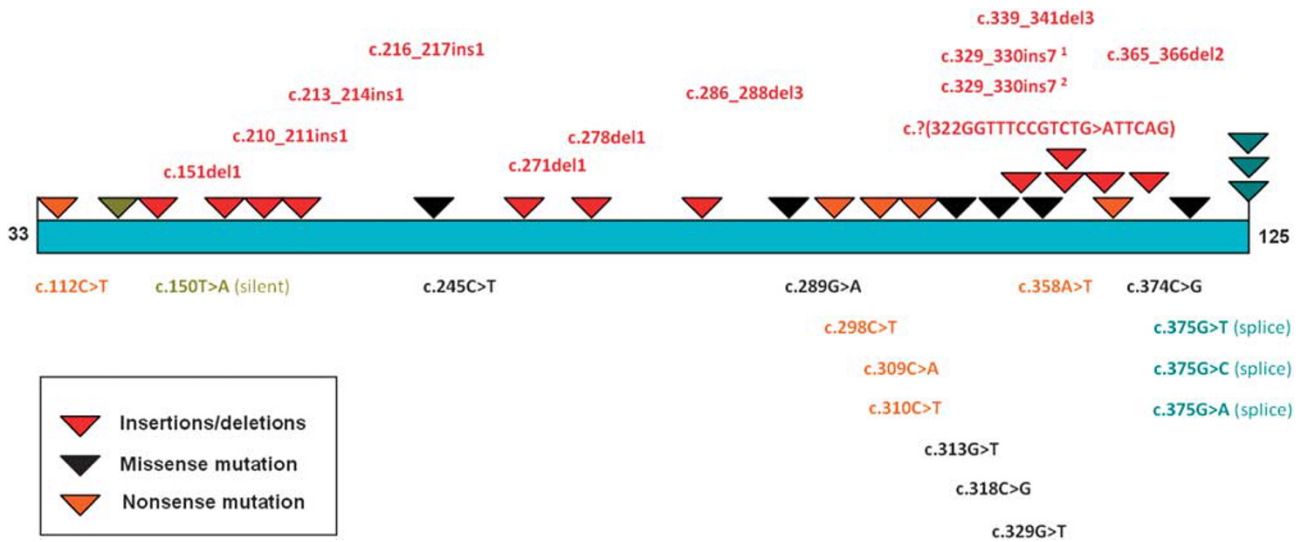

Figure 7. Schematic representation of *TP53* exon 4. In this representation, all germline *TP53* mutations included were registered at IARC database.[27] Upper: complex mutations, including insertions and deletions. Down: *TP53* point mutations including missense or nonsense mutations. Number 1 indicates HON001 family and number 2 indicates MEX001 family.

for 12 min at 95 °C, followed by a 10-cycle program (94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s), a 20-cycle program (89 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s) and a final 10-min extension at 72 °C. Genescan 400HD ROX size standard (Applied Biosystems) and 2 μl of PCR products were added to 24 μl of formamide (Invitrogen, Carlsbad, CA, USA). The mixture was separated by capillary electrophoresis in a 3730xl DNA Analyzer. Data were analyzed by using GeneMapper v4.0 software (Applied Biosystems). The same procedure was used to amplify a short fragment of exon 4 containing the mutation. Forward primer was also labeled with FAM. LOH was assessed by comparing the peak height for each allele amplified (188 bp corresponded to the WT allele and 195 bp to the mutated allele) in tumor versus blood DNA. Homozygosity was defined as the identification of only one allele in both blood and tumor tissue. Heterozygosity was defined as an allelic imbalance ratio of 0.5:2; a ratio <0.5 or >2.0 was defined as LOH.

Genetic profiling

Genetic profiling analysis used the 15 single tandem repeat loci included in the PowerPlex 16 kit (Promega, Madison, WI, USA). A multiplex PCR reaction was performed using fluorescent dye-linked primers and two-color detection according to the manufacturer's manual. The amplified PCR products were separated by capillary electrophoresis on a 3730xl DNA Analyzer and analyzed by using GeneMapper software.

Multiplex ligation-dependent probe amplification

MLPA of genomic blood and tumor DNA (100 ng) was performed by using the SALSA P056 TP53 MLPA Kit (MRC Holland, Amsterdam, the Netherlands), which contains probes for each of the 11 exons of the *TP53* gene on 17p13.1 and several probes for nearby exons telomeric and centromeric of the *TP53* gene. MLPA reactions were performed as recommended by the manufacturer. All fragments were separated by capillary electrophoresis on the 3730xl DNA Analyzer and analyzed by using GeneMapper software. CNV (that is, copy number that deviated from the population median) was measured by normalizing the target probe to all reference probes used in the run. The mean and standard deviation values for the probe of interest were calculated by using a panel of 12 unaffected blood donors (HRC-1 panel, Sigma, St Louis, MO, USA) to establish the *z* score for the tumor samples. We found that the peak height provided the most consistent results. Normal peaks showed a 0.8:1.2 ratio with normal controls, deletions showed a ratio <0.7, and duplications showed a ratio >1.3. A heat map was created by using the Spotfire DecisionSite for Functional Genomics software (Tibcoo Spotfire, Somerville, MA, USA).

Analysis of secondary structure

DNA folding was analyzed by using the DNA Mfold server (http://mfold.rna.albany.edu[12]) with DNA free energy parameters. Folding was predicted at 37 °C.

Immunohistochemical detection of p53 protein

Immunohistochemical detection of p53 expression was performed on deparaffinized tissue sections from ACT, sarcoma and breast tumors by using the avidin-biotin complex method. Slides were incubated with monoclonal antibodies to p53 protein (DO-7; Dako, Carpinteria, CA, USA; dilution 1:50). Staining was classified as negative (no staining), weak (up to 25% of cells stained), moderate (26-70% of cells stained) or strong (more than 70% of cells stained); cellular localization was classified as cytoplasmic or nuclear.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

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