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**Research Article**

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# Germline p53 Mutations Are Frequently Detected in Young Children with Rhabdomyosarcoma

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## Abstract

**We investigated the possibility that a proportion of children with sporadic rhabdomyosarcoma (RMS) carry constitutional mutations of the p53 tumor suppressor gene. 33 patients with sporadic RMS at two large outpatient pediatric oncology clinics submitted blood samples. Genomic DNA was extracted from peripheral blood leukocytes and PCR was used to amplify exons 2–11 of the p53 gene. Amplified genomic DNA was screened for the presence of germline p53 mutations using single-strand conformation polymorphism (SSCP) analysis. The DNA sequence of those samples that showed aberrant migration of bands on SSCP analysis was determined to identify the precise nature of the gene mutations. Patient records were reviewed to assess clinical correlates of the mutant p53 carrier state. Heterozygous constitutional mutations were detected in 3/33 patient samples screened. Two of these missense mutations are located in exon 7 and one in exon 8 of the p53 gene. The presence of mutations was not correlated with tumor histology, stage, or site. However, an association between young age at diagnosis and presence of a constitutional p53 mutation was noted: 3/13 children under the age of 3 yr at diagnosis carried mutations, whereas none of 20 children over 3 yr of age at diagnosis harbored a detectable constitutional mutation. These results in children with RMS corroborates previous findings in other clinical settings suggesting that the mutant p53 carrier state may predispose individuals to malignancy at an early age. Although this study did not assess whether the mutations were preexisting or new germline alterations, assessment of close relatives of RMS patients for cancer risk and predictive genetic testing may be indicated. (*J. Clin. Invest.* 1995. 95:1606–1611.) Key words: Li-Fraumeni syndrome • rhabdomyosarcoma • p53 tumor suppressor gene • germline mutation**

## Introduction

The p53 tumor suppressor gene, located on the short arm of human chromosome 17 (1, 2), encodes a 53-kd nuclear phosphoprotein that functions as a negative regulator of cell proliferation (3, 4). p53 is a transcription activating factor that acts as a "checkpoint" control late in $G_1$ to block the progression of cells through the cell cycle, particularly in the presence of DNA damage (5–9).

Alterations of the p53 gene or its encoded protein are the most common genetic abnormalities observed in human cancers (10, 11), having been associated with virtually every sporadically occurring malignancy (12, 13). The presence of heterozygous p53 mutations in an individual's constitutional cells confers a high risk for the development of a diverse set of malignancies (14). Such germline p53 mutations were initially identified in affected members of families with the Li-Fraumeni syndrome (LFS)[1] (15, 16). This autosomal, dominantly inherited disorder is characterized by the presence of soft tissue and osteosarcomas, early onset breast cancers, adrenocortical carcinomas, brain tumors, and acute leukemias in affected family members (17–20). Other features of LFS include the early age at diagnosis of tumors and the propensity of affected individuals to develop multiple primary tumors (14, 19, 20). The relationship between LFS and constitutional p53 mutations is complex. Although the majority of LFS families analyzed to date harbor germline p53 mutations in the coding region of the gene (15, 16, 21, 22), some LFS families lack detectable mutations (23, 24), and constitutional mutations have been identified in some cancer patients without a family history of malignancy (25–31).

The presence of a childhood sarcoma is the primary criterion for the diagnosis of LFS. In addition, despite potential bias through ascertainment methods, rhabdomyosarcoma (RMS) is the most frequently diagnosed sarcoma in this syndrome. Based on these observations, we elected to study a series of unselected pediatric RMS patients from two large institutions in order to determine the frequency of constitutional p53 mutations in this population and to correlate these findings with any distinct characteristics of these patients or their tumors. We chose to study patients who do not have family histories suggestive of a cancer-predisposing syndrome, so that the frequency of constitutional p53 mutations in patients with apparently sporadic disease could be assessed.

Genomic DNA extracted from peripheral blood lymphocytes of children with RMS was screened using PCR and single-strand conformation polymorphism (SSCP) analysis. All coding exons of the gene were analyzed. Those samples that showed altered electrophoretic mobility compared with the wild-type controls were further analyzed by dideoxynucleotide sequencing to characterize the alterations. The results of this analysis

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1. *Abbreviations used in this paper:* LFS, Li-Fraumeni syndrome; RMS, rhabdomyosarcoma; SSCP, single-strand conformation polymorphism.

are discussed in the context of the histologic subtype, tumor site, grade, sex, and age at diagnosis. In addition, the implications of the identification of germline p53 mutations in this population of patients is discussed with respect to the role of predictive genetic testing for the development of cancer.

## Methods

*Patient selection and clinical data.* Informed consent was obtained from the parents of all eligible minor patients (< 18 yr of age) and all eligible patients who were now adults. In addition, minor patients above age 12 yr gave consent after an explanation of the study. Patients with RMS, or a history of RMS, seen consecutively in the outpatient pediatric oncology clinics at the Dana-Farber Cancer Institute in Boston, MA, or The Hospital for Sick Children in Toronto, Ontario, were asked to participate in the study. Those patients with LFS, neurofibromatosis, second cancers, or a first-degree relative with cancer were excluded. The study group included newly diagnosed patients, patients on active treatment, and those in long-term follow-up. Age at diagnosis, sex, tumor histology (alveolar, embryonal), tumor site, grade (32), and current clinical outcome were obtained and recorded for each patient.

*Preparation of genomic DNA.* After signed informed consent was obtained, peripheral blood was drawn into EDTA-containing vacutainer tubes from each patient. White cells were isolated using a standard phenol–chloroform extraction method. To prepare high molecular weight genomic DNA, white blood cells were resuspended in high TE buffer (100 mM Tris, 40 mM EDTA, pH 8.0). The cells were then lysed by adding an equal volume of high TE buffer containing 0.2% SDS. After two extractions with TE-saturated phenol and one with chloroform/isoamyl alcohol (25:1), the DNA was precipitated with 1/10 volume of 4 M ammonium acetate and an equal volume of isopropanol. The DNA was redissolved in 10 mM Tris, pH 7.5, 1 mM EDTA and stored at 4°C at a concentration of 50 ng/$\mu$l.

*PCR-SSCP analysis and DNA sequencing.* Nine sets of primers were generated to amplify DNA fragments spanning exons 2–11 of the p53 gene. The primer sequences and lengths of the amplified fragments have been previously published (33, 34). PCR was performed using 250 ng of template DNA in 50 mM Tris-HCl, pH 8.6, with 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 250 ng of each primer, 1 $\mu$l of [$^{32}$P]dCTP (3,000 Ci/mmol) diluted 1:10, and 2.5 U of Taq polymerase (AmpliTaq; Cetus Corp., Emeryville, CA) in a 50-$\mu$l total reaction volume. The reaction conditions for the thermocycler (model 480, Perkin-Elmer Corp., Norwalk, CT) were 94°C (45 s), 55°C (45 s), and 72°C (45 s) for 35 cycles. The reaction was initiated with a 10-min incubation at 85°C and terminated with a 7-min incubation at 72°C followed by 3 min at 94°C. 5 $\mu$l of the PCR product was added to an equal volume of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol). The samples were denatured at 85°C for 5 min and loaded immediately onto an acrylamide–Tris-buffered EDTA nondenaturing gel. The gels contained 4.5–9.0% acrylamide and 2–10% glycerol, depending on the exon fragment being analyzed. Electrophoresis was performed at 25 W for 6–7 h or at 10 W for 15–17 h at room temperature. The gel was blotted onto 3M filter paper (Fisher, Toronto, ON), dried, and exposed to x-ray film (Eastman Kodak Co., Rochester, NY) with an intensifying screen at −70°C for 4–72 h. Each fragment was analyzed using two or three electrophoretic conditions, again depending on the specific fragment being analyzed. Each gel carried both positive and negative controls to ensure that known mutations could be detected. A fresh aliquot of DNA from each sample determined to be abnormal by detectable band shifts on SSCP analysis was amplified with the SSCP primers encompassing the respective abnormal region. Fragments were directly subcloned into a T-tailed pBSK vector, and at least six independent clonse were sequenced in both directions by the Sanger dideoxide method with a Sequenase 2.0 kit (US Biochemicals/Amersham Corp., Arlington Heights, IL).

## Results

*Clinical characteristics of patients.* 36 children with RMS or a history of RMS were seen at the two centers during the study period. Three patients were ineligible for the study: one had a family history consistent with LFS, one had a first-degree relative with cancer, and one carried a coincident clinical diagnosis of neurofibromatosis type 1. 33 patients were therefore entered into the study; family history and clinical history for all 33 revealed no LFS, no first-degree relative with cancer, no history of neurofibromatosis, and no history of second cancers. The clinical data for all 33 participants screened are outlined in Table I. The mean age at diagnosis of RMS was 60.1 mo (range 6 mo to 18 yr). There were 12 females and 21 males. Primary sites of origin are 11 genitourinary system, 8 head and neck, 4 orbital, 8 extremity, 1 abdominal, and 1 arising from the diaphragm. Histologic subtype was embryonal in 20 patients, alveolar in 11 patients, and mixed or undifferentiated in 2 patients. Clinical groups were determined according to the criteria of the International Rhabdomyosarcoma Study IV (32) and are outlined for each patient in Table I.

*Mutation analysis by SSCP and DNA sequencing.* SSCP analysis identified 7/33 genomic DNA samples with altered band patterns. Four of these proved to represent previously documented sequence polymorphisms (34, 35) (data not shown). Three patients were shown to be heterozygous for specific germline p53 mutations, in that DNA sequence analysis revealed the presence of both a wild-type allele and an allele containing a point mutation. These results are illustrated in Fig. 1 and summarized in Table II. The mean age of onset of disease for the noncarriers was 73.8 mo. The three carriers of mutant p53 tended to be younger (18, 19, and 29 mo), as determined by a two-sided Wilcoxon rank sum test ($P = 0.065$).

Patient 312 was 19 mo of age at diagnosis of a group III embryonal RMS of the vagina. An A to G switch at the second position of codon 235 in exon 7 yielded an amino acid change from asparagine to serine. 19 yr after diagnosis, the patient is free of recurrence or second cancer and has no interval occurrence of cancer in the family. Patient 338 was 29 mo of age at diagnosis of a group II alveolar RMS of the right thigh. A G to C switch at the second position of codon 306 in exon 8 predicts an amino acid change from arginine to proline. The patient is now 2 yr from diagnosis with no history of recurrence or second cancer and no significant family history of cancer. Patient 359 was 18 mo of age at diagnosis of a group II embryonal RMS of the orbit. A T to A switch at the first position of codon 227 in exon 7 predicts an amino acid change from serine to threonine. The patient is now 6 yr from diagnosis, with no history of recurrence or second cancer. Of note, the family history of this patient is significant for a paternal uncle with testicular carcinoma at age 40, although there is no history of malignancy in any first-degree relatives. Although functional analyses of these mutations were not performed, each has been previously reported to occur as acquired alterations in sporadic tumors (13, 22), suggesting that they are not likely to represent polymorphisms.

## Discussion

RMS is the most common sarcoma of childhood and accounts for 10–15% of all childhood cancers (36). The etiology of this tumor is unknown, but several lines of evidence suggest that a

*Table I. Clinicopathologic Characteristics of Patients*

| Patient | Sex | Age at diagnosis | Site | Group | Stage | Histology | Outcome |
|---|---|---|---|---|---|---|---|
| 093 | M | 36 | Extremity | III | III | A | DOD |
| 307 | M | 157 | Bladder | III | III | E | DOD |
| 310 | M | 220 | Paratestical | I | I | A | A/W |
| 311 | M | 5 | Orbit | I | I | U | DOD |
| 312 | F | 19 | Vaginal | I | I | E | A/W |
| 313 | M | 8 | Bladder | III | III | E | A/W |
| 314 | F | 30 | Parapharyngeal | III | III | E | A/W |
| 332 | F | 198 | Diaphragm | IV | IV | E | DOD |
| 333 | M | 108 | Prostate | IV | IV | E | A/W |
| 334 | M | 150 | Maxilla | III | II | E | DOD |
| 335 | M | 60 | Abdomen | III | III | A | A/W |
| 336 | M | 22 | Bladder | IV | IV | E | A/W |
| 338 | F | 29 | Extremity | II | II | A | A/W |
| 347 | M | 30 | Orbit | III | II | E | A/W |
| 349 | F | 114 | Nasopharyngeal | III | III | M | A/W |
| 350 | F | 72 | Masseter | IV | IV | E | A/D |
| 355 | F | 60 | Extremity | III | III | A | A/W |
| 359 | M | 18 | Orbit | II | I | E | A/W |
| 360 | M | 16 | Bladder | II | III | E | A/W |
| 373 | F | 63 | Middle ear | I | II | E | A/W |
| 408 | F | 156 | Vagina | III | III | A | A/W |
| 424 | M | 72 | Paratestical | II | I | E | A/W |
| 431 | M | 126 | Extremity | IV | IV | A | DOD |
| 434 | F | 38 | Spine | IV | IV | A | A/D |
| 435 | M | 36 | Extremity | III | III | A | A/D |
| 453 | M | 35 | Axilla | III | II | A | A/W |
| 454 | M | 6 | Palate | III | I | E | A/W |
| 455 | F | 19 | Pelvis | III | III | E | A/W |
| 457 | M | 104 | Axilla | III | III | A | DOD |
| 458 | F | 129 | Sinus | III | II | E | A/W |
| 459 | M | 79 | Orbit | III | I | E | A/W |
| 460 | M | 26 | Bladder | III | II | E | A/W |
| 461 | M | 38 | Pharynx | III | II | E | A/W |

Characteristics of the study population. Age at diagnosis is given in months. Stage and group are based on International Society of Pediatic Oncology Union Internationale Centre le Cancer soft tissue sarcoma clinical staging system (53), and the International Rhabdomyosarcoma Study IV classification. A, alveolar; E, embryonal; U, undifferentiated; M, mixed (alveolar/embryonal). A/W, patient is alive without evidence of disease; A/D, patient is currently alive with evidence of active disease on therapy; DOD, patient has died of disease.

proportion of patients with RMS are genetically susceptible to the development of the tumor. In 1969, Li and Fraumeni first defined an increased risk of cancers, especially breast cancer, in the relatives of patients with RMS (17, 18). Subsequent studies of RMS patients and their families have revealed an increased relative risk of early onset breast cancer in mothers of patients with RMS (37). This association has not been seen consistently in mothers of other childhood cancer patients. RMS is one of the tumors observed as a second cancer in survivors of childhood cancer (38) and is the most common sarcoma described in LFS (19, 20). A study of RMS tumor samples revealed a high frequency of alterations at the p53 tumor suppressor locus (39, 40). The current study provides genetic evidence that RMS, in a subset of patients, is associated with the carrier state of a constitutionally altered allele of the p53 tumor suppressor gene.

Previous surveys have sought to identify the clinical situations associated with increased risk of harboring a germline p53 mutation. Among families with LFS, the risk of carriage of a constitutional p53 mutation has been variable among studies. Initially, it had been demonstrated that 5/5 families with classic LFS harbored mutations (15), yet subsequent studies have been able to demonstrate mutations only in 70% of LFS families (14, 22, 23, 25, 41). Toguchida et al. (26) analyzed the entire coding sequence of the p53 gene in 196 sarcoma patients of all ages, most of whom had osteosarcoma. 15 of these patients were specifically recruited to the study because of an unusual family history of cancer. Eight individuals with germline p53 mutations were found, although only two of these were patients without an unusual family history of cancer. The authors did not note whether a tendency to early onset of disease in the mutant p53 carriers existed. Among women with breast cancer, germline p53 mutations have been reported to be rare, with Sidransky et al. and Børresen et al. finding only 2/337 women carrying mutations (29, 30). Of children and young adults who develop second malignant neoplasms after surviving childhood cancer,

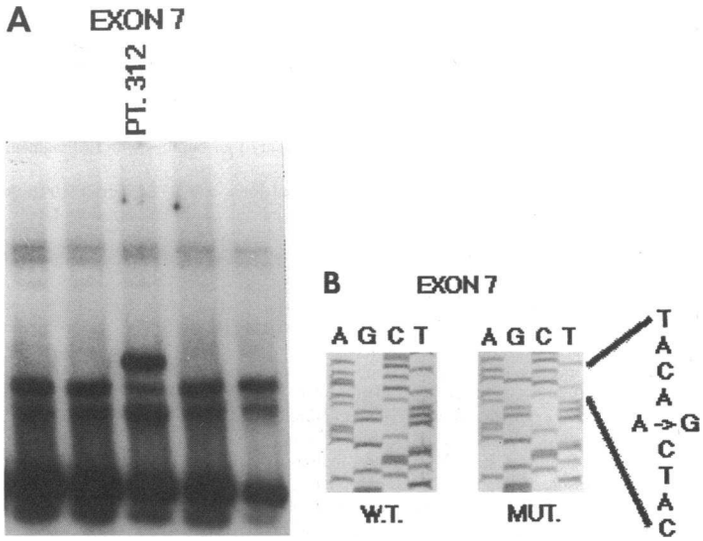


*Figure 1.* (*a*) SSCP analysis of the exon-7 fragment of the p53 gene from DNA extracted from peripheral blood leukocytes of patient 312. Note the extra band (band shift) in the lane carrying the patient's sample as compared with flanking wild-type controls. The presence of this shift is highly suggestive of a sequence alteration within the amplified gene fragment. (*b*) DNA sequence of the exon-7 fragment of the p53 gene from patient 312. The sequence is read from the bottom of the gel. A nucleotide switch from A to G at the second position of codon 235 (AAC → AGC) yields an amino acid change from asparagine to serine. *A,* adenine; *G,* guanine; *C,* cytosine; *T,* thymine; *W.T.,* wild-type sequence; *MUT.,* mutant sequence.

4/59 harbored germline p53 mutations. Adrenocortical carcinoma, an extremely rare tumor of childhood except in the setting of Beckwith-Wiedemann syndrome or LFS, has been associated with the p53 carrier state in 3/6 children studied (42). None of these individuals had a family history of cancer. In another survey, 2/6 children with adenocortical carcinoma in LFS families harbored germline p53 mutations (43). A survey of individuals with childhood acute lymphoblastic leukemia revealed 1 carrier of a germline p53 mutation among 25 patients studied (44). A recent survey of 235 unselected children with osteosarcoma found 7 (2.5%) who harbored mutations (45). Three of these children have no family history of cancer. In summary, the risk of carriage of a p53 mutation for a cancer patient without a strong family history of malignancy, a second cancer, or adrenocortical carcinoma is very low.

We report here that 3/33 children (9.1%) with RMS without a family history of cancer or second cancers harbor constitutional p53 mutations. This represents the highest proportion of patients to be described to date, except those with adrenocortical carcinoma who carry mutations but who have no suggestive family history or second cancers. All mutations were missense and predict amino acid substitutions. Gender, tumor site, histology, and clinical group showed no correlation with the presence or absence of mutations. However, 3/13 children (23%) diagnosed before the age of 3 yr carried p53 mutations, whereas 0/20 children 3 yr or older at diagnosis harbored mutations. The association between young age at diagnosis and genetic predisposition has been observed in other settings, such as in retinoblastoma and Wilms' tumor (46, 47). A larger sample size may allow stronger correlations between clinical and biologic factors, as well as a more stable estimation of frequency of mutations; however, the concepts that patients with RMS are at risk for p53 mutations and that a negative family history for malignancy does not eliminate this risk have been established by this study.

*Table II. Characteristics of Germline p53 Mutations in Three Patients*

| Patient | Exon | Codon | Basepair change | Amino acid switch |
|---|---|---|---|---|
| 312 | 7 | 235 | AAC → AGC | Asparagine → serine |
| 338 | 8 | 306 | CGA → CCA | Arginine → proline |
| 359 | 7 | 227 | TCT → ACT | Serine → threonine |

Characteristics of germline p53 mutations in three patients harboring heterozygous mutant p53.

The three mutations found have been previously described in somatic tumor tissue, but not in constitutional samples (13, 22). Of interest, one of the three patients was noted to have an alveolar RMS, a tumor associated with a nonrandom chromosomal translocation, t(2;13) (48). The fact that this patient carried a p53 mutation suggests that tumorigenesis of alveolar RMS is a multistep process that may be associated with genetic alterations beyond the characteristic translocation.

This study was designed as a survey, rather than a screening and predictive testing analysis. Thus, we did not examine the parents or siblings of the RMS patients for p53 mutations and are therefore unable to assess the rate of de novo versus inherited mutations in this group. It is possible that family members of those children found to harbor mutations may be carriers who have not, as yet, manifested disease. Because predictive testing of unaffected relatives has specific medical, social, and ethical implications, this screening is being carried out within the setting of carefully monitored genetic counselling–based studies at both participating institutions, for those family members who desire it.

Children with RMS who were not found to harbor germline p53 gene mutations in this series may yet have a genetically based increased risk for malignancy. Because SSCP is a technique with < 100% sensitivity, it is likely that not all p53 mutations were detected in the patient DNA samples. Some patients may carry wild-type p53 alleles, but inherit a predisposition for the development of cancer through a different gene or through a mechanism that functionally inactivates wild-type P53 protein. Such a scenario has been described in a patient from an LFS family who had no identifiable p53 gene mutation, but who demonstrated increased immunostaining of P53 protein in constitutional cells, suggesting abnormal stabilization of the protein (24). Other candidate genes for RMS predisposition would include the murine double minute gene, *mdm-2,* which

encodes a protein that complexes with P53 and may inhibit its function (49, 50). In fact, MDM-2 has been shown to be amplified or overexpressed in some sarcoma subtypes (50–52). Finally, children with RMS may also develop their disease as a consequence of alterations of other cancer-associated genes such as the neurofibromatosis-1 gene.

The previously observed finding of early onset RMS in LFS families together with the relatively high frequency of germline p53 mutations in early onset RMS observed in this cohort suggests a broader definition of LFS, as well as possible biologic differences between childhood RMS of early and late onset. These observations may in turn lead to novel therapeutic approaches to childhood RMS and could influence the clinical management of RMS survivors and family members.

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