Germ-line and somatic p53 gene mutations in multifocal osteogenic sarcoma

(multiple tumors/p53/cancer predisposition/tumor suppressor genes)

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ABSTRACT Multifocal osteogenic sarcoma patients without familial histories of increased tumor predisposition were examined for mutations in the highly conserved regions of the p53 gene. p53 point mutations were found in tumor DNA from each of the four patients we examined. A germ-line p53 mutation was detected in one of these patients, and a further rearrangement of the residual wild-type allele was detected in tumor tissue. p53 germ-line mutations can contribute to the enhanced changes including point mutations manifest in patients with multifocal osteosarcoma.

Mutations of tumor-suppressor genes have been found in two different human cancer syndromes: hereditary retinoblastoma, which is characterized by germ-line mutations of the RB gene, and the Li-Fraumeni syndrome, which is associated with germ-line mutations of the p53 gene (1–4). In families with these syndromes, individuals who carry one defective copy of a tumor-suppressor gene seem prone to develop primary cancers in somatic cells in which the remaining functional allele becomes inactivated (5). A more frequent manifestation of enhanced tumor susceptibility is the occurrence of multiple tumors in the same individual (6). Multifocal osteogenic sarcoma (mOS) is diagnosed when bone tumors occur at two or more sites that are commonly associated with “primary disease” (7–9). These tumors can arise as synchronous or metachronous lesions with or without metastatic visceral involvement (10, 11). The most common location of OS is the metaphyseal region of bones in the upper and lower extremities, while multifocal patients in whom metastatic disease develops almost invariably have such foci in their lungs (7).

OS is known to occur in patients with the Li-Fraumeni syndrome (12) and in transgenic mice carrying a mutant p53 allele (13). Also, sporadically occurring OS is frequently associated with rearrangements of the p53 gene (14–16). Somatic mutations of the p53 gene in tumors examined to date occur most frequently in a region between exons 5 and 9, coinciding with the most highly conserved domains of the p53 gene (17, 18). In contrast, the p53 germ-line mutations described in Li–Fraumeni syndrome families occur in a small region of exon 7 between codons 245 and 258 (3, 4). We utilized SSCP (single-strand conformation polymorphism) analysis, a recently developed technique for detection of DNA changes in patients (19), to examine exons 5–9 of the p53 gene in genomic DNA from tumor and normal tissues of four patients with mOS.

MATERIALS AND METHODS

DNA was isolated from frozen tumor specimens and peripheral blood lymphocytes (PBLs) of patients 1 and 4 and from paraffin-embedded specimens of biopsies taken from patients 2 and 3 as reported (20, 21). We monitored by histologic examination all of the specimens from which DNA was prepared to ensure that they were void of detectable contaminating tissue. DNA from patients 1 and 4 was not analyzed directly, rather it was first amplified by polymerase chain reaction (PCR) with primers P3 and P4 as described by Nigro et al. (22), and the expected 2.9-kilobase (kb) primary PCR product we obtained was used for subsequent analysis. For SSCP, a second amplification of this 2.9-kb PCR product was performed by using 1/1000th volume of the first PCR reaction product in a 10-μl PCR reaction mixture with 5 pmol of different sets of primers specific for exons 5–9 of the p53 gene (see below), 1 μCi (37 kBq) of [α-32P]dCTP (DuPont/ NEN; specific activity, 3000 Ci/mmol), and 1.25 mM magnesium chloride. Thirty-five cycles of 94°C (30 sec), 58°C (60 sec), and 72°C (60 sec) were performed, and the amplified products were treated for SSCP as described by Orita et al. (19). For DNA sequence analysis, 1/100th volume of the 2.9-kb PCR product was used as a template in a PCR reaction containing 20 pmol of the primers specific for exons 5–9. These PCR products were purified by polyacrylamide gel electrophoresis and evaluated for alterations in nucleotide sequence by a modification of the dyeoxy chain-termination method with these same exon-specific primers (20). The DNA sequence results were confirmed by sequencing from both strands using two different DNA templates that were independently synthesized by PCR amplification of genomic DNA.

DNA from patients 2 and 3 served as a template for PCR reactions in which primers specific for exons 5–9 were utilized to amplify exon-specific DNA fragments. These fragments were examined by SSCP analysis, and the nucleotide sequence was determined as described above.

A restriction enzyme analysis for exon 6 of the p53 gene amplified from normal and tumor tissue of patient 2 and from a normal control was performed with Taq I and Mae II. After amplification, PCR products were precipitated with ethanol, digested with restriction enzymes in the appropriate buffer, separated on 2% agarose gels, and visualized by ethidium bromide staining (20).

The primers used for PCR and DNA sequencing were 5'-GAHTTTCAAACCTGTC-3' and 5'-CTGGGGACCTGGGCAAC-3' for exon 5, 5'-ACAGGGCTGTGCCCCAGGGTT-3' and 5'-AGTTCGAAACCACTGCACTCC-3' for exon 6, 5'-CCAAAGGCCACTGCCCTC-3' and 5'-GAGGCAAGCATGGCTTG-3' for exon 7, 5'-CTCTGACTGTCCTTCTGTTC-3' and 5'-TGAATCGACCCATAACTG-3' for exon 8, and 5'-GAGATTTGCTCAGTATCCAC-3' and 5'-AA-GACTGTACCTGGAGGTGT3' for exon 9.

Abbreviations: OS, osteogenic sarcoma; mOS, multifocal OS; SSCP, single-strand conformation polymorphism; PBLs, peripheral blood lymphocytes.

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RESULTS

SSCP analysis of the p53 gene revealed evidence of DNA changes in tumor tissue from all four MOS patients and in the normal tissues of two. The nucleotide sequence of abnormal DNA fragments was determined, and the mutations detected are shown in Table 1. Data from the two patients with constitutional p53 changes shown in lane 1 of Fig. 1. Fig. 1A demonstrates the SSCP pattern of PCR reaction products corresponding to exon 8 of the p53 gene from a normal individual. The slowest migrating band corresponds to one strand from each of the two alleles. The band below this corresponds to the other strand of the two normal alleles (marked by a thin arrow), and the fastest moving band is denatured double-stranded PCR product. In DNA from normal tissue of patient 1 (Fig. 1A, lane 4), we detect both bands corresponding to normal single strands and an extra band (marked by a thickened arrow), suggesting the presence of two different alleles. In tumor tissue (Fig. 1A, lane 3) only the mutated allele is recognizable, indicating a loss of the normal allele.

The tumor specimen shown in lane 3 of Fig. 1A is from OS tissue removed from the left tibia of a 14-yr-old boy 5 years after a histologically similar tumor was diagnosed in his right tibia. We also examined DNA from this first tumor and from a late lung metastasis and found the SSCP pattern of DNA from these tissues to be indistinguishable from that observed in lane 3 (data not shown). We could not detect any evidence by SSCP of a p53 alteration in DNA from PBLs of the patient’s mother or sister (data not shown). The patient’s father could not be located. As the only known case of cancer in the patient’s family had been an OS in the son of the father’s cousin, we examined DNA from PBLs of this cousin and found no evidence for a mutation in the p53 gene (Fig. 1A, lane 2). The DNA sequence of exon 8 from normal tissue of patient 1 showed a single point mutation, a C → T transition, in position 1 of codon 282 resulting in the substitution of a tryptophan residue for an arginine (Fig. 2A). As can be seen, the normal band was retained with half of the expected intensity in DNA from the patient’s PBLs, which is consistent with constitutional heterozygosity. No evidence for the wild-type allele was seen in tumor DNA (Fig. 2B). The nucleotide sequence of exon 8 of the p53 gene in DNA from PBLs of the patient’s mother and sister as well as the patient’s father’s cousin showed only the wild-type sequence (data not shown).

Fig. 1B shows the results of SSCP analysis examining exon 6 of the p53 gene in DNA from tumor and normal tissue of patient 2 with synchronous MOS. This patient, whose family history did not indicate an enhanced predisposition to cancer, presented with MOS involving several different bones. In normal tissue from this patient (Fig. 1B, lane 3), four bands could be seen, suggesting the presence of two different alleles of the p53 gene one normal (bands a and e) and one apparently mutated (bands c and f). DNA from a tumor of patient 2 (Fig. 1B, lane 2) contained two alleles with altered mobility (bands c and f and bands b and d) and no bands corresponding to the normal allele. The DNA sequence of exon 6 from normal tissue of patient 2 (Fig. 3A) showed both an A and G nucleotide at the third position in codon 213. In tumor tissue (Fig. 3B), the change in codon 213 was maintained in one allele (see below), while an A → G transition at position 2 of codon 214 was also detectable. The tumor tissue remained heterozygous at this position for the normal nucleotide. While the nucleotide change at codon 213 did not change the amino acid (arginine), the mutation at codon 214 resulted in the substitution of an arginine for a histidine.

To determine whether these two different DNA sequence changes in p53 were on the same or different alleles, we utilized restriction enzyme analysis of PCR amplification products corresponding to exon 6 of the p53 gene (Fig. 4). In normal and tumor tissue from patient 2, the change in codon 213 leads to the loss of a TaqI site in one allele (TCGA → TCGG), while the other allele is digested. In tumor tissue a
Fig. 4. Restriction enzyme analysis of exon 6 of the p53 gene from normal lane 1 and tumor tissue lane 2 of patient 2 and from a normal control lane 3. bp, Base pairs.

new Mae II site, which requires the presence of a wild-type nucleotide (A) in codon 213, was created by the mutation in codon 214 (ACAT → ACCT). Therefore, the two DNA sequence changes in the tumor of patient 2 involve different p53 alleles.

DISCUSSION

Cancer patients who survive their first tumor are known to be at increased risk for developing a subsequent malignancy in both the same and in different organs from the one in which their first tumor developed. Multiple tumors also occur in individuals affected by syndromes associated with enhanced tumor incidence (6). Indeed, the finding of multiple retinoblastomas at the time of presentation is a hallmark of hereditary retinoblastoma (23). We sought to determine whether mutations in a known tumor suppressor gene, p53 gene, could be identified in patients with a nonfamilial tumor syndrome characterized by the occurrence of several apparently independent bone tumors. The occurrence of somatic p53 mutations in four patients with mOS and of a germ-line mutation in one of them provided strong evidence implicating the p53 gene in the pathogenesis of mOS.

The specific germ-line mutation found in patient 1 has been described previously only as a somatically acquired mutation in tumor tissue (24) and has not been reported in patients with the Li–Fraumeni syndrome (3, 4). The occurrence of this mutation in normal tissue and the loss of the normal allele in this patient’s tumor are consistent with current genetic models of tumor suppressor genes in which the altered structure of one allele determines a predisposition to cancer (5). The DNA sequence change was observed in normal tissue from patient 2 did not modify the primary amino acid sequence of the p53 protein encoded by the affected allele, and it is likely that this change is a rare DNA polymorphism (25).

Previous reports have shown that 27% of human osteosarcomas have rearrangements of the p53 gene (14–16). The frequency of p53 point mutations in osteosarcomas has not been determined, but our data indicating that all four of the mOS patients we examined had point mutations in the p53 gene suggest that mOS will represent a subgroup of bone tumors with a very high incidence of p53 mutations. It seems likely that at least two different cancer predisposition syndromes can be associated with constitutional p53 mutations. Although too few cases have been examined to determine whether the mutations associated with Li–Fraumeni and mOS will be recognized as functionally distinct, our findings present an important paradox that is likely to be more frequently encountered—namely, different clinical syndromes whose pathogeneses share common genetic features.

The absence of cancer susceptibility in the family of patient 1 raises the possibility that other cancer patients who present with multiple, nonfamilial tumors will carry germ-line p53 mutations. If improved therapy enhances the survival of such patients, their characterization will be of importance, as it is likely they will transmit an enhanced cancer predisposition to their offspring.

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