Alterations of the \( p53 \), \( Rb \) and \( MDM2 \) genes in osteosarcoma

Abstract Molecular defects affecting tumor-suppressor genes are an important step in the genesis of sarcomas. For example, inheritance of a defective \( Rb \) or \( p53 \) gene predisposes the carrier to develop osteosarcoma, among other malignancies. In this study, we have assessed the occurrence of \( p53 \), \( Rb \) and \( MDM2 \) alterations in the same samples of osteosarcomas, along with representative samples of various other sarcomas. Point mutations of the \( p53 \) gene were found in 13 of 42 osteosarcomas and 1 of 8 leiomyosarcomas, and gross rearrangement of the \( p53 \) gene was demonstrated in 5 of 37 osteosarcomas. The retinoblastoma susceptibility gene (\( Rb \)) was either rearranged or deleted in 7 of 37 osteosarcomas, 1 of 8 leiomyosarcomas, and 4 Ewing sarcomas. Remarkably, 5 of the osteosarcomas having \( Rb \) alterations also had \( p53 \) mutations. Amplification and overexpression of the \( MDM2 \) oncogene may lead to increased \( MDM2-p53 \) binding resulting in inactivation of \( p53 \) function. A two- to threefold increase in the copy number of \( MDM2 \) was detected in 7 of 37 samples, 5 of which were osteosarcomas. Amplification of the \( MDM2 \) gene occurred independently of \( p53 \) mutation; one sample having threefold amplification of \( MDM2 \) also had a \( p53 \) mutation. In summary, 34 alterations of the \( p53 \), \( Rb \) and \( MDM2 \) genes were found in 26 of 42 (62%) osteosarcomas.

Key words \( p53 \) · \( Rb \) · \( MDM2 \) · Osteosarcoma · Sarcoma

Introduction

Osteosarcoma is the most frequent bone malignancy in children, its occurrence peaking around age 14. Multiple molecular lesions are likely to underlie progression from normal bone to osteosarcoma. Alterations of tumor-suppressor genes, especially those coding for the retinoblastoma-susceptibility product (\( Rb \)) and \( p53 \), have been implicated as important events in this progression (Fuchs and Winkler 1993).

Retinoblastoma has been traced to the loss of function of both \( Rb \) alleles; patients inheriting one inactivated \( Rb \) locus develop bilateral retinoblastomas with high penetrance and are also at risk for secondary malignancies (Fung et al. 1987; Friend et al. 1986; Lee et al. 1988). The most common second malignancy in bilateral retinoblastoma is osteosarcoma (Abramson et al. 1979). Alterations of the \( Rb \) locus have also been demonstrated in sporadic osteosarcoma. Loss of heterozygosity affecting chromosome 13q, the site of the \( Rb \) gene, is detected in 60% of osteosarcomas (Toguchida et al. 1988; Scheffer et al. 1991; Yamaguchi et al. 1992; Wadayama et al. 1994). Gross structural alterations of the \( Rb \) gene, including rearrangements or deletions, are seen in about 30% of osteosarcomas (Wadayama et al. 1994; Araki et al. 1991; Reissmann et al. 1989; Scholz et al. 1992; Ozaki et al. 1993). Point mutations of the \( Rb \) gene have been found in less than 10% of osteosarcomas (Wadayama et al. 1994). Expression of \( Rb \) protein is absent in half of all osteosarcomas (Xu et al. 1989; Shew et al. 1989). The \( Rb \) gene product has a pivotal role in cell-cycle control; it binds to transcription factors of the E2F family until it is phosphorylated by the CDK4/cyclin-D complex at the restriction point in late G1 phase of the cell cycle (Weinberg 1995). Loss of \( Rb \) function removes a critical negative control of DNA replication.
The *p53* gene is rearranged in 10%-20% of human osteosarcomas, the first known consistent alteration of this gene in human cancers (Masuda et al. 1987; Miller et al. 1990). Alterations of *p53*, especially mutations, are one of the commonest genetic alterations detected in cancer (Greenblatt et al. 1994; Soussi et al. 1994). Sarcomas, including osteosarcomas, are a defining tumor of the Li-Fraumeni familial cancer syndrome, which has been traced to inheritance of mutant *p53* genes (Srivastava et al. 1990; Malkin et al. 1990; Toguchida et al. 1992b). Loss of heterozygosity affecting chromosome 17p near the *p53* locus has been described in 75%-80% of osteosarcomas (Scheffer et al. 1991; Yamaguchi et al. 1992; Toguchida et al. 1989). Mutations are detected in 25%-30% of osteosarcoma tumors (Straton et al. 1990; Toguchida et al. 1992a; Iavarone et al. 1992;Andreassen et al. 1993). Normal *p53* functions to inhibit cell-cycle progression and/or activate apoptosis (Hartwell and Kastan 1994; Oren 1994). Consequently, mutation of *p53* inhibits these functions (Hartwell and Kastan 1994; Oren 1994).

Tumor-suppressor function is also targeted by interactions with viral antigens. In fact, the *p53* protein (*p53*) was originally discovered because it binds simian virus 40 (SV40) T antigen in infected cells (McCormick and Harlow 1980; Lane and Crawford 1979). Hypothetically, viral antigens mimic normal cell constituents that regulate *P53*, effecting viral transformation. For example, binding of *P53* by *MDM2* protein has been proposed as an alternative way in which *P53* function can be disrupted (Momand et al. 1992; Oliner et al. 1992). *MDM2* was originally isolated as a mRNA coded in amplified DNA in murine double minutes, hence the name mouse double minute 2 (Fakhrazadeh et al. 1991). Amplification of *MDM2* has been described in osteosarcoma, but it is especially common in liposarcomas (Oliner et al. 1992).

We have examined a large collection of osteosarcomas, and representative samples of several other sarcomas, for alterations of *Rb*, *p53* and *MDM2* in the same samples. Gross alterations, such as rearrangements and deletions, of the *p53* and *Rb* genes were detected by Southern blotting. Rearrangement of the *p53* gene has been previously examined in many of these samples (Miller et al. 1990). The copy number of the *MDM2* gene was measured by comparing its signal with that of the vitamin D$_3$ gene, in turn compared to that of normal DNA. Small alterations of the coding region of the *p53* gene were detected by single-strand conformation polymorphism (SSCP) and defined by sequencing. Using SSCP allows detection of most base changes that occur. Previous studies have determined that these genes are altered in a significant proportion of sarcomas. Our goal was to correlate alterations of these genes with sarcomas.

### Materials and methods

#### Samples

A total of 81 samples from 68 sarcoma patients were examined and are described in Table 1; 11 of the osteosarcoma samples were maintained as explants. Cell-line DNA was used as control for the presence of mutations as described below. The cell line SAOS-2, which we previously showed not to contain *p53*, was used to control for artifactual amplification (Masuda et al. 1987). The cell line OsaCl, which has previously been demonstrated to have *MDM2* amplification, was used as a positive control for *MDM2* amplification. For detection of *MDM2* amplification normal bone marrow DNA was run in one lane of all blots as an index for normal copy number. All samples were analyzed by the polymerase chain reaction and SSCP, while 57 samples from 50 patients were analyzed by Southern blot hybridization.

#### Amplification, SSCP and sequencing of DNA

Coding exons of *p53* were amplified and the presence of mutations was detected by SSCP. Cell lines with known *p53* mutations that were used as controls included HOS (codon 156, proline) and CEM (175, histidine) for exon 5, Jurkat (196, end) for exon 6; SW837 (234, Gin) for exon 7, and A431 (273, His) for exon 8 (Romano et al. 1989; Cheng and Haas 1990; Nikro et al. 1989; Harlow et al. 1985). Primers were based on GenBank entry X54156 (Buchman et al. 1988). Oligonucleotide primers and product size after PCR were, for exons 2 and 3 (codons 1-33), TCCTTGATCGTCTTTTCAACCTAC and ACGGCCAGGCATTGCTCATGG yielding a fragment of 298 bp; for exon 5 (codons 126-186), CCTCTGACGCTTTCAGCCAACACCT and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 6 (codons 187-224) CACTGATTTGCTTGAAGTGGTG CGCCCACTGTTCTACCA yielding a fragment of 214 bp; for exon 7 (codons 332-425) TCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 9 (codons 26~306), CCTATC and TTAACCCCTCCTCCCAGAGACC giving a fragment length of 278 bp; for exon 10 (codons 307-331), TGGAACTTTCCACTTG yielding a fragment of 116 bp; for exon 11 (codons 34-125), CCTCTGACGCTTTCAGCCAACACCT and ATGTTGAAACCCAGACCTCAGCGC for a fragment of 144 bp; for exon 7 (codons 225-261), CTTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 9 (codons 307-331), TGGAACTTTCCACTTG yielding a fragment of 116 bp in length; for exon 10 (codons 332-367), CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 368-393), CATCTCTC and TTAACCCCTCCTCCCAGACCGC for a fragment of 162 bp; for exon 11 (codons 394-421), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 422-450), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 451-480), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 481-510), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 511-540), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 541-570), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 571-600), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 601-630), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 631-660), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 661-690), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 691-720), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 721-750), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 751-780), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 781-810), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 811-840), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 841-870), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 871-900), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 901-930), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 931-960), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp; for exon 11 (codons 961-990), CCTCAGATGTGCTTTTCAACCTAC and CTGCCCACTGTTCTACCA yielding a fragment of 166 bp.

Southern blot hybridization

Samples were digested with *Ran*II, separated on 1% agarose and subsequently blotted onto Biotrans nylon membranes. The presence and extent of *p53* rearrangements were determined as previously described; the *p53* Neor probe corresponds to exons 2-5 of the *p53* gene (Miller et al. 1990). The *Xba*1/*HindIII* fragment of pFL4MDM2, a kind gift from B. Vogelstein, was used to probe for