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Disruption of the Murine p53 Gene by Insertion of an Endogenous Retrovirus-like Element (ETn) in a Cell Line from Radiation-Induced Osteosarcoma

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The mammalian genome harbors a large number of endogenous retroviruses and retrovirus-like elements. Increasing evidence is found that such elements can be activated and act as insertional mutagens. The activation of endogenous retroviral elements can be induced by a variety of environmental factors including irradiation. We have observed the insertion of a murine endogenous retrovirus-like ETn element into intron 4 of the p53 gene in an osteosarcoma cell line derived from a radiation-induced osteosarcoma. The insertion resulted in a p53–ETn–p53 fusion mRNA, a novel form of p53 mutation. This is the first report on insertion of an endogenous retroviral element into the p53 tumor suppressor gene. The data suggest that activated endogenous retroviruses and retrovirus-like elements might pose an enhanced risk for individuals exposed to noxae, which activate endogenous retroviral elements.

Endogenous retroviruses and retrovirus-like sequences constitute more than 10% of the mammalian genome (*1–3*). Upon activation retroviral RNA is expressed and potentially reverse transcribed into proviral DNA, which is able to reintegrate at different sites in the host genome. It is well documented that new integration of somatically acquired retroviruses can activate cellular oncogenes and thus contribute to tumor formation (for reviews see *4, 5*). A less frequently observed consequence of retroviral integration is the disruption and subsequent inactivation of cellular genes (*4*). Whereas inactivation of an essential cellular gene may be lethal for the affected cell, for the whole organism loss of the affected cell may be without consequence. In contrast, an accidental inactivation of a tumor supressor gene may distort the well preserved balance between proliferation and differentiation in the progeny of the affected cell. The high number of retroviruses and retrovirus-like elements within the mammalian genome are an immense source of endogenous mobile elements, which upon activation could act as insertional mutagens.

Expression of endogenous retroviral sequences can be induced by a variety of environmental factors including external or internal irradiation *in vitro* and *in vivo* (*6, 7*). Irradiation of Balb/c mice with bone-seeking radionuclides has led to dose-dependent activation of murine leukemia viruses during the early latency period (*8*), and endogenous ecotropic proviruses were found in the cellular genome of 60% of the resultant osteosarcomas (*9, 10*). A striking feature of murine radiation-induced osteosarcomas, and cell lines derived therefrom, were gross alterations of the genomic region harboring the tumor suppressor gene p53 (*11*).

Alterations of the p53 gene have been found in a variety of human and animal tumors (for reviews see *12–14*), including osteosarcomas (*15–20*). p53 was also a target of insertional inactivation in leukemia cells, which had been experimentally infected with exogenous murine leukemia viruses (*21–25*). Alterations of p53 in osteosarcomas reported to date apparently were not due to retrovirus action (*26*). A functional p53 gene product is critical for regulation of the cell cycle, differentiation, and cell death (*27–29*). In addition, accumulating evidence is found for a role of p53 in guarding the genome from DNA damage (*30*). p53 protein binds specifically to double-stranded DNA (*31*) and can act as regulator of transcription both to promote the transcription from some genes and to reduce transcription from other genes (*32*).

Functional inactivation of p53 can be caused by various mechanisms, most commonly by subtle point mutations or small deletions in evolutionary highly conserved regions of the p53 gene (for examples see *33, 34, 26*). In this report we show evidence for insertion of an endogenous retrovirus-like element into the p53 gene leading to a p53–ETn–p53 fusion mRNA, a novel kind of p53 mutation.

We have investigated mutations in p53 gene exons from cell lines derived from radiation-induced murine osteosarcomas (*11*) by PCR analysis of p53 mRNA. Total RNA of six cell lines from radiation-induced osteosarcomas (OS-43, OS-46, OS-47, OS-48, OS-49,

Sequence Data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. U06639.

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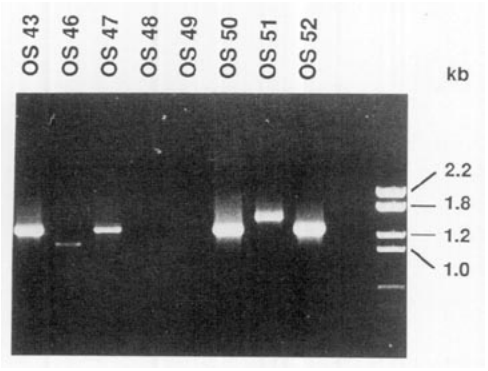

FIG. 1. PCR analysis of p53 c-DNA of eight mouse osteosarcoma cell lines. RNA preparation, reverse transcription, and PCR were performed according to standard protocols (*51, 52*) or with kits according to instructions of the manufacturer. Briefly, PCR conditions were as follows: 94/58/72°C 1 min each, 40 cycles, 2.5 units Perkin–Elmer Taq polymerase per reaction. PCR primers (10 pmol per reaction) were used to amplify the entire translated region between exon 1 and 11 of the p53 gene. PCR products were separated on 1% agarose gels. PCR primer (p53 exon 1): 5′ GGAATTCCCAGCAGGGTGTCACGCTT. PCR primer (p53 exon 11): 5′ CAGGTACCAAGGGACCGGGAGGATTG.

OS-51) and two cell lines from spontaneous osteosarcomas (OS-50, OS-52) was reverse transcribed. The translated region of p53 exons was amplified using oligonucleotide primers for exons 1 and 11. PCR products approximately of the expected normal size [1300 bp, (*35*)] were identified in two of the cell lines derived from radiation-induced tumors (OS-43, OS-47) and in both of the cell lines derived from the spontaneous osteosarcomas (OS-50 and OS-52; Fig. 1). No PCR product could be generated from cell lines OS-48 and OS-49. The OS-46 PCR product was smaller than expected from a normal p53 gene. In contrast, cell line OS-51 showed a PCR product of 1.5 kb, some 0.2 kb larger than the normal size.

Further PCR analysis of the 1.5-kb product from cell line OS-51 with various combinations of exon specific primers localized the altered region to exons 4 to 6. To further characterize this mutation the PCR product from OS-51 DNA, obtained in the first amplification step, was reamplified with a pair of oligonucleotide primers specific for p53 exons 4 and 7 and cloned into the vector pGEM3. Sequencing of the PCR product showed an insertion of 183 bp of a p53 unrelated sequence between exon 4 and 5 of p53 (Fig. 2). Database searches revealed that the p53 unrelated sequence is identical with sequences from the ETn family of mouse transposons from position 249 to 431, comprising sequences from the LTR and from the untranslated region 5′ of the gag gene (*36, 37*). The presence of intact p53 exon 4 and 5 sequences in the cDNA suggested that ETn insertion has taken place in the intron 4. Therefore, the integration site in p53 intron 4 was analyzed in genomic DNA from cell line OS-51 (as described in the legend to Fig. 2b). The genomic p53–ETn junction PCR products were sequenced and showed wild type p53 intron sequences of 301 bp 5′ to the integration site and 428 bp 3′ of the integration site. Therefore, the ETn integration occurred approximately in the center of the 729-bp intron 4. A 6-bp cellular target site duplication (CAGCCT), found flanking the ETn element, is characteristic for integration by retrotransposition (*38*).

The genome of ETn elements in Balb/c mice comprises about 5.7 kb and is present in about 200 copies. ETn elements apparently do not contain open reading frames (*39*). Most of the copies in the mammalian genome are also of this structure. In the cell line OS-51, both 5′ and 3′ LTRs of the ETn element were present. PCR of genomic DNA using primer pairs spanning p53 exons 4 and 5 (embracing the ETn element) failed to produce amplification products in OS-51. Assuming an ETn genome of the size of 5.7 kb, the failure to generate PCR products containing the whole ETn genome would be expected. Therefore, both the presence of the 5′ and 3′ ETn LTR sequences in the p53 gene and the failure to generate PCR products containing the whole ETn element indicate, although do not prove, that a full size ETn element might have been integrated.

Although ETn insertion took place within an intron of the p53 gene, ETn sequences were found in cDNA representing p53 mRNA. This is due to the use of the p53 exon 4 splice donor site and a cryptic splice acceptor site in the ETn 5′ LTR at position 248. This is reminiscent of the insertional mutation by ETn of the gene of the skeletal muscle chloride channel in myotonic mice (*40*) where ETn sequences were spliced using a cryptic splice acceptor site in the 5′ LTR and further splice donor sites from the ETn element. A similar insertional mutation by ETn has been observed within intron 2 of the Fas antigen gene of lpr mice (*41*). The ETn insertions into the skeletal muscle chloride channel gene and the Fas antigene gene both resulted in premature termination of mRNAs at the polyadenylation signal of the ETn LTR. In contrast, the p53 mRNA in cell line OS-51 is not terminated by the abnormal splicing. In the OS-51 cell line, a second splice event occurred between a canonical splice donor site in the ETn region downstream of the 5′ LTR at position 432 and the regular splice acceptor site of p53 intron 4. The ETn sequences spliced into p53 mRNA represent an open reading frame without stop codons. Also, the splicing does not lead to a shift of the p53 reading frame. The steady state level of mRNA from the mutated p53 gene in cell line OS-51 was virtually undetectable by Northern blot analysis (*11*). Interestingly, mouse p53 intron 4 sequences have been suggested to play a role in tissue specific expression of the p53 gene (*42*). Therefore, the insertion of the ETn element

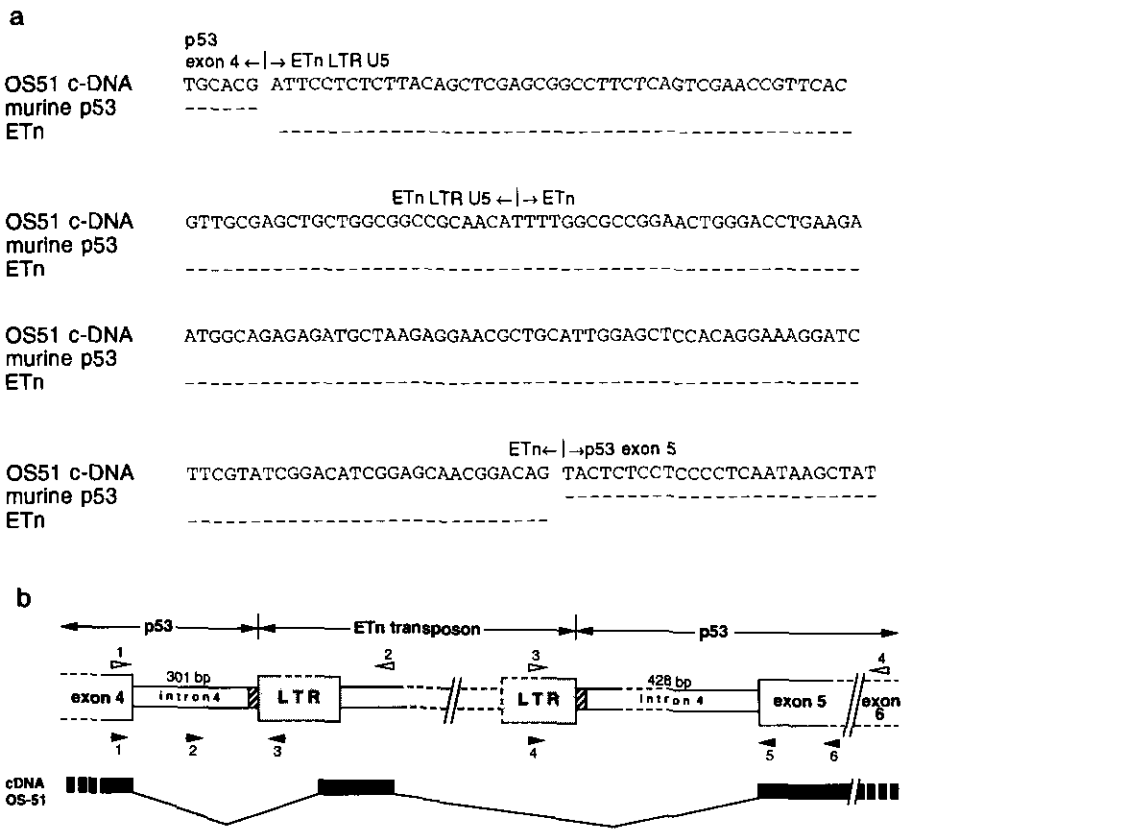

FIG. 2. ETn transposon insertion into the p53 gene in osteosarcoma cell line OS-51. (a) Sequence analysis of p53 cDNA derived from cell line OS-51. The sequence is compared with the sequences of murine p53 (*35*) and ETn (*54*). The PCR product obtained by using PCR primers for p53 exon 1 and 11 (Fig. 1) was reamplified using PCR primers for exons 4 and 7 and cloned into the vector pGEM3. The sequencing primer used for cDNA sequencing was Seq-primer 1a (p53 exon 4): 5′ GGAATTCCTGCAGTCTGGGACAGC. (b) Schematic representation of the p53 splice variant due to the ETn insertion into p53 intron 4. Genomic DNA of the cell line OS-51 was amplified with two pairs of primers. One pair, PCR primers 1 and 2, allowed amplification of part of the p53 intron 4, including the junctions to p53 exon 4 and the ETn 5′ LTR. With the second pair of primers, PCR-primers 3 and 4, the sequence from the ETn 3′ LTR to p53 exon 6 was amplified. The p53 exon/intron junctions were sequenced using the sequencing primers Seq-primers 1 and 6. Seq-primer 5 was used to extend sequencing of p53 intron 4. The p53/ETn junctions were sequenced using the sequencing primers Seq-primers 2, 3, and 4. The sequences of the PCR and sequencing primers are given below (the 5′ ends of all primers contain *Eco*RI or *Kpn*I restriction sites). The binding sites of the primers are indicated (PCR primers, open arrowheads; Seq-primers, closed arrowheads). Solid lines represent sequenced parts and broken lines mark supposed parts of ETn and p53, respectively. Target site duplication is shown as hatched boxes. PCR-primer 1 (p53 exon 4): 5′ GGAATTCCGCATTCTGGGACAGCCAAGTCTG. PCR-primer 2 (ETn 5′ noncoding region): 5′ GCGGTACCTGGAGCTCCAATGCAGC. PCR-primer 3 (ETn 3′ LTR): 5′ GCGAATTCCAGAGATTATTCGGCGG. PCR-primer 4 (p53 exon 6): 5′ TAGGTACCCGGATAAGATGCTGG. Seq-primer 1 (p53 exon 4): 5′ GGAATTCCGCATTCTGGGACAGCCAAGTCTG. Seq-primer 2 (p53 intron 4): 5′ GCGAATTCGGGACGTGGAACTCTCT. Seq-primer 3 (ETn 5′ LTR): 5′ GCGGTACCGTGGCAGAACGAATGAG. Seq-primer 4 (ETn 3′ LTR): 5′ GCGAATTCCAGAGATTATTCGGCGG. Seq-primer 5 (p53 exon 5): 5′ GCGGTACCTTGAGGGGAGGAGAGTA. Seq-primer 6 (p53 exon 5): 5′ CCGGTACCGAGCAGCGCTCATGGTGGGGGC.

may have resulted in transcription suppression of the altered p53 gene.

Expression of normal p53 RNA was not found in cell line OS-51 by Northern blotting or PCR analysis. This is in accordance with the finding that the OS-51 cell line has lost both normal p53 alleles, as was reported in a previous paper (*11*). In addition, using an antibody which recognizes wild type and mutant p53 protein, in cell line OS-51 no p53 protein could be demonstrated (data not shown).

The insertion of the ETn element observed in the cell line must have occurred at an early stage in carcinogenesis of the primary tumor. Both OS-51 cell line and transplant tumor OTS-80 (*11*) were derived from the same primary osteosarcoma. DNA from cell line OS-51 and from transplant tumor OTS-80 gave PCR products of identical length and of the expected size using ETn 3′ LTR and p53 exon 6 or exon 7 primers. Furthermore, the presence of the 5′ ETn region in both cases could be demonstrated by PCR using primers for exon 4 and ETn 5′ noncoding region. DNAs from cell lines lacking the ETn insertion were consistently negative with these primers.

Several cases of insertion of endogenous transposable elements into murine or human cellular genes have been described (*2, 40, 41, 43–50*). The somatic inactivation of a suppressor gene is of particular interest for tumorigenesis in man. In fact, the putative sup-

pressor gene APC was inactivated in a human colon cancer by insertion of a LINE-1 element (*51*).

At present, the insertion of the endogenous ETn element into the p53 tumor suppressor gene seems to be a rare event. However, we cannot exclude that the large number of various retroviruses and retrovirus-like elements, which can be activated spontaneously by environmental risk factors such as carcinogens and irradiation, may impose a considerable tumor risk. Possibly, in individuals significantly exposed to these factors, endogenous retroviral elements could play an as yet underestimated role in tumor development.

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Note added in proof. After this manuscript was finished, a report by Wu *et al.* (*55*) was published showing a very similar form of splicing ETn sequences into the mRNA of the Fas apoptosis gene.

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