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Inactivation of the *p53* Tumor Suppressor Gene via a Novel Alu Rearrangement

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Abstract

Inactivation of the *p53* tumor suppressor gene is a common finding in human cancer. In most cases, inactivation is due to a point mutation in the gene, but rearrangement of the *p53* gene is sometimes observed. We analyzed the inactivation of *p53* in the human pancreas cancer cell line Hs766T, which harbors a structural alteration in the *p53* gene. This inactivation was found to be the result of a complex deletion/insertion event involving at least two different Alu elements. The rearrangement eliminated exons 2–4 from the *p53* gene, whereas a 175-bp Alu fragment was inserted between the breakpoints of the deletion. DNA sequence analysis of this Alu fragment revealed that it is identical to an Alu element in intron 1 of the *p53* gene. This is the first report of *p53* inactivation due to a rearrangement involving Alu elements. This type of inactivation may go unnoticed when only traditional methods to detect *p53* alterations are used.

Introduction

Mutation of the *p53* tumor suppressor gene is one of the most common defects associated with human cancer. Under normal circumstances, the p53 protein has specific DNA binding properties through which it regulates the transcription of genes involved in cell cycle regulation and apoptosis. In cancer cells, the inactivation of *p53* is usually the result of a point mutation in exons 5–8, leading to an amino acid change in the p53 protein (1). Although these *p53* mutations are dominant over the wild-type protein, loss of the normal *p53* allele (loss of heterozygosity) is also a common finding in *p53* mutant cells. Not all *p53*-associated human cancers have a high frequency of point mutations. For instance, about 50% of osteosarcomas have rearranged *p53* genes, whereas only 20% harbor point mutations in the *p53* gene (2). The rearrangements found in osteosarcomas disrupt the normal *p53* sequence and, combined with larger chromosomal deletions, lead to cells that do not express *p53* (*p53*-null cells). Because the current methodology is aimed at the detection of point mutations in *p53*, the inactivation of *p53* via large deletions may go unnoticed (1). Specifically, immunohistochemical staining will only detect mutant p53 protein, whereas an analysis of PCR products by single-strand conformational polymorphism and DNA sequencing may detect sequences derived from admixed normal cells when the tumor is homozygously deleted for *p53*. This is not an unlikely scenario: one of the first reports on *p53* inactivation in human lung cancer showed truncated mRNAs and *p53* gene rearrangements in 7 of 30 cell lines (3).

One possible mechanism by which rearrangements may occur is by recombination between repetitive elements in the genome (4). In some cases, recombination may be driven by the interaction of homologous or nonhomologous sequences within such elements, because they could provide opportunities for recombination initiated by DNA alterations such as double strand breaks (4, 5).

Approximately 40% of the human genome consists of repetitive DNA; the main form is the Alu repeat element, of which an estimated 500,000 copies account for about 10% of all human DNA (6). Alu elements were initially discovered by the digestion of human DNA with the *Alu*I restriction endonuclease. The prototype sequence has a total length of about 280 bp, consisting of two similar monomers separated by an oligo-dA tract. The average inter-Alu homology is about 87%.

Alu-rich sequences can be hot spots for genomic instability (6). One well-studied example is that of gene inactivation by germ-line rearrangement in the low-density lipoprotein receptor gene (7). Two clusters of highly homologous Alu elements make this gene particularly prone to gene rearrangements. Alu-associated recombination occurring in the germ line has also been found in human familial cancer (8). At present, there are only a few reports in which Alu elements were found in or near chromosomal breakpoints in somatic cells derived from human cancers. In some of these cases, the Alu elements appear to have been participants in homologous recombination. Examples are the *ALL-1* oncogene in acute myeloid leukemia (9) and the *tre* oncogene in Ewing's sarcoma (10).

The possible involvement of Alu-associated events in the inactivation of the *p53* tumor suppressor gene has not been investigated until now. This gene spans approximately 20 kb of genomic sequence, of which approximately 2.5 kb are encoded in 11 exons. *p53* harbors 13 single Alu elements and 4 clusters of rearranged Alu elements dispersed throughout the gene (GenBank accession number, X54156; Fig. 1). Small, intragenic deletions in the *p53* gene have been reported in a Li-Fraumeni syndrome family (11) and have recently been reported in a human glioblastoma cell line (12), but Alu elements were not involved in these deletion events. We report the molecular analysis of a small deletion/insertion event spanning *p53* exons 2–4 in a pancreas cancer cell line and suggest that it resulted from a complex rearrangement event involving multiple Alu elements in the *p53* gene. This is the first example of *p53* inactivation by a mechanism involving Alu elements.

Materials and Methods

Cell Line and DNA Preparation. The human cell lines Hs766T and Y79 were obtained from the American Type Culture Collection. Placenta DNA was used as a source of normal DNA (Sigma, St. Louis, MO). DNA was isolated by incubating 10^6 cells in 500 μl of lysis buffer [10 mM Tris (pH 8.0), 0.2% Tween 20, and 100 μg/ml proteinase K] for 20 h at 56°C. Before PCR, the proteinase was inactivated by incubation at 95°C for 10 min.

Long PCR of p53. The *p53* gene was divided into three segments that were amplified separately using long PCR. The *p53* genomic region spanning exons 2–9 was amplified using TaqPlus long PCR polymerase (Stratagene, La Jolla, CA) with primers F11 (5′-CAGAGATTGCAGGCTGAGAATGAC-3′) and R15 (5′-CTCCATCGTAAGTCAAGTAGCATC-3′). Amplification was done in a two-step protocol, cycling for 35 rounds at 95°C for 10 s and 68°C for 5 min. The predicted length of the F11-R15 PCR product was 4021 bp. The 5′ genomic portion of *p53* was amplified using primers F2 (5′-TCCTGGTAGGAGGCGGAACTC-3′) and R11 (5′-GTCATTCTCAGCCTGCAATCTCTG-

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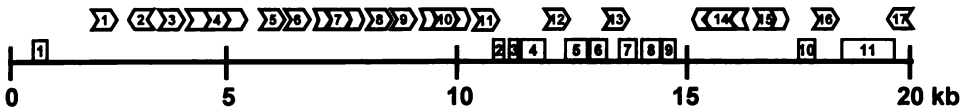

Fig. 1. Schematic representation of the *p53* gene. Exons 1–11 of *p53* (□) and the Alu elements found in the *p53* gene (*open arrows*) are indicated. Note that Alu 4, 7, 10, 14, and 15 are rearranged clusters, and that Alu 2, 14, and 17 are in the reverse orientation.

3′), with a predicted length of 8826 bp. The 3′ genomic part of *p53* was analyzed with primers F15 (5′-GATGCTACTTGACTTACGATGGAG-3′) and R18 (5′-GTCCTGGGTGCTTCTGACGCAC-3′), with a predicted length of 3781 bp. For analysis of the intron 1 Alu element, *p53* was amplified with primers F8 (5′-CTGTCTGTAGACCTGTGTCCCTC-3′) and R11 using the conditions described above.

Analysis of PCR Products. For DNA sequence analysis, the region spanning exons 2–5 was amplified with primers F11 and 5AS (5′-CAACCAGC-CCTGTCGTCTCTC-3′) using Taq polymerase (Perkin-Elmer Corp., Norwalk, CT) for 35 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The predicted length of this PCR fragment was 2249 bp. The PCR product from Hs766T was purified with QIAquick PCR columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA sequence analysis was done using an ABI Prism DNA sequencing kit (Perkin-Elmer Corp.) and analyzed on an ABI fluorescent sequencer. For sequence analysis of the 5′ Alu elements, the F8-R11 TaqPlus PCR product was purified using QIAquick PCR columns and analyzed as described above. DNA sequence comparisons were performed with the Wisconsin Package Version 9.0 (Genetics Computer Group, Madison, WI). Genome-wide searches were performed at the National Library of Medicine using the BLAST Version 2 algorithm.

Results

The pancreas cancer cell line Hs766T contains a large deletion that was first reported as an aberrant hybridization pattern on a *p53* DNA blot (13). Subsequent analyses provided evidence that this deletion spans exons 2–4, because these exons could not be PCR-amplified from Hs766T (14). To investigate the possible involvement of Alu elements in the inactivation of the *p53* gene in human cell lines, we chose to further analyze the *p53* alteration in Hs766T.

The *p53* gene was analyzed by the amplification of three separate long PCR fragments in pancreas cancer cell line Hs766T, retinoblastoma cell line Y79, and human placenta DNA. The PCR fragment spanning *p53* exons 2–9 was of the expected 4 kb in Y79 and in the normal human DNA but showed a truncated amplification product of approximately 2 kb in Hs766T (Fig. 2). No normal-sized amplification product was found in Hs766T, indicating loss of the other allele of *p53*. A minor amplification product of about 2 kb was seen in the normal control DNA; this fragment was not present in subsequent analyses and presumably represents a PCR artifact. The 5′ PCR fragment migrated at the expected length of about 8.5 kb. Similarly, the 3′ part of *p53*, which harbored exon 10 and part of exon 11, yielded the expected PCR product of about 4 kb for Hs766T, Y79, and normal human DNA (data not shown).

Molecular Analysis of Genomic *p53* Spanning Exons 2–5. To further characterize the deletion event in Hs766T, the region corresponding to *p53* exons 2–5 was amplified and subjected to DNA sequence analysis. This region would result in a 2.2-kb PCR fragment in wild-type *p53*. The DNA sequence analysis revealed that exons 2–4 had been deleted from the Hs766T *p53* gene, and that several Alu elements were present in and around the breakpoint. The DNA sequence of this area is shown in Fig. 3. At the 5′ end, the DNA sequence in Hs766T is identical to the published *p53* sequence up to nucleotide position 11348 (GenBank accession number, X54156). The DNA sequence then continues with a 175-bp fragment (*p53* nucleotide position 8737–8911) of an Alu element that was apparently inserted into the *p53* gene. After this inserted Alu fragment, the DNA sequence is again identical to the published *p53* sequence starting at nucleotide position 12993. Previous reports have indicated that Hs766T harbors a homozygous G to A point mutation at codon 181 of exon 5, changing an arginine to histidine (13). However, bidirectional DNA sequence analysis of Hs766T exon 5 did not show this mutation.

A schematic representation of the *p53* deletion/insertion event in Hs766T is shown in Fig. 4. The 5′ breakpoint is located 10 bp from the end of Alu element 11 in the *p53* sequence (at position 11347), whereas the 3′ breakpoint occurs within the last nucleotides of intron 4 (at position 12993), approximately 100 nucleotides after Alu element 12.

Analysis of Alu Elements in and around the Hs766T p53 Deletion. The newly identified Alu sequence that was inserted into *p53* was compared with all known Alu elements in the human genome by the BLAST Version 2 algorithm. Interestingly, this comparison led to a closest match with an Alu element within intron 1 of the *p53* gene. The matching Alu element had 173 of 175 nucleotides (98%) identical to a part of Alu 8 in the published sequence of *p53* (nucleotide positions 8703–8982), whereas the next nearest match had only 147 of 175 nucleotides (86%) identical with the inserted Alu fragment. All Alu elements involved in the *p53* rearrangement are highly homologous: (*a*) the identity between Alu 8 and Alu 11 is 79% (225 of 284 bp); (*b*) the identity between Alu 11 and Alu 12 is 75% (214 of 286 bp); and (*c*) the identity between Alu 8 and Alu 12 is 76% (217 of 285 bp).

To establish the origin of the inserted Alu fragment in the Hs766T *p53* deletion, we analyzed Alu 8 from *p53* in Hs766T, Y79, and human placenta DNA. The sequence of the 175-bp inserted Alu fragment was identical to Alu element 8 from the Hs766T *p53* gene. Compared to the published *p53* sequence, two thymines (at positions 8759 and 8797) are replaced by cytosines in Hs766T. The change to a cytosine at position 8797 was also found in Y79 and in placenta DNA, but these samples had a thymine at position 8759.

Discussion

Inactivation of the *p53* tumor suppressor gene in human cancer is usually due to a single point mutation leading to an amino acid change in the p53 protein (1). Such mutations have a dominant negative effect

Fig. 2. Long PCR of the *p53* coding region in Hs766T, Y79, and placenta DNA. The normal-sized amplification product of about 4 kb is visible in Y79 and placenta DNA, but the PCR product of Hs766T is only about 2.3 kb. *Lane M*, marker Lambda cut with *Hin*dIII; *Lane 1*, Hs766T; *Lane 2*, Y79; *Lane 3*, placenta DNA.

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                                                  11347
                                                    |
p53 intron 1    ACTGTGCTCC AGCCTAGGTG ACAGAGAGAG  actccatctc aaaaaaaaaa aaaaatacag
                                                  8737
                                                    |
                gggcttgggg gctcacgcct gtaatcccag  CACTTTAGGA GGCCAAGGGG GGCGGATCAC


                CTGAGGTCGG GAGTTCAAGA CCAGCCTGAC CAACATGGTG AAACCCCGTC TCTACTAGAA
Alu 8 fragment
                GTACAAAATT AGCCAGGTGT GGTGGCACAT GCTTGTAGTC CTAGCTACTC AGCAGGCTGA
                                                8911
                                                  |
                GGCAGGAGAA TCATTTGAAT CCGGG  aggag gttgcagtaa gcggagatag tgccactgta
                                                12993
                                                  |
p53 intron 4    gtttgtttct ttgctgccgt gttcc  AGTTG CTTTATCTGT TCACTTGTGC CCTGACTTTC
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Fig. 3. DNA sequence of the deletion/insertion site in the *p53* gene in Hs766T. The *p53* DNA sequence of Hs766T is indicated in *uppercase letters*, whereas the flanking sequences not present in Hs766T are indicated with *lowercase letters*. The partial *chi* sequence CCAG is *underlined*. A 175-bp fragment from Alu 8 was inserted between the breakpoints in introns 1 and 4. All sequences involved in the intron 1 breakpoint correspond to Alu DNA. The intron 4 breakpoint involves Alu 8 and a sequence 100 bp downstream from Alu 12.

over the wild-type protein. In addition, some point mutations may lead to the generation of STOP codons causing premature termination of protein translation or even occur in consensus splice sites, leading to aberrant *p53* mRNAs that do not encode functional protein. It has been reported that *p53* microdeletions (those that involve 3 or more bp) at the sites of small repeats are more common in pancreas cancer than in other cancer types (14).

From a mechanistic point of view, these small genetic alterations are different from those causing larger deletions or even a completely rearranged *p53* gene. Gross genetic changes in *p53* have been reported in several human cell lines and primary human tumors, especially from lung and osteosarcomas, and there is one report indicating a large deletion of *p53* in a patient from a Li-Fraumeni syndrome family (2, 3, 11). Such cases of *p53* rearrangement are of interest for mechanistic, evolutionary, and physiological reasons. Homologous recombination that might occur between repeated elements in the genome is an obvious source of deletions. Alu elements are good potential substrates because of the large numbers of Alu elements in the genome and because they share significant homology. Also, Alu elements have been implicated in germ-line rearrangements in human familial cancer syndromes (8).

We analyzed a large deletion spanning exons 2–4 of *p53* in pancreas cell line Hs766T at the molecular level. Previous studies had shown that this cell line had a deletion of approximately 2 kb in the *p53* gene (13), and that it had lost exons 2, 3, and 4 (14). The 5′ breakpoint of the deletion occurred in an Alu element (Alu 11 in the *p53* gene), whereas the 3′ breakpoint was 110 bp downstream from a second Alu element (Alu 12) within *p53*. In addition, a 175-bp Alu fragment was inserted between the breakpoints of the deleted area. This additional Alu fragment showed a high homology with another reported intron 1 Alu element (Alu 8). Further sequence analysis revealed that the 175-bp Alu insert was actually identical to Alu 8 in intron 1 of *p53*.

There are two possible explanations for the observed *p53* deletion/insertion event in Hs766T involving Alu elements. The first would involve retrotransposition, by which multiplication of an Alu element

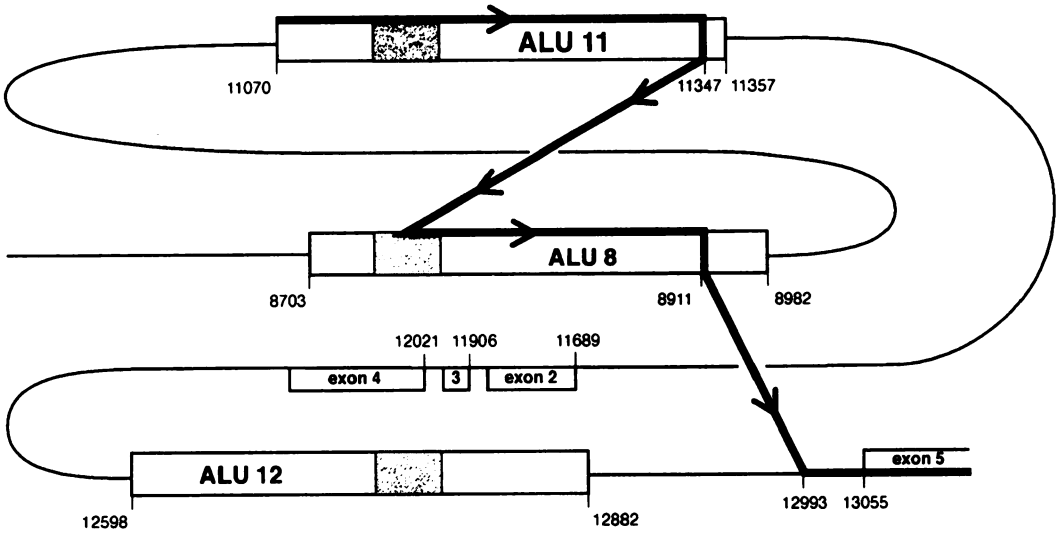

Fig. 4. Model indicating the three Alu elements and their possible involvement leading to the rearrangement and inactivation of the *p53* tumor suppressor gene observed in the pancreas carcinoma cell line Hs766T. The DNA sequences present in Hs766T are indicated by *thick lines*, whereas the regions that became deleted (including exons 2–4 and Alu 12) are indicated by *thin lines*. The three Alu elements □ and the recombination hot spots within them ■ are indicated.

occurs in a fashion similar to that seen for retroviruses. Retrotransposition is thought to be responsible for the generation of new, evolutionary "young" Alu elements, for instance, those of the Alu-Sb2 class (15). At present, however, there is no evidence for retrotransposition of Alu elements in somatic cells. In addition, if retrotransposition were responsible for the deletion/insertion in Hs766T, one would expect at least some degree of target site duplication associated with the retrotransposition event. However, no evidence for such a mechanism is present in either the Hs766T *p53* DNA sequence or in the sequence differences found in this cell line. A more attractive explanation of the observed deletion/insertion event would be through homology-driven recombination in which the homology between different Alu elements leads to an exchange of genetic material. Under this model, the *p53* deletion/insertion in Hs766T would have been a complex rearrangement because it involved at least two separate Alu elements within the *p53* gene. A schematic representation of this rearrangement is shown in Fig. 4.

Several properties of the Hs766T deletion/insertion sequence are of interest. The 5′ part of the inserted Alu fragment starts with the last 13 bp of a 26-bp core sequence that was reported to be a hot spot for Alu-associated recombination (16). This sequence includes the 5′-GCTGG-3′ motif that forms the first 5 bases of the 5′-GCTGGTGG-3′ prokaryotic recombination hot spot *chi* sequence (17). The actual 5′ breakpoint does not fall within this consensus sequence but rather at the 3′ end of a short GA dinucleotide repeat. There are three occurrences of another 4-bp motif of *chi* (5′-CCAG-3′) at or near the breakpoints of the Hs766T deletion/insertion. This motif has been reported to be located near nonhomologous recombination breakpoints of the immunoglobulin loci. Also, one-ended homology-driven recombination has been demonstrated for *Escherichia coli* and indirectly in mammalian cells (18).

Although the position of the 5′ Alu 8 breakpoint falls within the 26-bp core sequence, there is no clear evidence that any *chi*-related sequence was directly involved in the observed deletion/insertion of the Hs766T *p53* gene. We also did not find any homology between the Alu elements and the intron 4 sequence starting 3′ from the deletion/insertion breakpoint.

In addition to the larger genetic alterations, it has been reported that Hs766T harbors a mutation in *p53* exon 5 at codon 181 (CGC to CAC, changing an Arg to His; Ref. 13). Such a mutation would be of interest because *p53* inactivation has recently been linked to increased intrachromosomal homologous recombination (19). However, we could not confirm this exon 5 mutation in Hs766T, a result that is in agreement with another report on the status of *p53* in this pancreas cancer cell line (14). From a theoretical point of view, a point mutation in *p53* would eliminate the need to inactivate *p53* through other mechanisms.

One additional property of the sequences around the *p53* deletion/insertion is that the breakpoint occurred at the 3′ end of an almost perfect 12-bp-long GA dinucleotide repeat. It has been reported that pancreas cancer has an increased incidence of small deletions and insertions at homocopolymer tracts (repeats of purines or pyrimidines of at least 4 bases; Ref. 14). Such tracts may form dinucleotide repeats such as the GA repeat located at the *p53* breakpoint in Hs766T. In a recent report on *p53* inactivation in a case of glioblastoma, it was shown that a CA dinucleotide repeat (STS D17S960) was located close to the breakpoint of a somatic rearrangement that inactivated the *p53* gene (12). It would be of interest to determine whether such motifs provide structures that might initiate recombination. Motifs referred to as at-risk motifs have been identified that stimulate recombination including large inverted repeats comprised of genes and Alu elements in yeast and mammalian cells (20).[2] Whereas DNA divergence might be expected to reduce the interaction between Alu repeats, a DNA double break has been shown to greatly stimulate recombination between highly divergent direct repeats (5).

In conclusion, we analyzed a deletion/insertion event in *p53* in a pancreas cancer cell line at the molecular level and demonstrated that the recombination event involved at least two separate Alu elements. Although the inactivation of *p53* by genetic recombination appears to be infrequent in most human cancers, the true frequency of *p53* inactivation may be underestimated when only traditional assays for *p53* mutation are used.

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[2] Unpublished data.