Methylation in the p53 Promoter Is a Supplementary Route to Breast Carcinogenesis: Correlation between CpG Methylation in the p53 Promoter and the Mutation of the p53 Gene in the Progression from Ductal Carcinoma In Situ to Invasive Ductal Carcinoma

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SUMMARY: Aberrant methylation in the CpG sites located in the promoter region of several tumor suppressor genes has been reported in various types of cancers. However, the methylation status of the p53 promoter has not been clearly determined and no information is available on its role in breast cancer. The aim of the study was to determine the presence and timing of the methylation of CpG sites in the p53 promoter, in the progression from ductal carcinoma in situ to invasive cancer. We also explored the correlation between the CpG methylation of the p53 promoter and p53 mutation during the progression of breast cancer. The corresponding lesions of both the invasive and noninvasive types were microdissected in paraffin-embedded tissue of 26 breast carcinomas. Bisulfite-modified DNA sequencing for methylation status in the p53 promoter was carried out, and double-strand DNA sequencing was performed in the promoter region and exons 4 to 9 of the p53 gene. CpG site methylation in the p53 promoter was detected in three cases (11.5%). Two noninvasive and three invasive lesions harbored CpG methylation in the p53 promoter. Methylations in more than one site were observed in three lesions, all of which contained methylation in two sites. The methylated CpG sites were located near the AP1 and YY-1 binding sites and at the YY-1 binding site. The p53 mutation was not found in the lesions where methylation in p53 promoter region was evident. In 16 cases (61.5%), neither methylation nor p53 mutation was detected. We conclude that the methylation in the p53 promoter region is found in the breast cancer irrespective of the status of invasion, and that the hypermethylation in the p53 promoter region is an alternative pathway to tumorigenesis where there is no p53 gene mutation. (*Lab Invest 2001, 81:573–579*).

Alterations of the methylation pattern of DNA are common in cancer cells and are capable of directly modifying carcinogenesis. Cytosine methylation is thought to contribute to the formation of point mutations in tumor suppressor genes in somatic and germline cells (Bird, 1992; Holliday and Grigg, 1993). CpG islands are rich in the CpG dinucleotide, often associated with genes, and are normally unmethylated in cells. Hypermethylation in a promoter region represents a substitute for mutations in the coding region that eliminate tumor suppressor gene function (Merlo et al, 1995).

The p53 promoter region has been sequenced and basal promoter activity localized to an 85 bp region that is indispensable for full promoter activity (Bienz-Tadmor et al, 1995; Tuck and Crawford, 1989). The p53 promoter has five putative binding sites for transcriptional factors. Putative binding sites for YY-1/NF-1, NF-kB, and the basic helix-loop-helix family of transcriptional factors arise within this minimal 85 bp region extending into exon 1 (Ginsberg et al, 1990; Reisman et al, 1993; Tuck and Crawford, 1989). The p53 promoter also contains an AP-1 site, approximately 100 bp upstream from the minimal 85 bp region (Kirch et al, 1999) and a PAX 5 binding site within the noncoding exon 1 (Stuart et al, 1995).

Aberrant methylation in the CpG islands located in the promoter region of several tumor suppressor genes has been reported in colorectal (Herman et al, 1995), breast (Graff et al 1995), renal (Herman et al,

1994), and gastric (Lee et al, 1997) cancers. Introduction of CpG methylation into the human p53 promoter down-regulated the transcriptional activity of p53 as assessed by in vitro cultured cells transfected with a plasmid construct (Schroeder and Mass, 1997). However, the methylation status of the p53 promoter has not been clearly determined and there is no information available on the methylation status of p53 promoter in breast cancer tissue.

The aim of the current study was to determine the presence and timing of the methylation of the CpG sites in the p53 promoter region, where the transcriptional activity is down-regulated by CpG methylation (Schroeder and Mass, 1997) in the progression from ductal carcinoma in situ (DCIS) to invasive ductal cancer by means of microdissection and bisulfite mapping of DNA. Additionally, we investigated the correlation between the hypermethylation of the p53 promoter and p53 mutation in the carcinogenesis of breast cancer.

Results

Mutation and Methylation in the Promoter Region of the p53 Gene

Before examining the methylation status of the promoter region, corresponding sequences under investigation were examined for any mutation. The 400 bp promoter sequence of the p53 gene, spanning from −212 to +188, was amplified in 26 carcinomas by two successive PCR reactions and the sequence was analyzed. There were no apparent mutations within the promoter region of p53, including the noncoding exon 1, in any of the 26 cases.

DNA extracted with bisulfite modification was compared to DNA extracted without modification to test the validity of the technique. The sequences of p53 promoter region with bisulfite modification were concordant with those without manipulation, except that unmethylated cytosines were converted into thymidines (Fig. 1). None of the 13 normal breast tissue samples had methylation on the CpG dinucleotides (Fig. 1).

The methylation profile within the same promoter region was ascertained by bisulfite mapping in control normal breast tissue, DCIS, and invasive ductal carcinoma. Sequence analysis revealed that CpG site methylation in the p53 promoter region appeared only in 3 of 26 cases (11.5%). Two noninvasive lesions (3-Non, 6-Non) and three invasive lesions (3-Inv, 6-Inv, 21-Inv) harbored CpG methylation in the p53 promoter. Two noninvasive lesions had comedo and solid histology, separately. Methylation at more than one CpG site was observed in three cases, all of which showed two methylations. Of the 16 potential sites, the methylation sites were restricted to −195, −192, −132, and −105. Interestingly, all of the three cases were methylated at the −132 site. Of these four CpG sites, the −195 to −192 sites were located just upstream of the AP1 binding site, the −130 site was located just upstream of the YY-1 binding site, and the −103 CpG was located in the YY-1 binding site (Fig. 2, Table 1).

Correlation between Hypermethylation of the p53 Promoter and p53 Mutation

As shown in Table 1, we analyzed the correlation between the methylation status of the promoter and the mutation of the p53 gene. The frequency of breast cancers with p53 gene mutations was 26.9% (7 of 26 cases). The mutations were observed in 5 of 19
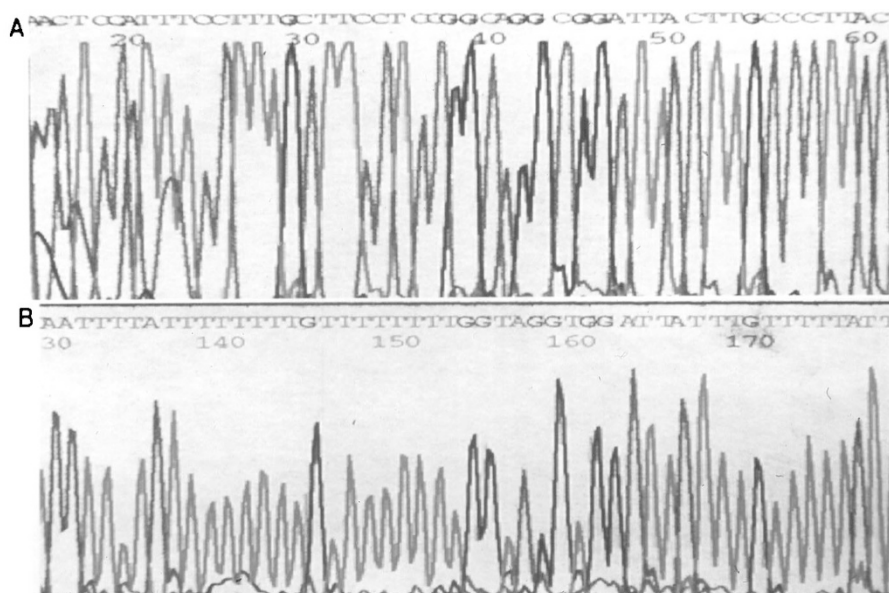

Figure 1.

Bisulfite sequencing of the wild-type p53 promoter region in the normal breast lobule. The promoter sequence of the wild-type p53 gene was treated with bisulfite and the corresponding region was amplified by two rounds of PCR using the specific primer sets (C to T, G to A). The PCR products were sequence analyzed for both strands. Each C in the CpG sites was changed to T by the bisulfite treatment. (A) Before bisulfite-modification. (B) After bisulfite modification.
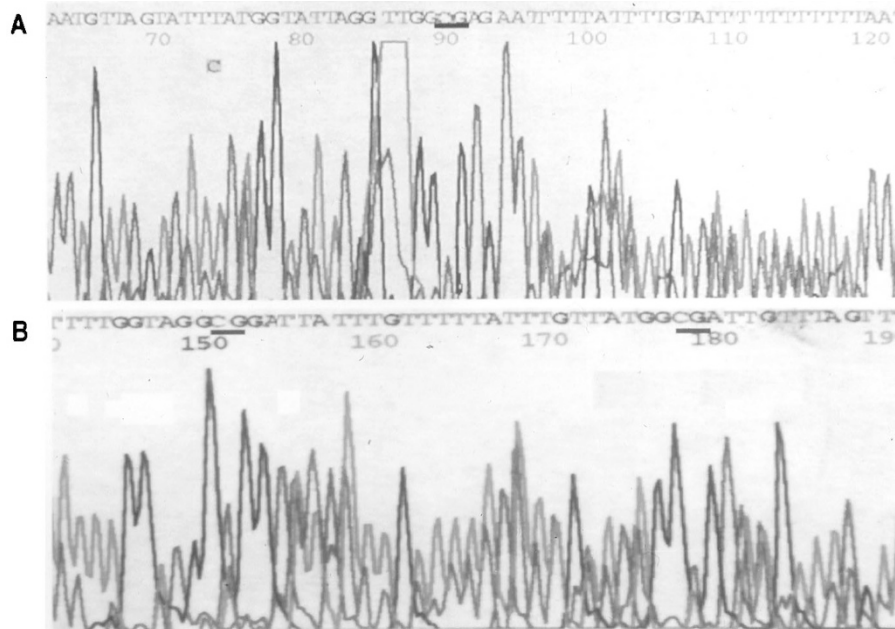
Figure 2.
Bisulfite sequencing of the p53 promoter region for cancer tissue. The promoter sequence was modified with bisulfite and the analogous region was amplified by two rounds of PCR using the specific primer sets (C to T, G to A). The PCR products were sequence analyzed for both strands of DNA. The methylated CpG sites are *underlined*. (A) Single CpG site methylation (−192) in the #3-Non sample. (B) Double CpG site methylation (−132 and −105) in the #6-Inv sample.

noninvasive lesions (26.3%) and in 6 of 21 invasive lesions (28.6%). Interestingly, the methylation of the promoter seemed independent from mutation in the p53 gene. No mutation in the p53 gene was found in the five lesions where CpG methylation in the promoter was evident, whereas the lesions with mutation(s) in the p53 gene bore no methylation at the 16 promoter CpG sites. In the remaining 16 cases (61.5%), neither methylation nor p53 mutation was identified (Table 1).

Discussion

p53 is a tumor suppressor and it plays a pivotal role in the fate of stressed cells and in eliminating cells that have sustained genetic damage. Damage to the p53 gene with other mutant genes may cause cells to proliferate unchecked. Inactivation of p53 can occur through several mechanisms, including loss of p53 alleles or deletions, insertions, or point mutations in the p53 gene (Hollstein et al, 1991).

Another mechanism of p53 inactivation that was observed in cases with no deletion or mutation is the epigenetic hypermethylation of the regulatory region. Abnormal promoter hypermethylation is observed in many tumor types and is associated with transcriptional inactivation and silencing of defined tumor suppressor genes in human cancers (Gonzalez-Zulueta et al, 1995; Merlo et al, 1995). For that reason, promoter region hypermethylation represents an alternative to coding region mutations in eliminating tumor suppressor gene function (Merlo et al, 1995).

Most studies have indicated a correlation between the hypermethylation of the p53 promoter and a drop in the transcriptional activity in cultured cells (Pogribny

et al, 2000; Schroeder and Mass, 1997). However, the presence and the timing of CpG methylation of p53 promoter in breast cancer tissue, especially during the tumorigenesis of breast carcinoma has not been as well investigated.

The hypermethylation status of the promoter region of p53 gene was detected in 3 of 26 cases (11.5%) (Fig. 2, Table 1). The two noninvasive and three invasive components harbored CpG site methylation in the p53 promoter. The methylation sites were restricted to sites −195, −192, −132, and −105 of the 16 potential sites. To the best of our knowledge, this is the first demonstration of CpG site methylation in the p53 promoter in breast cancer tissue, especially in specifically microdissected samples of both noninvasive and invasive lesions.

The four CpG sites found to be methylated were located near the AP1 binding site (−195 and −192 CpG), near the YY-1 binding site (−130 CpG), and in the YY-1 binding site (−103 CpG) (Table 1). The methylation was observed in the CpG site of the YY-1/NF-1 binding site (6-Non, 6-Inv, Fig. 2). Both YY-1 and NF-1 bind a composite element within the proximal promoter of the human p53 gene. NF-1 proteins are a heterogeneous family of proteins that regulate the cell-specific expression and function-specific expression of genes (Jackson et al, 1993). In the mammary gland, NF-1 plays a critical role in activating milk protein gene promoters during lactation (Watson et al, 1991). However, differentiation of the mammary gland and milk protein gene expression is inhibited by oncogenic transformation of mammary epithelium with Ha-v-ras (Andres et al, 1988). Furthermore, there is a down-regulation of NF-1 level by

Table 1. Methylation of CpG Sites in p53 Promoter and Mutation of p53 and Its Promoter Region in Noninvasive and Invasive Lesions

Number	NG[a]	Histology	Methylation	Mutation of p53 promoter	p53 mutation	
IDC with DCIS (total: 14 cases)						
1-Non	3	Comedo	None	None	Ins T at 14043	Frameshift
1-Inv	2	IDC	None	None	Ins T at 14043	Frameshift
2-Non	3	Solid	None	None	CGC→CCC	Arg72Pro[c]
					ATG→TTG	Met246Leu
2-Inv	3	IDC	None	None	CGC→CCC	Arg72Pro[c]
					ATG→TTG	Met246Leu
3-Non	2	Comedo	+(−192,−139)[b]	None	None	
3-Inv	2	IDC	+(−195,−192)[b]	None	None	
6-Non	3	Solid	+(−105)[b]	None	None	
6-Inv	2	IDC	+(−132, 105)[b]	None	None	
7-Non	1	Cribriform	None	None	Del T at 12033	Frameshift
7-Inv	2	IDC	None	None	Del T at 12033	Frameshift
9-Non	2	Comedo	None	None	Ins T at 14488	Frameshift
9-Inv	2	IDC	None	None	Ins T at 14488	Frameshift
Pure DCIS (total: 5 cases)						
17-Non	3	Comedo	None	None	AAG→AGG	Lys291Arg
Pure IDC (total: 7 cases)						
21-Inv	2	IDC	+(−132)[b]	None	None	
23-inv	2	IDC	None	None	ATG→TTG	Met246Leu
25-inv	3	IDC	None	None	GCC→GTC	Ala159Val

NG, nuclear grade; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ; Non, noninvasive; Ins, insertion; Inv, invasive; Del, deletion.

[a] Only lesions that have shown either methylation or mutation are illustrated. "None" denotes absence of the methylated CpG or the p53 mutation.

[b] Numbering scheme taking the major transcriptional site as +1, which corresponds to #837 from NCBI, Genbank database, accession number X54156.

[c] DNA polymorphism.

Ha-v-ras (Nebl et al, 1994) and this might inhibit milk protein gene expression. Recently, it was demonstrated that a depletion or low level of NF-1 was observed in the majority of breast tumor samples (Nayak and Das, 1999). Based on our results, it is conceivable that methylation of the recognizable NF-1 binding site in the p53 promoter may inhibit the binding of NF-1 and elicit the carcinogenesis in the breast cancer.

Methylation-specific PCR was developed for assessing the methylation status of virtually any CpG-rich region, making use of no methylation-sensitive restriction enzymes. Methylation-specific PCR is markedly more sensitive than other techniques based on restriction enzyme mapping with Southern blot analysis (Herman et al, 1996; Wong et al, 1997). However, it is not able to differentiate which CpG sites are methylated (or unmethylated) or able to detect how many methylations occur, especially in the situation where many CpG sites are adjacent to each other. These can be major drawbacks in using methylation-specific PCR, because it is difficult for this technique to discover the exact CpG dinucleotides that are involved in the transcriptional activity of the promoter of the critical gene. To overcome this obstacle, we used DNA sequencing with bisulfite modification to obtain an accurate picture of hypermethylation in the p53 promoter region.

Venkatachalam et al (1998) demonstrated that a reduction in wild-type p53 gene dosage without mu-

tation is sufficient to promote tumorigenesis. In our work, a concurrent p53 mutation was not found in five lesions with hypermethylation of the p53 promoter region. This result is consistent with our hypothesis that methylation in the p53 promoter region and, thereby, reduced expression of the gene, contributes to the carcinogenesis of breast cancer.

It was not determined how many CpG methylations in the p53 promoter were required to trigger tumorigenesis. Pogribny et al (2000) recently showed that a single-site methylation that occurs 216 nucleotides upstream from the 85 bp minimal promoter region, suppressed promoter activity by 85% and was associated with transcriptional inactivation in the folate/methyl-deficient model of mouse hepatocarcinogenesis. We observed two lesions with methylation of only a single CpG site, which is concordant with their results.

However, neither p53 mutation nor the hypermethylation in the p53 promoter region was observed in 15 of 26 total cases (57.7%). This suggests that it is unsuitable to elucidate the carcinogenesis of the breast cancer exclusively in the view of the methylation status of the p53 promoter and the mutation of the p53 gene.

In conclusion, we demonstrated that CpG methylation in the p53 promoter region is found in breast cancer, irrespective of the status of invasion and that methylation in the p53 promoter region is an alterna-

tive pathway to neoplastic progression in the case of p53 gene mutation.

Materials and Methods

Tissue Selection and Microdissection

Paraffin-embedded tissues were obtained from 26 patients with breast cancer who had undergone mastectomy in Boramae City Hospital (Seoul, Korea) from January 1997 through February 1999. They were classified as DCIS (5 cases), invasive carcinoma with an intraductal component (14 cases), and pure invasive ductal carcinoma (7 cases). None of the patients received preoperative chemotherapy or radiotherapy. In each case, appropriate tissue blocks of normal lobules (13 lesions), DCIS (19 lesions), and invasive lesion (21 lesions) were selected. The term 'case' was used to describe the tumor sample with noninvasive and/or invasive component, whereas 'lesion' referred to either the identifiable noninvasive or invasive component in the tissue section. The microdissection for the specific lesions was carried out as previously described (Zhuang et al, 1995). Briefly, unstained 5-μm-thick sections were cut from the paraffin embedded blocks, deparaffinized twice with xylenes, rinsed twice with 95% ethanol, and stained with eosin. The adjacent sections were stained with hematoxylin and eosin. The essential lesions were dissected under a microscope using 26 gauge needles (Fig. 3). Microdissected samples were transferred into a 1.5 ml microtube containing 40 μl of lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% Tween 20, 200 μg/ml of proteinase K), incubated at 55° C for 72 hours, and divided into two tubes for p53 mutation assay and methylation assay of the p53 promoter region.

Bisulfite DNA Sequencing of the Promoter Region of the p53 Gene

For the methylation assay of the CpG sites in the p53 promoter, bisulfite modification was carried out as described previously with minor modifications (McDonald and Kay, 1997). Twenty microliters of DNA solution extracted from the microdissected tissue using Proteinase K were digested by *Hin*dIII at 37° C for 3 hours, 0.5 μg of *Escherichia coli* (*E. coli*) tRNA was added, precipitated by ethanol, and dissolved in 40 μl of distilled water. The DNA was denatured by adding 4.4 μl of 3 M NaOH and incubated for 15 minutes at room temperature. The denatured DNA was mixed with 200 μl of 40% sodium bisulfite (Sigma, St. Louis, Missouri) and 25 μl of 10 mM hydroquinone (Sigma), covered with a drop of mineral oil, and incubated on a Perkin Elmer (Foster City, California) 480 Thermal Cycler at 55° C for 16 hours. The DNA was desalted using a QIAEX II gel extraction kit (Qiagen, Valencia, California), and eluted in 40 μl of distilled water. The samples were desulfonated in 4.4 μl of 3 M NaOH at 37° C for 15 minutes. The samples were precipitated by the addition of 2.5 volumes of 100% ethanol and dissolved in 20 μl of distilled water. The promoter region was amplified by two rounds of PCR. The PCR products were purified using QIAEX II (Qiagen), subcloned into the pGEM-T vector (Promega, Madison, Wisconsin), and then transformed into *E. coli* DH5α. Colony PCR was performed to identify positive colonies harboring the p53 DNA fragment. Briefly, each *E. coli* colony was suspended in 500 μl of colony lysis buffer (20 mM Tris-HCl pH 8.5, 2 mM EDTA, 1% Triton X-100), and PCR was performed using 1 μl of the lysed *E. coli* and the universal forward and reverse primers. The PCR condition was 30 cycles at 94° C for 30 seconds, 55° C for 1 minute, and 72° C for 30
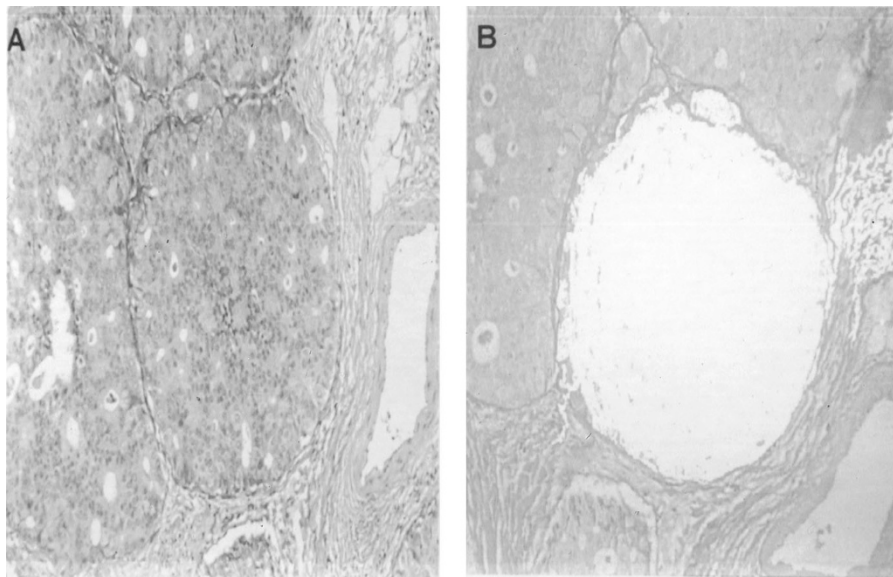

Figure 3.
Microdissection of noninvasive breast epithelial lesions (A) before microdissection, stained with hematoxylin and eosin, and (B) after microdissection, stained with eosin.

seconds. The recombinant plasmids were purified from the *E. coli* and the p53 region was sequence analyzed using the universal forward and reverse primers. The sequences of the primers used for the amplification of the bisulfite-treated promoter were as follows: for primary PCR 5'-TTATAGTTTTGGTTTGTAGAAT (up) and 5'-TAACTCAAAAAAAACTCATCAA (down), for nested PCR 5'-TTTTTATTTTAAAATGTTAGTA (up) and AT-CAAATTCAATCAAAAACTTA (down).

Sixteen CpG dinucleotides were located in the promoter fragments corresponding to −212 through +108 (taking the major transcriptional site as +1 in numbering). These nucleotides would be equal to 631 through 950, according to NCBI GenBank database accession number X54156. The pattern and presence of methylation at 16 CpG sites was analyzed in this data (Fig. 4).

PCR for the Promoter and Exons 4 through 9 of the p53 Gene

Reagents for each PCR reaction consisted of 0.2 mM deoxynucleotide triphosphate, 1.5 units of Pwo poly-

Figure 4.

(A) Schematic view of the p53 promoter region, showing the *Hind*III site and CpG site (*dotted line*). (1) Numbering scheme taken from NCBI GenBank, accession number X54156. (2) Numbering scheme with the transcriptional start site as +1. (B) The nucleotide sequence of the p53 promoter region was adopted from GenBank accession number X54156. The CpG sites (*bold*) in the sequence are serially numbered from 1 to 16 and indicated above the sequence. The putative binding sites for AP-1, YY-1/NF-1, NF-kB, Myc/Max, and Pax are also shown (*underlined*).

merase (Roche Molecular Biochemicals, Indianapolis, Indianapolis), 30 pmol of primer, 1x PCR buffer, and 1 μl of tissue sample in 50 μl of reaction volume. The PCR mixture was covered with a few drops of mineral oil and amplified on a Perkin Elmer 480 Thermal Cycler. After incubation at 94° C for 3 minutes, typical tissue reactions for primary PCR were cycled 25 times at 94° C for 30 seconds, 50° C for 60 seconds, and 72° C for 30 seconds. A nested PCR was performed to amplify exons 4 to 9 (except for exon 7, which was adequately amplified at the primary PCR). One microliter of primary PCR product was reamplified with nested primers in 50 μl of PCR mixture. The condition for the nested PCR was the same as that of the primary PCR, as described above. The sequences of primers used in the study were as follows: for the p53 promoter: primary 5'-TCACAGCTCTGGCTTGCAGA (up) and 5'-CCACTCACCCCCAAACTCGC (down), nested 5'-TTTCCACCCCAAAATGTTAG (up) and 5'-CTGCCCCACCCCCAGCCCCA (down); for exon 4: primary 5'-TGAGGACCTGGTCCTCTGACTG (up) and 5'-CCAAACTCGCTAAGTCCCCA (down), secondary 5-CCTCTGACTGCTCTTTTCAC (up) and 5-CATTGAAGTCTCATGGAAGCCA (down); for exon 5 and 6: primary 5-CCAGTTGCTTTATCTGTTCA (up) and 5-CTCACCCGGAGGGCCACTGA (down), secondary 5-CTTGTGCCCTGACTTTCAACTC (up) and 5-CAACCACCCTTAACCCCTCCTC (down); for exon 7: 5'-CGTCTAGAGGCCTGTGTTGTCTCC (up) and 5'-CGGTCGACGGTGGCAAGTGGCTCC (down); for exon 8 and 9: primary 5-AGGACCTGATTTCCTTACTG (up) and 5-TTGAGTGTTAGACTGGAAAC (down), secondary 5-ATTTCCTTACTGCCTCTTGCTT (up) and 5-TAGACTGGAAACTTTCCACTTG (down).

PCR products were checked on 2% agarose gels stained with ethidium bromide. PCR products were extracted with phenol/chloroform and followed by ethanol precipitation. The DNA was dissolved in 20 μl of distilled water and subjected to electrophoresis on a 2% agarose gel. The specific DNA fragments in the gel were purified using QIAEX II gel extraction kit (Qiagen), and dissolved in 15 μl of distilled water.

Direct Double-Strand DNA Sequencing

For the PCR-amplified DNA, DNA sequencing was carried out using the fluorescence cycle sequencing method, by means of an automatic DNA sequencer (377 DNA Sequencer, PE Applied Biosystems, Foster City, California). Both strands of DNA sequence were analyzed using 1 to 2 μl of DNA (approximately 50 ng). DNA sequencing was performed twice from separate PCR reactions. Primers used in nested PCR were applied as sequencing primers. The sequences were aligned with the wild-type p53 sequence by using the Clustal w (version 1.5) program (EMBL, Heidelberg, Germany), and the presence of CpG site methylation and mutation in p53 promoter region and exons 4 to 9 of p53 were investigated.
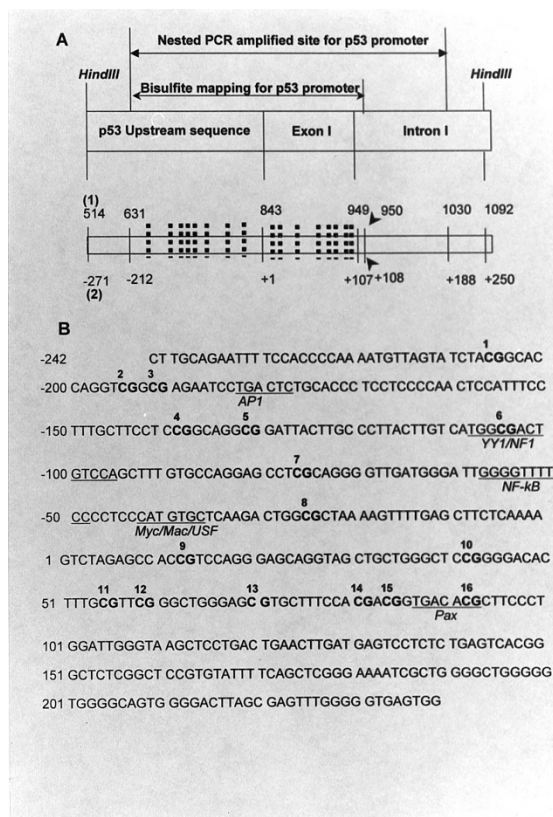
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