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Mutational analysis of *p73* and *p53* in human cancer cell lines

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***p73* is a candidate tumor suppressor gene with substantial DNA and protein homology to the *p53* tumor suppressor gene. We have investigated two hypotheses: (a) *p73* is mutated in diverse types of human cancer, and (b) *p73* is functionally redundant with *p53* in carcinogenesis so that mutations would be exclusive in these two genes. The entire coding region and intronic splice junctions of *p73* were examined in 54 cancer cell lines. Three lung cancer cell lines contained mutations that affected the amino acid sequence. One amino acid substitution was in a region with homology to the specific DNA binding region of *p53* and two microdeletions were outside the region of homology. Two of the cell lines with *p73* mutations also carried *p53* mutations. Although our results are inconsistent with the two hypotheses tested, *p73* mutations may contribute infrequently to the molecular pathogenesis of human lung cancer.**

Keywords: p73; mutation; deletion

Introduction

Recently, Kaghad *et al*. (1997) reported a novel protein, *p73*, that shares considerable sequence homology with *p53*, localizes to 1p36, an area where the loss of heterozygosity (LOH) has been reported in neuroblastoma, malignant melanoma, hepatocellular carcinoma and breast cancer. In neuroblastoma, a high frequency of LOH at the *p73* locus was detected and *p73* was expressed monoallelically. Furthermore, *p73* protein was significantly reduced in most neuroblastoma cell lines. *p73β*, which is a shorter, alternately spliced form of *p73*, interacts homotypically and with *p53* by the yeast two-hybrid system, suggesting that *p73* forms homo- and hetero-oligomers (Kaghad *et al*., 1997). When DNA damage occurs, *p53* accumulates, transcriptionally transactivates $p21^{WAF1}$, and blocks cell cycle progression at the G1 check points prior to DNA replication (Harper *et al*., 1993; El-Deiry *et al*., 1993; Xiong *et al*., 1993). Although *p73* is not activated by DNA damage, *p73* also transcriptionally transactivates $p21^{WAF1}$, and cell growth is suppressed by reintroducing a *p73* expression vector into a *p73* non-expressing cell line (Kaghad *et al*., 1997). Overexpression of *p73* can induce apoptosis in SAOS2 cells lacking *p53* (Jost *et al*., 1997). The structural and functional similarity between *p73* and *p53* suggested two hypotheses: (a) *p73* is mutated in diverse types of human cancer, and (b) *p73* is functionally redundant with *p53* in carcinogenesis so that mutations would be exclusive in these two genes. We studied *p73* alterations in 54 cell lines from diverse types of human cancer. Among these, the mutational status of *p53* was known in 45 of these cell lines. Three *p73* mutations were found in lung cancer cell lines, indicating the possibility that *p73* mutations may contribute to lung carcinogenesis.

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Results

Genomic clone isolation and p73 *intron sequencing*

One BAC clone (124N18) that was isolated by PCR screening contained all 13 *p73* coding exons. From this BAC clone, intron sequences adjacent to the exons were determined. The sequence data has been deposited into GenBank (Accession AF077616 through AF077628).

PCR–SSCP and the p73 *sequencing analysis*

Twelve of the 54 cell lines showed either base substitutions or deletions when compared to the coding regions and splice junctions of the wild type *p73* gene. Base substitutions were found at eight positions (Table 1). The altered nucleotides and cell lines in which they occured were the following: 519 T to C (HUH7, MIA PaCa-2 and NCI-H292), 735 G to A (MDA-MB-468 and NCI-H292), 790 G to T (NCI-H1155), 1008 C to T (COLO 320DM, LS 174T and M24), 1047 T to C (NCI-H292, COLO 320DM, LS 174T and M24), 1671 G to A (SK-HEP-1), 1689 T to C (HEP 3B), and 1830 G to A (NCI-H292, COLO 320DM, LS 174T and M24). Among these, three are T to C transitions, three are G to A transitions, one is a C to T transition and one is a G to T transversion. Seven of eight base substitutions are in the third codon, and are silent, i.e., the amino acid coded for is unchanged. Only one base substitution, nucleotide 790 G to T, causes an amino acid substitution at codon 264 from Gly to Trp (Figure 1a and b). Among those cell lines showing base substitutions, HEP 3B, HUH7, MDA-MB-468, NCI-H292, NCI-H1155 (Figure 1b), and COLO 320DM were homozygous or hemizygous for the altered allele. As an example, the *p73* mutation in NCI-H1155 is shown in Figure 1a and b. MIA PaCa-2, SK-HEP-1, LS 174T and M24 cell lines are heterozygous (data not shown).

Two cell lines have microdeletions in the coding region of *p73*. A-427 has a deletion of 12 bases in exon 13 (nucleotides 1808 through 1819), which causes a change at amino acid 603 from Gly to Asp, and then the next four amino acids are deleted (Figure 2a and b). In the other cell line, MDS92, deletions occur in exon 10 where nucleotides 1251 and 1252 are deleted, nucleotide 1253 is unaltered, and nucleotides 1254 through 1257 are also deleted (Figure 3a and b). This results in the deletion of amino acids 418 His and 419 Gly in exon 10, while still conserving the reading frame. These two cell lines have only the altered allele (Figures 2 and 3).

Table 1 *p73* nucleotide sequence alterations in the entire coding region with splice junctions using 54 cancer cell lines

Cell line name	*Histology*	p73 *status*	p53 *status*
HCT 116	Colon cancer	WT	WT
DLD1	Colon cancer	WT	C→T (241S→F)
SW620	Colon cancer	WT	CGT→CAT (273R→H)
HT-29	Colon cancer	WT	CGT→CAT (273R→H)
SW480	Colon cancer	WT	CGT→CAT (273R→H), CCC→TCC (309P→S)
COLO 320DM	Colon cancer	1008 C→T, 1047 T→C, 1671 G→A, 1830 G→A (all silent)	CGG→TGG (248R→W)
SW48	Colon cancer	WT	CGG→TGG (248R→W)
WiDr	Colon cancer	WT	CGT→CAT (273R→H)
LS 174T	Colon cancer	1008 C→T, 1047 T→T&C, 1671 G→G&A, 1830 G→G&A (all silent)	WT
RKO	Colon cancer	WT	WT
SW403	Colon cancer	WT	
866MT	NSCLC	WT	TGT→TGA (229C→stop)
A2182	NSCLC	WT	WT
NCI-H292	NSCLC	519 T→C, 735 G→A, 1047 T→C, 1830 G→A (all silent)	WT
Calu6	NSCLC	WT	CGA→CGT (196R→stop)
A427	NSCLC	Deletion of 12 bp in exon 13	WT
Calu-1	NSCLC	WT	Deletion
NCI-H358	NSCLC	WT	Deletion
NCI-H1155	NSCLC	790 G→T (R→W)	CGT→CAT (273R→H)
NCI-H157	NSCLC	WT	G→T (298E→stop)
NCI-H596	NSCLC	WT	GGC→TGC (245→C)
A549	NSCLC	WT	WT
NCI-N417	SCLC	WT	GAG→TAG (298E→stop)
MDS92	SCLC	Deletions of 2 and 4 bp in exon 10	ATG→ATA (237M→I)
NCI-H446	SCLC	WT	WT
NCI-H146	SCLC	WT	WT
NCI-H82	SCLC	WT	
NCI-H526	SCLS	WT	Splice junction of intron3 G→C
MDA-MB-468	Breast cancer	735 G→A (silent)	CGG→TGG (273R→H)
T-47D	Breast cancer	WT	CTT→TTT (194L→F)
MCF7	Breast cancer	WT	
Hs 578T	Breast cancer	WT	GTC→TTC (157V→F)
ZR-75-1	Breast cancer	WT	
HA22T/VGH	HCC	WT	Deletion
HUH4	HCC	WT	121 S→stop
HEP 3B	HCC	1689 T→C (silent)	Deletion
HUH7	HCC	519 T→C (silent)	TAT→TGT (220C→stop)
SK-HEP-1	HCC	1671 G→G&A (silent)	WT
HepG2	Hepatoblastoma	WT	WT
HB611	Hepatoblastoma transfected HBV	WT	
THLE-5B	SV40 immortilized liver cell	WT	WT
MIA PaCa-2	Pancreas cancer	519 T→T&C (silent)	CGC→TGG (248R→H)
Capan-2	Pancreas cancer	WT	CGT→CAT (273R→H)
AsPC-1	Pancreas cancer	WT	CGT→CAT (273R→H)
FaDu	Oral cancer	WT	CGG→CTG (248R→L)
SSC-4	Oral cancer	WT	CCC→CTC (151P→L)
M9K	Mesothelioma	WT	WT
M24	Mesothelioma	1008 C→C&T, 1047 T→T&C, 1671 G→G&A, 1830 G→G&A (all silent)	WT
CCRF CEM	T-cell lymphoblastic leukemia	WT	
H9	T-cell lymphoma	WT	
SK-0V-3	Ovarian cancer	WT	
CaSki	Cervix carcinoma	WT	
HCE7	Esophagus cancer	WT	C→T (278P→S)
118 MG	Glioblastoma	WT	G→A (213R→Q)

NSCLC: non-small cell lung cancer. SCLC: small cell lung cancer, HCC: hepatocellular carcinoma. The Adenine residue of the first Methionine is 1 in nucleotide number of *p73*. *p53* status are retrieved from references (Bodner *et al*., 1992; Caamano *et al*., 1993; Hollstein *et al*., 1990; Hsu *et al*., 1993; Kastrinakis *et al*., 1995; Kovack *et al*., 1991; Lehman *et al*., 1991; Metcalf *et al*., 1992; Mitsudomi *et al*., 1992; Murakami *et al*., 1991; Nigro *et al*., 1989; Peinado *et al*., 1993; Rodrigues *et al*., 1990; Ruggeri *et al*., 1992; Russell *et al*., 1995; Somers *et al*., 1992). Blanks in *p53* status are not known

Discussion

We have analysed the entire sequence of the *p73* coding region, including splice junctions in 54 cell lines from diverse types of human cancers. Eight base substitutions and two microdeletions were detected. These alterations are concentrated in 5 exons (4, 6, 8, 10 and 13). In this analysis, we termed the first coding exon as exon 1. Among eight base substitutions, seven are silent and only one occurring in exon 6, results in an amino acid substitution. Although mutations of CpG sites are frequent in *p53* (Greenblatt *et al*., 1994), we did not detect any *p73* mutations at CpG sites in these cell lines. However, the distribution of 5-methylcytosine at CpG sites in the *p73* gene is unknown. Two reports have described genetic polymorphisms in the *p73* gene (Mai *et al*., 1998; Nomoto *et al*., 1998) at the following nucleotides: 519 (T to C), 1008 (C to T),

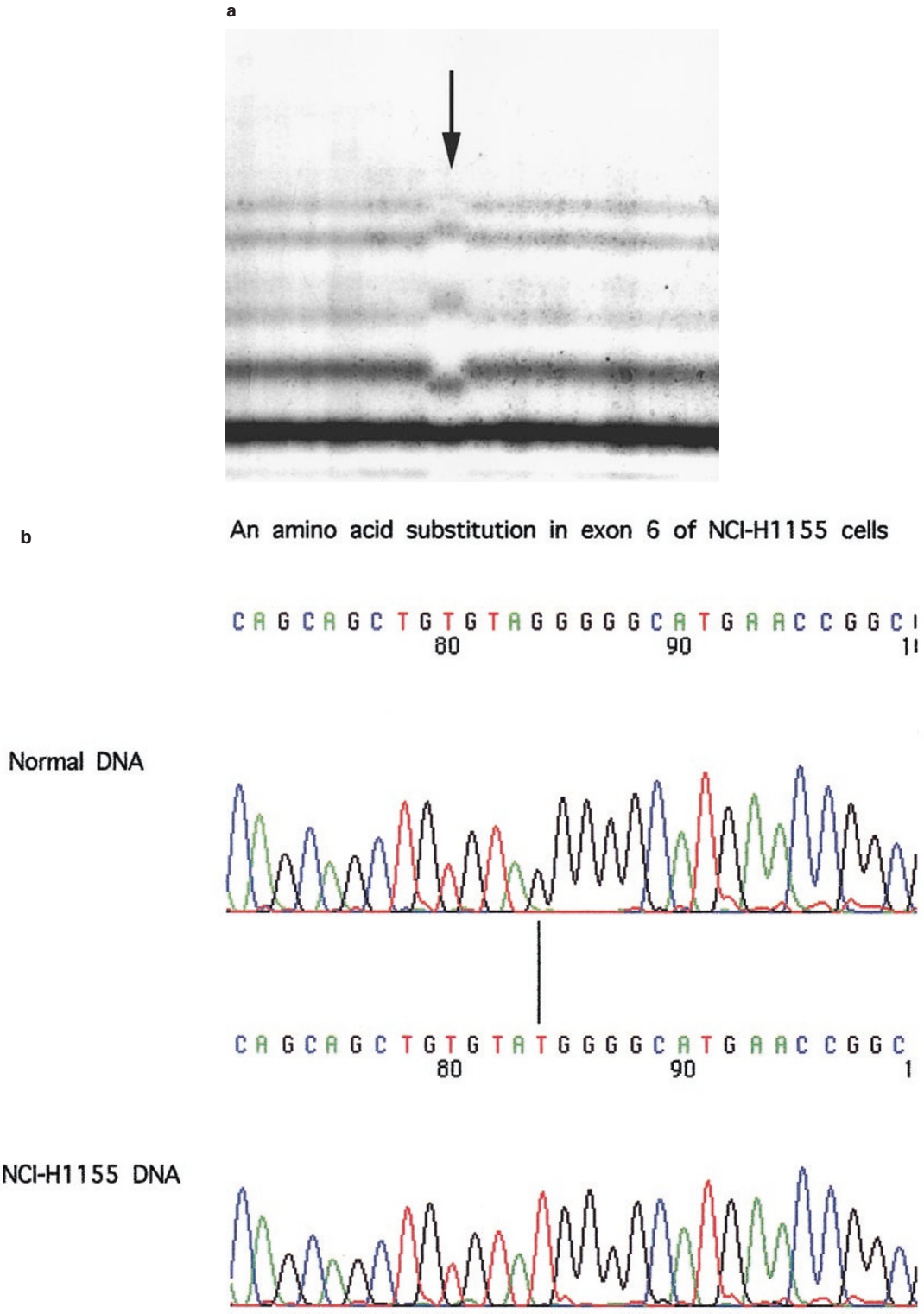

Figure 1 (**a**) PCR–SSCP analysis of *p73*, exon 6. An arrow indicates the shifted bands in NCI-H1155 cells. (**b**). Sequencing analysis of *p73*, exon 6 of NCI-H1155 DNA. An arrow indicates an altered base versus normal DNA

1047 (T to C), 1671 (G to A), and 1830 (G to A). The remaining silent nucleotide substitutions at 735 (G to A), and at 1689 (T to C) have not been reported previously. The missense mutation at nucleotide 790 (G to T) that leads to an amino acid substitution and two deletions has not been reported previously. We speculate that these are cancer-related genetic alterations. The two microdeletions in *p73* occur outside of the DNA binding domain, which is a similar and common finding in the *p53* gene (Greenblatt *et al*., 1994). The 12 bp deletion in *p73*, exon 13 of A-427 cells (nucleotides 1808 through 1819) (Greenblatt *et al*., 1996), is involved in a direct repeat with nucleotides 1796–1807. The 2 and 4 bp deletions in exon 10 of MDS92 cells are associated with a 6 bp interspaced repeat. The exon 10 deletion is complicated in that a 6 bp deletion occurs, but leaves one nucleotide intact, while still conserving the reading frame. Interestingly,

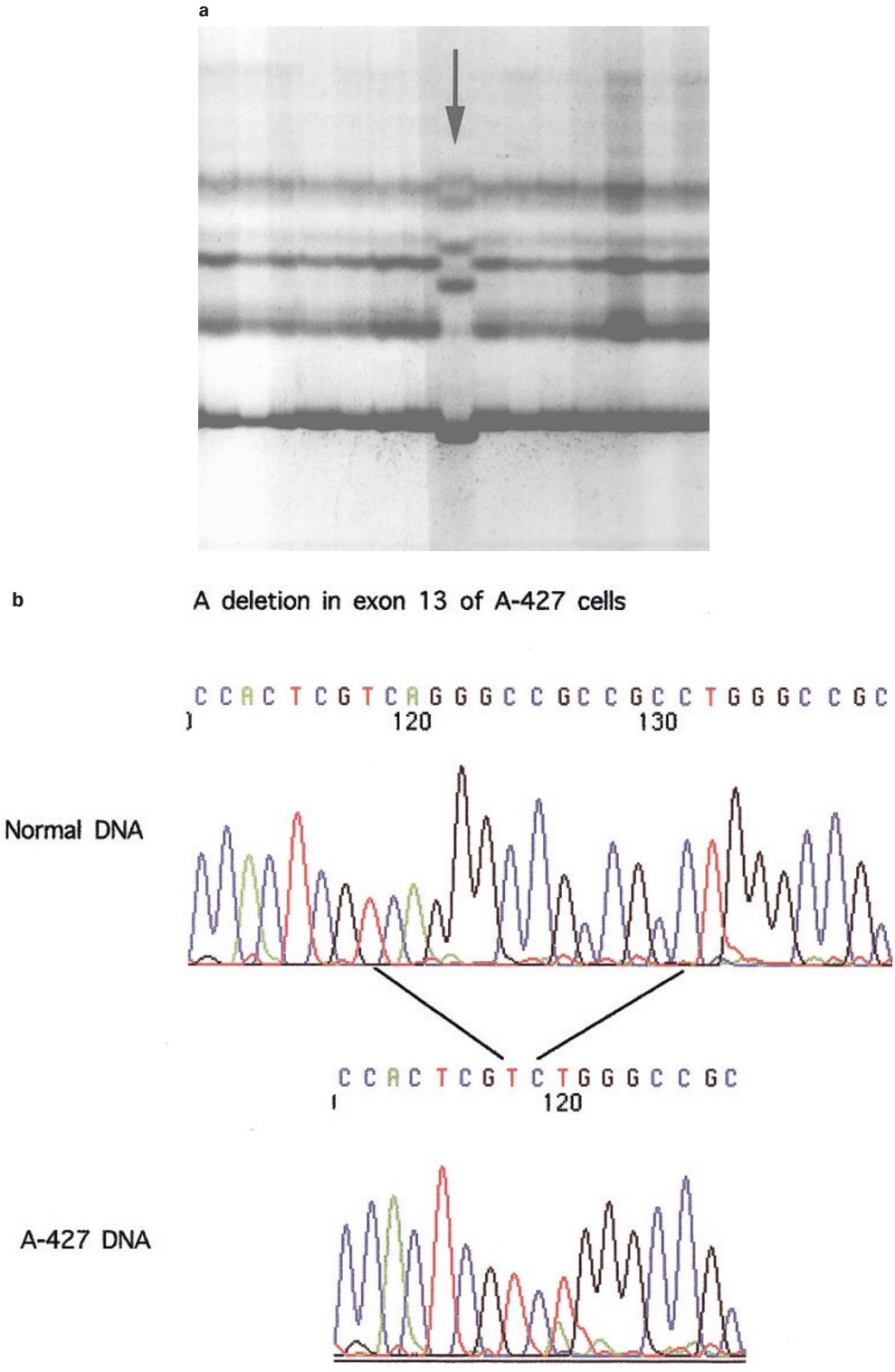

Figure 2 (**a**) PCR–SSCP analysis of *p73*, exon 13. An arrow indicates the shifted bands in A-427 cells. (**b**) Sequencing analysis of *p73*, exon 13 of A-427 DNA. A 12 bp deletion is shown versus normal DNA. Note that the sequences are of the anti-sense strands

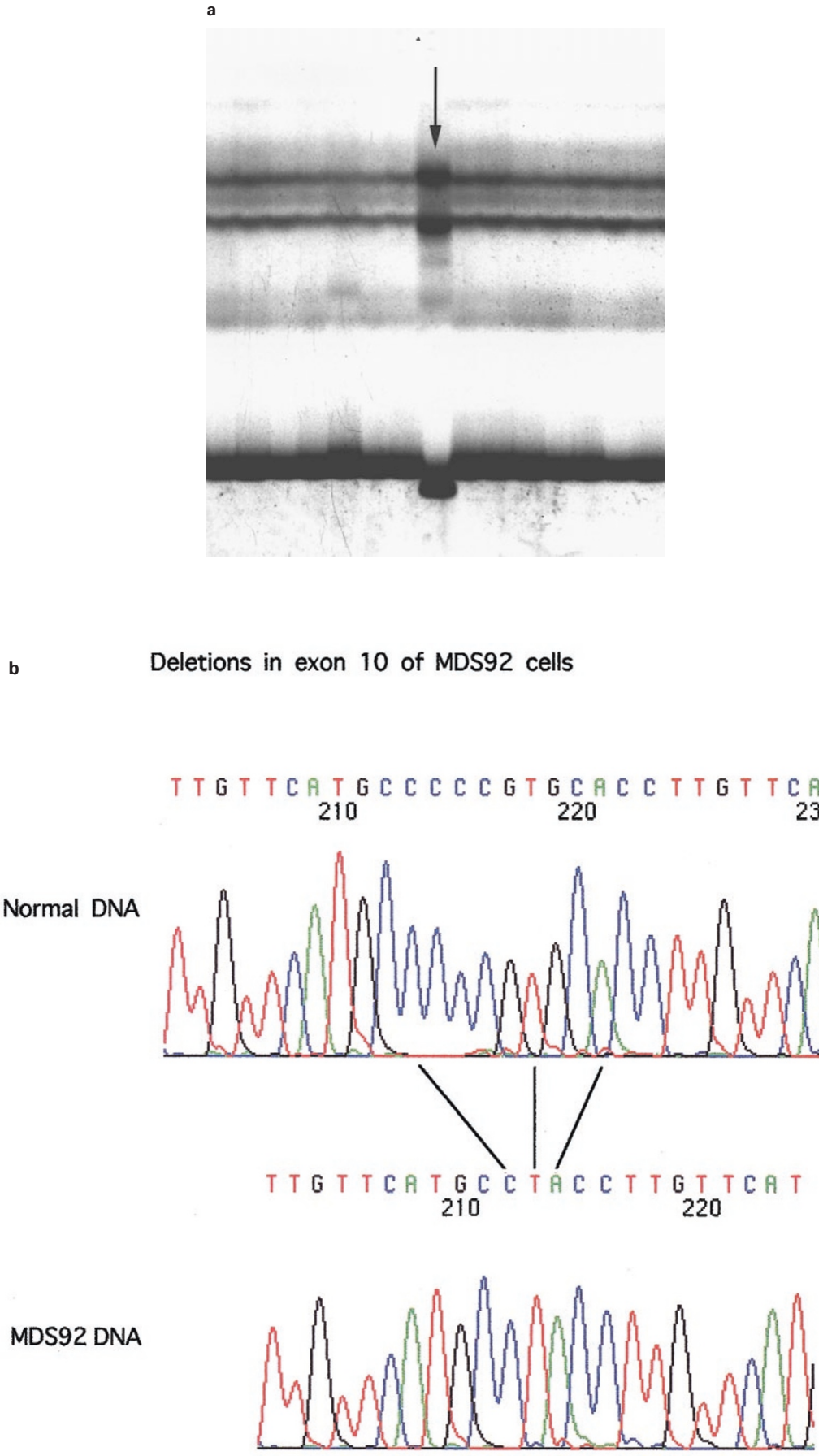

Figure 3 (**a**) PCR – SSCP analysis of *p73*, exon 10. An arrow indicates the shifted bands in MDS92 cells. (**b**) Sequencing analysis of *p73*, exon 10 of MDS92 DNA. Four bp and 2 bp deletions leave T intact

both deletions maintain the reading frame. The functional analysis of these *p73* mutations and the search for *p73* mutations in primary cancers are warranted.

p73 mutations in human cancers have not been reported previously. In the original report (Kaghad *et al*., 1997), 15 cell lines were analysed, but no *p73* mutations were found. In 106 primary prostatic carcinomas, no mutations were found (Takahashi *et al*., 1998): nor were *p73* mutations detected in two studies of primary lung cancers (Mai *et al*., 1998; Nomoto *et al*., 1998), but we detected one amino acid substitution and two deletions in a total of 17 lung cancer cell lines. Nevertheless, it is possible that the *p73* mutations occur in cell culture. These results indicate that additional studies of primary lung cancer samples for the *p73* mutational status are warranted. We also tested the hypothesis that *p73* and *p53* may be in the same tumor suppressor pathway, so that mutations would most likely occur in one but not both of the genes during carcinogenesis. *p53* mutations were detected in two of the cell lines with *p73* mutations, leading to amino acid alterations (Table 1), therefore, these results are inconsistent with that hypothesis. We conclude that *p73* mutations are uncommon in human cancers and that *p53* and *p73* are not members of the same tumor suppressor pathway.

Materials and methods

Genomic DNA preparation

Fifty-four human cancer cell lines (including 45 cell lines for which the *p53* mutational status had been determined) (Table 1) were grown in the recommended media. Cell line and normal liver DNAs were extracted using the Non-Organic DNA extraction Kit (Oncor), and dissolved in 10 mM Tris pH 8.0 as approximately 50 ng/μl.

Isolation of a genomic BAC clone containing the p73 *gene*

A genomic BAC library (Research Genetics) was screened by PCR using a primer set to amplify exon 13 of *p73*. The sense primer used was 5′CCTGAAGCAGGGCCACGACT. The anti-sense primer used was 5′TGCTTGCGGGCCTTGCAGTC.

Sequencing of the p73 *intronic sequences that neighbor the exons*

The long distance sequencer method (Hagiwara and Harris, 1996) was used to amplify genomic DNA by PCR using a gene-specific primer and the SP6 primer, specific to a kind of linker-adaptor 'vectorette'. The *p73* cDNA sequence was retrieved from GenBank. Amplified DNA was sequenced using the SP6 primer.

PCR–SSCP analysis

Thirteen primer sets were designed from the introns, 5′nontranscribed and 3′nontranscribed sequences to amplify all coding exons of *p73* with the splice junctions. For exon 1, 5′TGCAGAGCGAGCTGCCCTCGGA and 5′AGGCTAGCCCAGAGTGCCTCCCA. For exon 2, 5′CCACTCCAGTCCTCTTGCAGA and 5′TGACACCCAAACTGGGGACTGA. For exon 3, 5′GACGACTGACTGTGTGTGTTTC, and 5′CTCAGGGACTAGGGGAACTC. For exon 4, 5′CAGTTGGGACCACTGGTCTCA and 5′ATGCTGGGCAAAGTGCCACCGT. For exon 5, 5′GACCCGTACAGCTGACTGCA and 5′ACCTCTATGCACCTCTCTGAAG. For exon 6, 5′CCTGCAGGTCTCCATGACAGCT and 5′TTGGGGCTGCGTGCTGATGCTA. For exon 7, 5′CAGGGTTGAGCTCACAATTCTG and 5′TCCTCCCACACGCGTCCAGTT. For exon 8, 5′ACCCTCTGGTCCTGCCTGCTCA and 5′ACGACAGAGGTGAGGCAGGTCT. For exon 9, 5′TTCCCCACACTGATGGTGGGCTA and 5′AGAGATCTGCTCCTCTGTGCTCA. For exon 10, 5′CCTCCTGCCCAGAGGGTGGAA, and 5′AGGCTCCACCCATTCGCAGCA. For exon 11, 5′TGGATGCCCAGCCTGGCTGCCCTGAT and 5′CAGACAGGGTGACAGCACATGCTCAG. For exon 12, 5′AAGGCTCTTTGCCCTCCGGACA, and 5′AGCCAGGCCACTCTCAGAGAT. For exon 13, 5′TCCACTGCCCCCTGCCCCTAAT and 5′AGGCAGCTTGGGTCTCTGGGCGGT. PCR was performed using the GeneAmp XL PCR kit (PE Applied Biosystems) or the Advantage-GC Genomic PCR kit (CLONTECH), using the following conditions respectively: 40 cycles of 94°C for 40 s, 55°C for 30 s and 68°C for 2 min, or 40 cycles of 94°C for 40 s and 68°C for 3 min. One μl of PCR product was amplified for five additional cycles with 1 μl [α-^{33}P]dATP (3000 Ci/mmol, ANDOTEK). SSCP was performed as described (Orita *et al*., 1989). The remaining PCR products were stored at −20°C for sequencing.

Sequencing of PCR products

PCR products were sequenced as directed by the ABI PRISM BigDye Terminator Cycle Sequencing Kit using each intron primer pair bi-directionally.

Abbreviations

LOH, loss of heterozygosity; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; HCC, hepatocellular carcinoma: BAC, bacterial artificial chromosome.

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