Binding of wild-type and mutant forms of P53 protein from human tumors to a specific DNA sequence of the first intron of the H-*ras* oncogene

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Abstract. p53 is the most frequent target for genetic alterations in a wide variety of human cancers. The product of the p53 tumor suppressor gene binds to DNA and activates transcription from promoters containing its consensus binding site. In the accompanying paper we have found that P53 tumor suppressor protein recognizes specifically a transcriptional element within the human H-*ras* proto-oncogene (Spandidos DA, *et al*, Int J Oncol 7: 1029-1034, 1995). We transfected Saos-2 cells, which are p53-null cells, with plasmids encoding for the wild type (wt) and for one 'hot spot' mutant (mt) of the p53 gene (H 273). Using the resulted nuclear extracts for gel retardation assays, we showed binding of both the wild-type and the mutant form of p53 to the H-*ras* DNA. Furthermore, using nuclear extracts from head and neck tumors and from adjacent normal tissues in gel retardation assays, we found binding of both the wild-type and the p53 mutant in the same responsive element of the H-*ras* oncogene. These experimental results suggest a direct role of p53 in regulation of H-*ras*. Identification of cellular proto-oncogenes as mediators of the transcriptional effects of wild-type and mutant forms of p53 gene, will be a step towards a better understanding of the role of oncogenes and onco-suppressor genes in tumor promotion.

Introduction

The p53 gene is localized to 17q13 chromosome and encodes a 393 amino-acid protein which functions as a *trans*-acting transcriptional factor controlling the expression of genes important in transcription, DNA synthesis and repair (1,2), cell differentiation (3) and apoptosis (4). Wild-type p53 mediates these actions by binding specifically to certain sites found in human genomic DNA (5). All of these p53 responsive elements share a repeat of the loose consensus sequence, the ten base pair 5'-(Pu)$_3$C(A/T)(T/A)G(Py)$_3$-3'

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motif (5,6). Such sites have been located in regulatory elements of the *gadd45* (7), *waf-1/cip-1* (8), and *mdm-2* (9) genes. In order to bind these regions, oligomerization of the P53 protein is required, although monomeric P53 can also bind DNA, depending on the protein concentration and the nature of the P53 binding site (10,11).

It is not surprising that such a molecule, which stands in the crossroad of so many vital cellular functions is inactivated in more than half of all human tumors (12). The most important mechanism to overcome the p53 tumor suppressor barriers is alteration of the gene, mostly via mutations, although complex formation of wt p53 with viral and cellular gene products also abrogates p53 functions (13-16).

H-*ras* gene is a member of the family of *ras* proto-oncogenes. The gene product of H-*ras*, p21, is localized in the inner side of the cell membrane and acts in the normal signal transduction pathway as a GTP-binding protein. Genes of the *ras* family are implicated in human tumorigenesis both through mutations and overexpression (17). Since overexpression of activated *ras* genes may cause oncogenic transformation (18,19) and expression of the normal H-*ras* gene can act as an onco-suppressor (20), it is important to understand the regulation of *ras* genes. It has been shown that mutant p53 co-operates with *ras* for cellular transformation (21,22) and that activated H-*ras* is necessary to overcome the onco-supressor function of the wt-p53 (23,24).

Previous studies (25) identified within the H-*ras* gene three putative p53 binding half sites, two of which are contiguous and contain a ten base core which perfectly matches the p53 CON element (6).

In order to find a direct link between p53 protein forms and regulation of H-*ras*, we examined the binding of the 'core' H-*ras* p53 element to: (i) wt p53 (ii) p53 mutant His 273 and (iii) wt p53 Δ364-393, a truncated protein which is activated for DNA binding (10). We showed that all three forms of p53 bind the H-*ras*-p53 element. Binding of the p53 to the same sequence of the H-*ras* oncogene has been successfully tested in nuclear extracts from head and neck tumors, as well. These results indicate that p53 could also function through the transcriptional regulation of the H-*ras* oncogene.

Materials and methods

Recombinant plasmids. Plasmid pSV2hp53B encoding full-length human wild-type p53 contains the EcoRI-HindIII fragment of pGEM53wtB (26), blunt-ended and cloned in pSV2 vector cut with SalI-BglII. Plasmid pSV2hp53BHis273 was derived by site-directed mutagenesis (27) from pSV2hp53B and encodes full-length P53 protein with single point mutation; Arg 273 to His. Plasmid pGEMhp53BΔ364-393 encodes human P53 protein with a deletion spanning residues 364-393.

Cell culture and transfection. Human osteosarcoma (Saos-2) cells were propagated in RPMI medium containing 10% fetal calf serum, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Transfections were performed using the calcium phosphate technique (28), as modified (29). Briefly, semi-confluent exponentially growing cells were transfected with 40 μg of plasmid DNA. Cells were collected by scraping 2-3 days later and used for preparation of nuclear extracts.

Preparation of nuclear extracts. Nuclear extracts were prepared from biopsy material obtained from patients who had undergone head and neck surgery at the Hippokration Hospital, Athens, Greece, and from transfected and untransfected Saos-2 cells. The above tissues and cells were homogenized in hypotonic buffer (25 mM Tris HCl pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF). The nuclei were pelleted, washed with isotonic buffer (25 mM Tris HCl, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 0.2 mM sucrose) and lysed with an extraction buffer (25 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.1% Triton, 0.5 mM DTT, 0.5 mM PMSF). Nuclear debris was removed by centrifugation at 25,000 rpm for 1 h at 4°C. The protein concentration of the supernatant was measured by the method of Bradford (30).

Oligonucleotides and labelling. Oligonucleotide BC contains the optimal p53 binding site: 5'-CC-<u>GGGCA</u>-<u>TGTCC</u>-<u>GGGCA</u>-<u>TGTCC</u>-GGGCATGT-3' (26). Oligonucleotide BC-S21 contains 21 spacers between the p53 half sites: 5'-TAT-<u>GGGCA</u>-<u>TGTCC</u>- TATATATATGCGTATATAT AT-<u>GGGCA</u>-<u>TGTCC</u>-TAT-3' (26). *Eras* 2HS probe contains the two contiguous half sites with HindIII compatible ends: 5'-AGCTTGGCAC-<u>TAGCC</u>-<u>TGCCC</u>-<u>GGGCA</u>-<u>CGCCG</u>-TGGCGA-3'. MDM-2 oligonucleotide contains the p53 binding element 5'-GGCT-<u>GGTCA</u>-<u>AGTTG</u>-<u>CGTCC</u> GGCGTCGGCTGTCGGAG-<u>GAGCT</u>-A-<u>AGTCC</u> <u>TGACA</u>-<u>TGTCT</u>-CCAG-3' of *mdm*-2 gene (31). The pentanucleotide p53 binding repeats are underlined. SP-1 oligonucleotide was used as a p53-unrelated oligonucleotide competitor (32). Radioactive end labelling was performed using T4 polynucleotide kinase (Boehringer) and [γ-^{32}P]-ATP (33).

DNA binding assay. DNA binding reactions were carried out as described (31) with modifications (10). The samples were electrophoresed on 6% polyacrylamide gels. Gels were dried and exposed to X-ray film (RX Fuji) at -70°C. A 50 fold molar excess of unlabelled oligonucleotides, or 0.4 μg of anti-p53 antibody pAb 1801 (Oncogene Science, Inc., Manhasset, NY), or 0.4 μg of anti-p53 antibody DO-1 (kindly provided by Dr D.P. Lane, Dundee, UK) were included prior to the addition of the radiolabelled probe in the reaction mixture, where indicated, and incubated at 0°C for 10 min.

Western blotting. Western blotting was performed using 0.5 μg anti-p53 DO-1 antibody at a dilution of 1:200, using the ECL kit (Amersham), following the instructions of the manufacturer.

DNA extraction, LOH, PCR-SSCP analysis. Genomic DNA from head and neck tumors and adjacent normal tissues was extracted by methods previously described (33). The three microsatelite primers (D17578, TP53 and CHRNB1) and PCR conditions, used for loss of heterozygosity (LOH) studies on 17 chromosome have been previously described (34). The fragment of the p53 gene, containing exons 4 to 9 (2.9 kb), was amplified by the polymerase chain reaction (PCR), using primers and conditions as described previously (35,36). The first PCR product (amplified DNA size, 2.9 kb) was used for the nested PCR-SSCP analysis. Briefly, two microliters of the 2.9 kb product were combined with 5 μl 10X buffer, 1 μl dNTP mixture and 0.4 μM of both primers in a final volume of 50 μl (35,36). Five μl of the PCR-amplified product was added to an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) (United States Biochemicals, Ohio). Samples were denatured at 95°C for 10 min, placed immediately on ice and run on an 8% polyacrylamide nondenaturing gel. DNA was electrophoresed in 0.5X TBE running buffer at 12 to 15 W at room temperature. Gels were visualized using the silver staining technique (37).

Results

A computer search revealed that the human c-H-*ras* gene contains two contiguous putative p53 half sites (6,25). Transient transfection assays were used in this study to examine DNA binding activity of wild-type and mutated forms of P53 protein in the H-*ras* element. Endogenously produced wild-type and mutant p53 from head and neck tumors and adjacent normal tissue were also analysed for *in vivo* DNA binding.

Human osteosarcoma (Saos-2) cells, which have deleted endogenous p53 alleles (38), were transfected with plasmids coding the full length wild-type p53, wild-type p53 with a deletion which spans the last 30 amino-acid residues (Δ364-393), and full-length p53 protein with single point mutation: Arg 273 to His (His 273). The resulted nuclear extracts were tested for p53 expression with immunoblotting, using anti-p53 antibody DO-1 (Fig. 1). Extract from untransfected Saos-2 cells was used as negative control (lane 1). Extracts containing full-sequence wild-type and (Δ364-393) p53 (lanes 2 and 3), as well as mutant form H273 of the protein (lane 4), showed a detectable amount of P53 protein.

H-*ras*-p53 element was then tested for binding of the wild-type P53 protein. In Fig. 2, the control BC probe (lane 1)
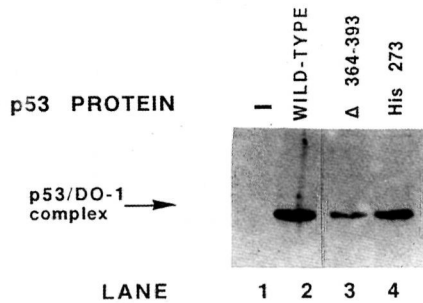
Figure 1. Western blot analysis of p53 expression. Nuclear extracts were prepared after transient transfection of Saos-2 cells with plasmids encoding full length wild-type p53 (lane 2), (Δ364-393) p53 (lane 3) and mutant His 273 (lane 4) Extract from untransfected Saos-2 cells was used as negative control (lane 1). Thirty μg of total protein were subjected to immunoblotting in each lane. Anti-p53 DO-1 antibody was used for immunoblotting, as described in Materials and methods.
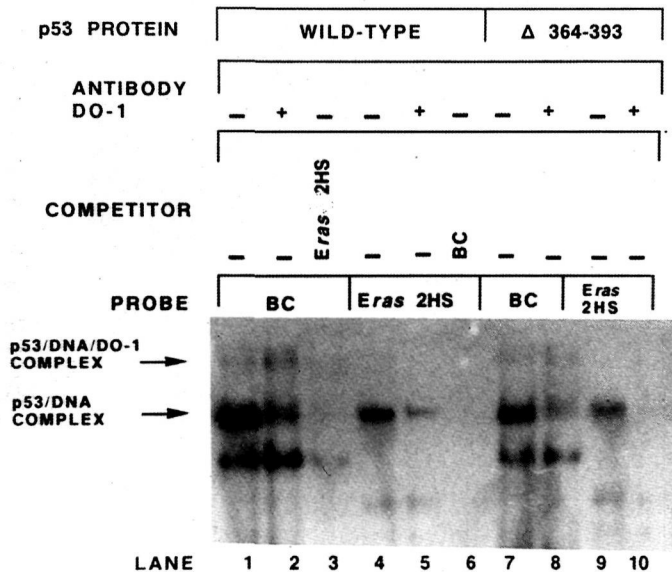

Figure 2. Sequence specific binding of wild-type p53 protein to the H-*ras* element. After transfection of Saos-2 cells, full length (lanes 1-6) and truncated (lanes 7-10) forms of p53 protein were used in DNA binding assay. Binding reaction mixture contained 20 μg of total protein, 0.01 pmoles of ^{32}P-labelled probes BC (lanes 1-3 and 7-8) and E*ras* 2HS (lanes 4-6, 9-10), 0.4 μg anti-p53 antibody (lanes 2, 5, 8, 10) and a 50 fold molar excess over the labelled probe of unlabelled competitors (lanes 3, 6). Protein-DNA complexes were separated by polyacrylamide gel electrophoresis and visualized by autoradiography. p53/DNA complex is indicated by an arrow.

Figure 3. Sequence specific binding of mutant form of p53 protein His 273 to the H-*ras* DNA. His 273 p53 mutant, was analyzed after transfection of Saos-2 cells. Binding reaction mixture contained 20 μg of total protein, 0.01 pmoles of ^{32}P-labelled probes BC (lanes 1-4), E*ras* 2HS (lanes 5-8) and a 50 fold molar excess over the labelled probe of unlabelled competitors (lanes 2-4 and 6-8 respectively). Protein-DNA complexes were separated as indicated in Fig. 2 legend. p53/DNA complex is indicated by an arrow.

reacted with BC (lane 7) and E*ras* 2HS probe (lane 9) and the P53/DNA complex was abolished by DO-1 antibody (lanes 8 and 10).

His 273 'hot spot' mutant was also tested for binding H-*ras*-p53 element. The p53 mutant was incubated with control BC (Fig. 3, lane 1) and E*ras* 2HS (lane 5) probes, indicating binding of mutated P53 protein. The retarded band in both control and E*ras* 2HS probes was abolished in the presence of molar excess of p53-related oligonucleotide competitors BC-S21 (lanes 2 and 6), MDM2 (lanes 3 and 7), E*ras* 2HS (lane 4) and BC (lane 8), implying the specificity of the p53-DNA complex.

Having established that all of the examined head and neck tumors and adjacent normal tissues had detectable P53 protein by Western blot analysis (data not shown), we tested the samples for binding to H-*ras* p53 element. Indicatively, five head and neck tumors and adjacent normal tissues were analysed. As shown in Fig. 4, a common retarded band was observed in the majority of the examined cases, indicating binding of the p53 protein to the H-*ras* responsive element. In all normal tissue samples we observed binding of p53 to the H-*ras*-p53 sequence. In contrast, 2 of the 5 tumor samples (Nos. 4 and 5) contained no p53 binding activity.

Specificity of the p53/*ras* DNA complex was shown by competition experiments, using nuclear extracts from tumor and normal tissue of patient No. 1 (Figs. 5 and 6, respectively). The labelled control BC-S21 (lanes 1-3) and E*ras* 2HS oligos (lanes 4-6), were incubated with a molar excess of unlabelled oligonucleotide competitors: with each other (Fig. 5, lanes 2,5) and with unrelated SP-1 oligonucleotide (lanes 3,6). Binding of p53 on E*ras* 2HS probe was abolished in the presence of excess BC-S21 competitor,

and the radiolabelled E*ras* 2HS probe (lane 2) were incubated with wild-type P53 protein. A common retarded band was observed, indicating binding of the protein to the probe. This band was supershifted/or abolished when anti-p53 DO-1 antibody was included in the reaction mixture (lanes 2 and 5) and abolished with competition of the probes with a molar excess of unlabelled oligonucleotides (lanes 3 and 6). Activated, truncated P53 protein p53Δ364-393 also
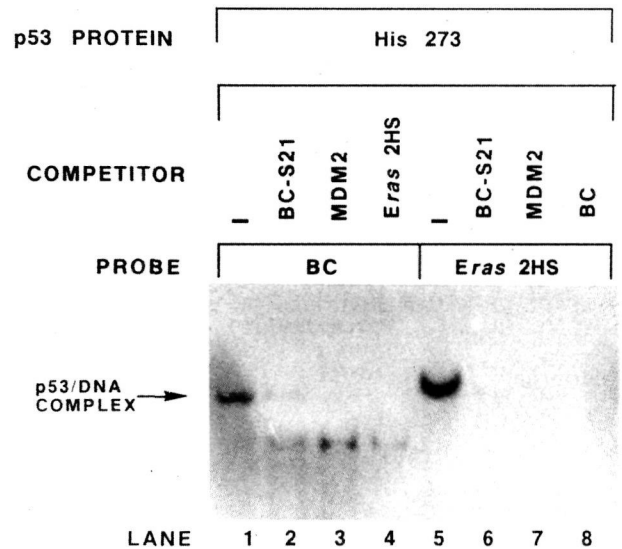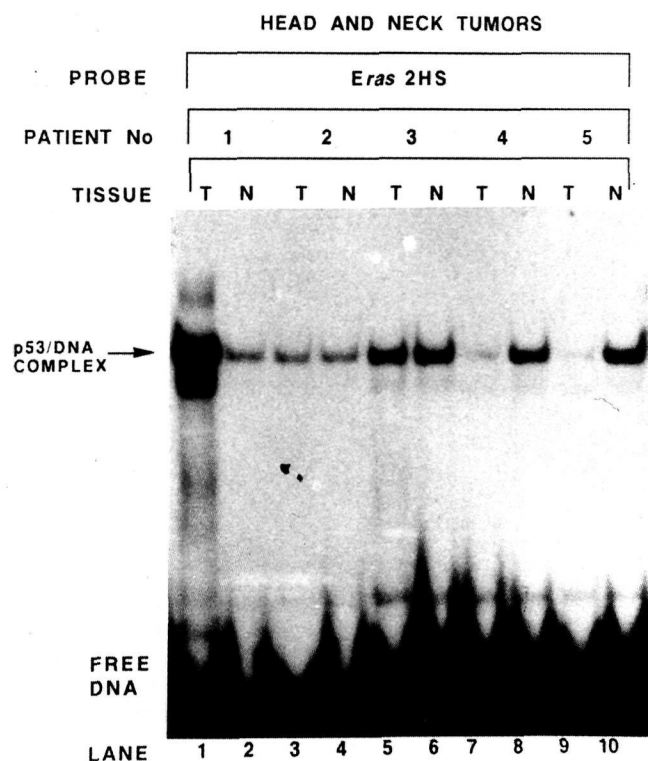
HEAD AND NECK TUMORS

Figure 4. Sequence specific binding of endogenously produced p53 protein from head and neck tumors to H-*ras* element. Nuclear extracts from head and neck carcinomas were incubated with 0.01 pmoles of γ-^{32}P-end-labelled probe E*ras* 2HS (lanes 1-10). Protein-DNA complexes were separated as indicated in Fig. 2 legend. Protein-DNA complex is indicated by an arrow. T, tumor; N, normal tissue.

Table I. Loss of heterozygosity analysis of five head and neck tumors in chromosome 17 using PCR microsatellite primers.

	Patient number				
	1	2	3	4	5
D175578	O	O	Ø	●	●
TP53	O	O	Ø	●	●
CHRNB1	O	O	Ø	?	●

o, constitutional heterozygosity with no loss of heterozygosity; ø, homozygous; ●, constitutional heterozygosity with loss of heterozygosity; ?, not done.

but was unaffected by the unrelated SP-1 competitor. Equivalent results were observed from the control BC-S21 probe.

We subsequently studied the formation of the DNA-protein complex between E*ras* 2HS or BC-S21 and the p53 nuclear extracts from head and neck normal tissue (Fig. 6, lanes 1 and 3). Inclusion of the anti p53 1801 antibody into the reaction mixture led to the disappearance of the specific retarded band (Fig. 6, lanes 2 and 4).
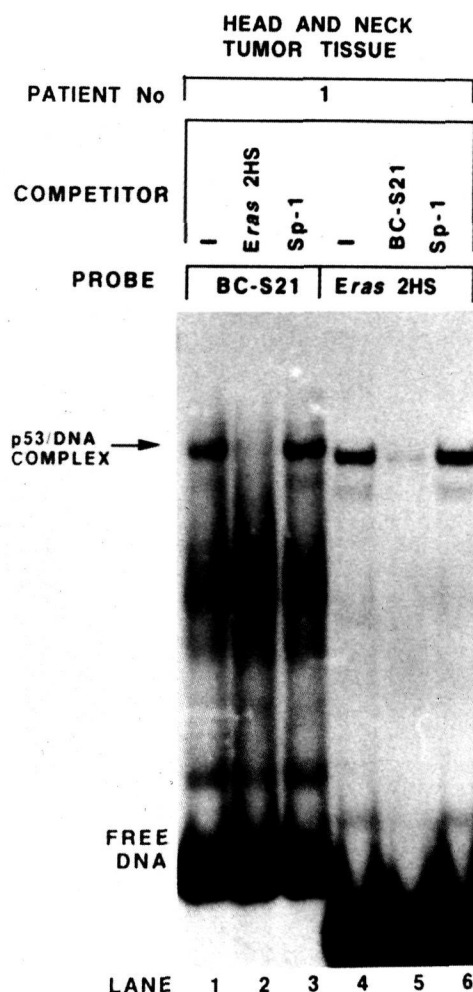
**HEAD AND NECK
TUMOR TISSUE**

Figure 5. Effect of competitor sequences on gel electrophoretic mobility assay. Nuclear extracts from head and neck tumor tissue (patient No. 1) were incubated with γ^{32}P-end labelled BC-S21 and E*ras* 2HS oligonucleotide hybrids. In competition assays, 50-fold molar excess of cold competitor oligonucleotides E*ras* 2HS, SP-1 (lanes 2, 3) and BC-S21, SP-1 (lanes 5, 6) respectively, were incubated with the nuclear extracts before addition of the probes. Protein-DNA complexes were separated as indicated in Fig. 2 legend. Specific retarded bands of interest are shown.

Furthermore, we have analyzed five head and neck tumor tissues for loss of heterozygosity (LOH) in chromosome 17. The results are summarized in Table I. Also we analyzed the same samples for mutations in exons 4 to 9 of the p53 gene, as the majority of previous studies have shown that the p53 mutations in human tumors are found in this region (12). We first determined the optimal conditions for PCR amplification of the 2.9 kb p53 gene fragment. In order to exclude mutations caused by *Taq* polymerase the SSCP analysis was carried out on two different occasions for each tumor sample. The SSCP results are shown in Fig. 7. Three of the tumor samples (patient Nos. 1, 4 and 5) showed different pattern of bands in comparison with corresponding normal samples. No mutations were found by this method in two other samples (patient Nos. 2 and 3).

Discussion

In this study we examined the responsiveness of a p53 binding element, located in the H-*ras* gene, to the wild-type,
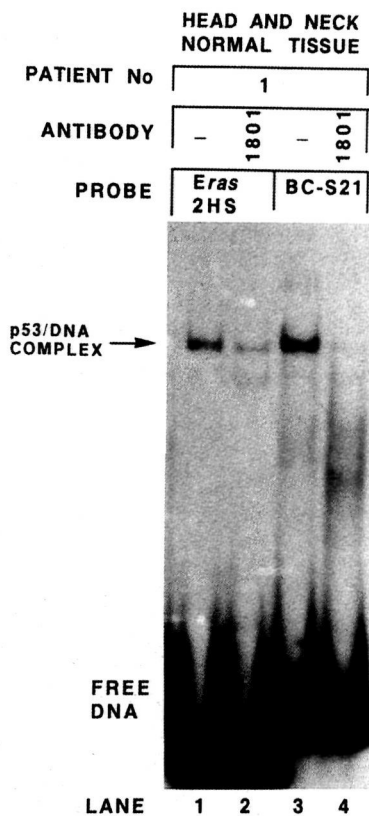
Figure 6. Effect of anti-p53 1801 antibody p53-*ras* DNA complex in electrophoretic gel mobility shift assay. Nuclear extracts from head and neck normal tissue (patient No. 1) were incubated with E*ras* 2HS oligonucleotide (lane 1) and BC-S21 oligonucleotide (lane 3). The anti-p53 antibody 1801 and nuclear extracts were incubated with E*ras* 2HS (lane 2) and BC-S21 (lane 4), respectively. Protein-DNA complexes were separated as indicated in Fig. 2 legend. Absence of the retarded band is shown. DNA-p53 complex is indicated by the arrow.

Figure 7. SSCP analysis of exons 6, 5 and 7 of p53 gene in head and neck specimens. Aliquots of 5 μl of the PCR amplified samples were loaded on an 8% polyacrylamide nondenaturing gel. DNA was electrophoresed according to the conditions described in Materials and methods. PCR products from tumor DNA (A, exon 6; B, exon 5; C, exon 7) gave different patterns compared to normal DNA. PN, patient number; N, normal, T, tumor; ds, double stranded; ss, single stranded. Arrows indicate the different bands.

a mutant and a truncated form of P53 protein. This element consists of two contiguous p53 half sites (1216-1235) at the 3' end of the first intron of the H-*ras* gene (25).

In order to examine the responsiveness of the element to each wild-type, mutant and truncated p53 protein, we transfected plasmids encoding wild-type p53, the mutant His 273 and the truncated (Δ364-393) form of p53 protein into p53-null Saos-2 cells. Nuclear extracts from the tranfectants were used in DNA binding experiments. Furthermore, we extended our studies *in vivo* using nuclear extracts from 5 head and neck tumors. Three of them possessed a mutant p53, two of which were accompanied by a deletion in the remaining allele.

Successful transfection was determined by immuno-blot analysis. Electrophoretic mobility shift assays with wild-type and truncated p53 transfected extracts, revealed specific binding of P53 protein to the H-*ras* element, without adding monoclonal antibody PAb421 into the reaction mixture. Other groups have already shown that wild-type p53 can bind to certain DNAs (6,26,39).

In addition, deletion of residues 364-393 has a similar effect in protein conformation as PAb421, because a negative regulatory eleme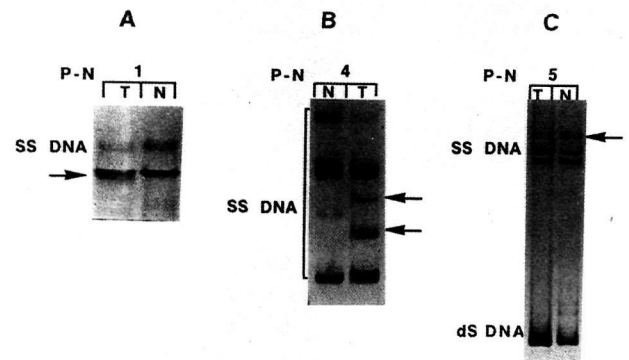nt of the protein is removed, shifting the conformation to the active state (26,40). The specificity of the retarded band, was confirmed by supershifting and/or abolishing when DO-1 antibody was included in the reaction. Furthermore, competition with molar excess of unlabelled related oligonucleotides abolished it.

Based on recent reports providing evidence for specific DNA binding of p53 mutants (39,41-47), we examined the binding of His 273 mutant, which is known to be a 'gain of function' mutant, to the H-*ras* DNA (43,46,48). Arg 273 is located in the loop sheet helix 2 motif which interacts with the major DNA groove and is primarily involved in DNA backbone contacts with phoshate groups (49). DNA binding experiments with His 273, revealed a retarded band similar to that of wild type and truncated (Δ364-393) p53, which was abolished when H-*ras* RE competed with p53 related oligonucleotides. The discrepancy between these results and earlier studies (25) may relate to the levels of P53 protein and/or the presence of cellular proteins in the nuclear extracts derived from tumor cells. It is possible that cellular factors induce DNA binding of the p53 mutant.

These experiments established this region of the H-*ras* gene as a specific p53 binding site and led us to a series of experiments focusing on the reaction of nuclear extracts derived from human tumors with H-*ras*-p53 RE. We examined five head and neck tumors, two of which (patient Nos. 4 and 5) had mutations in exons 5 and 7, respectively, accompanied by LOH in the p53 locus. Sample 1 (patient No. 1) had a mutation in exon 6 without LOH and the remaining two (patient Nos. 2 and 3) had no obvious p53 gene alterations.

DNA binding experiments revealed a retarded band, similar to that previously described in tumors 1, 2 and 3 and in all normal tissues, while in tumors 4 and 5 there was no indication of band shift. The P53-specific antibody PAb 1801 inhibited DNA binding confirming the identity of the DNA binding protein as P53. The ability of PAb 1801 to suppress DNA binding of P53 has been documented previously (6).

The biological explanation of these findings rely on the nature of the P53 protein in each of these tumors. In case 1 (patient No. 1), one p53 allele is mutant while the other is intact. Enhanced binding of P53 to the H-*ras*-p53 RE in this tumor sample probably relates to overexpression of the mutant P53 protein, combined with expression of wild-type P53. Probably the p53 mutant in this tumor retains its ability to bind DNA. In cases 2 and 3 (patient Nos. 2 and 3), p53 alleles are intact thus wt p53 binds the element. Finally, in cases 4 and 5 (patient Nos. 4 and 5), where one allele is lost, the remaining mt p53 is not capable of binding the consensus. The latter is enforced by the fact that in these specimens mutations were located in exons 5 and 7. Exons 5 and 7 form the L2 H1 L3 motif which interacts with the minor groove of the DNA. Mutations in these exons are considered to be among the most disruptive to p53 binding function.

Binding of the p53 to H-*ras* responsive element has been found with nuclear extracts from lung tumors, as well (Zoumpourlis *et al*, unpublished data).

In conclusion, we showed that the H-*ras* gene possesses a p53 responsive element which interacts with wt and mutant forms of P53 protein in human tumors. Regulation of H-*ras* cellular oncogene by p53 opens new perspectives in understanding normal signal transduction pathway and its altered pathways in carcinogenesis.

Acknowledgements

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