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Molecular analysis of *p53* gene in laryngeal premalignant and malignant lesions. p53 protein immunohistochemical expression is positively related to proliferating cell nuclear antigen labelling index

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Abstract This study was undertaken in order to investigate the molecular nature of the *p53* gene in 19 laryngeal squamous cell carcinomas and dysplasias. Moreover, we have examined the possible relationship between proliferating cell nuclear antigen (PCNA) expression and p53 protein detection status in 42 laryngeal premalignant and malignant lesions in which 14 of the 19 samples used in the molecular study were included. *p53* gene analysis was performed with the single-strand conformation polymorphism technique. PCNA was stained with the peroxidase/antiperoxidase immunohistochemical method using the monoclonal antibody PC-10. Data from previous work concerning p53 expression was used. We found that 9 of 12 of the immunohistochemically p53 positive (+) cases had mutations in exons 5 or 6. In the remaining immunohistochemically p53(+) and p53 negative (–) specimens there was no indication of sequence alterations. Furthermore, we did not observe any deletions in the chromosomal region 17p31.1 which encodes exons 4–8 of the p53 gene. The PCNA labelling index (LI) increased progressively with p53 protein detection status (percentage of cells immunohistochemically positive for p53). The difference between the group with the higher percentage of p53(+) cells and the others was statistically significant. These data show that although there is a discrepancy between immunohistochemical demonstration of p53 and molecular analysis, a large proportion of the former harbours the mutant form of the protein. In addition, p53 overexpression is positively correlated with PCNA LI, a finding which accompanies tumour progression.

Key words p53 mutations · Polymerase chain reaction · Single strand conformation polymorphism · Proliferating cell nuclear antigen · Laryngeal squamous cell carcinoma

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Introduction

p53 mutations seem to be the most common genetic abnormality associated with malignancy [14, 28]. Mutant *p53* gene product is characterized by a conformational change of the protein resulting in prolonged half life and stability which permits its immunohistochemicaly detection [13]. In a previous study [10] we examined the presence of p53 protein in laryngeal squamous cell carcinomas (LSCCs) and dysplasias and suggested that p53 overexpression is an early event in laryngeal oncogenesis and its altered expression is influenced by tobacco and human papillomavirus infection. Since several studies have shown discrepancies between immunohistochemical demonstration of p53 and direct sequencing of the gene [27], we proceeded to *p53* gene analysis of 19 laryngeal specimens with dysplasia or carcinoma included in our previously examined material. We used the single strand conformation polymorphism (SSCP) technique [22], in order to shed light on the molecular nature of p53 overexpression. Because of a number of controversial reports [6, 16, 20, 26], we also investigated the relationship between the expression of p53 and proliferating cell nuclear antigen (PCNA), a 36 kDa nuclear protein involved in DNA synthesis and related to S phase [19]. We examined 42 laryngeal premalignant and malignant lesions in which 14 of 19 cases, analysed at molecular level, were included.

We used data from our previous work [10] concerning p53 expression and we stained PCNA with the peroxidase/anti-peroxidase (PAP) immunohistochemical method using the monoclonal antibody PC-10.

Material and methods

Forty-seven formalin fixed, paraffin embedded laryngeal specimens with dysplasia or SCC were selected from the material included in our previous report [10]. Nested polymerase chain reaction (PCR)-SSCP analysis was performed in 19 specimens which consisted of 6 dysplasias (2 grade I, 1 grade II and 3 grade III) and 13 carcinomas (5 grade I, 4 grade II and 4 grade III; Table 1). Five of these cases concerning grade II dysplasias and carcinomas were not included in the PCNA study. Adjacent normal laryngeal tissue from each examined specimen was included. PCNA study was carried out in all 42 selected laryngeal specimens, which included 22 LSCCs (13 grade I and 9 grade III) and 20 dysplasias (10 grade I and 10 grade III; Table 1). Since the aim of this part of the study was not to investigate PCNA expression as a proliferation index correlated with the differentiation grade of the lesions, we examined only low and high grade (grade I and III) LSCCs and dysplasias where pathological evaluation is clearer and more discrete. We placed our specimens into three groups according to p53 detection status: 18 cases with moderate or marked p53 expression (positivity 10–50% or >50% of the cells; group 1), 10 cases with weak p53 immunoreactivity (positivity <10% of the cells; group 2) and the remaining 14 p53 negative (–) cases (group 3).

Molecular analysis was performed on adjacent 5 μm sections of the paraffin embedded tumour tissue. Serial 5 μm sections were processed and the first section was stained with haematoxylin and eosin to visualize the extent of the tumour cells within each sample. The boundaries of the malignant tissues were delineated microscopically and excess stromal tissues were excised from paraffin sections on the slide using sterile surgical blades, so that only neoplastic cells were included. The remaining neoplastic tissue was scraped with a sterile blade and collected into 1 1.5 ml microcentrifuge tube. DNA was extracted as previously described [9].

Nested PCR-SSCP analysis was performed only on the cases which gave p53 immunohistochemical positivity in more than 50% of the cells, in order to enhance the sensitivity of the method. Of the 17 p53(+++) cases from the previous study [10], 5 were excluded due to inadequate amount of tissue. In addition, two p53(–) cases were included in the molecular study. We analysed exons 4–8 as the majority of previous studies have shown that the mutations in human tumours are found in this region [11]. First we determined the optimal conditions for PCR amplification of the 2.9 kb *p53* gene fragment (Fig. 1) [7] and for the nested PCR amplification of the individual exons, 4–9 (Fig. 1). In order to exclude mutations caused by *Taq* polymerase, the SSCP analysis was carried out on two different occasions for each tumour sample. The primers for the *p53* gene containing exons 4–9 (2.9 Kb) were amplified by PCR using the primers listed in Table 2. The PCR reac-

Table 1 Number of specimens examined in the *p53* gene analysis and proliferating cell nuclear antigen (*PCNA*) study according to histological diagnosis and grade of differentiation (*SSCP* single strand conformation polymorphism)

Grade of differentiation	Dysplasias			Carcinomas		
	I	II	III	I	II	III
PCNA study	10		10	13		9
SSCP	2	1	3	5	4	4
Total number of specimens	10	1	10	13	4	9

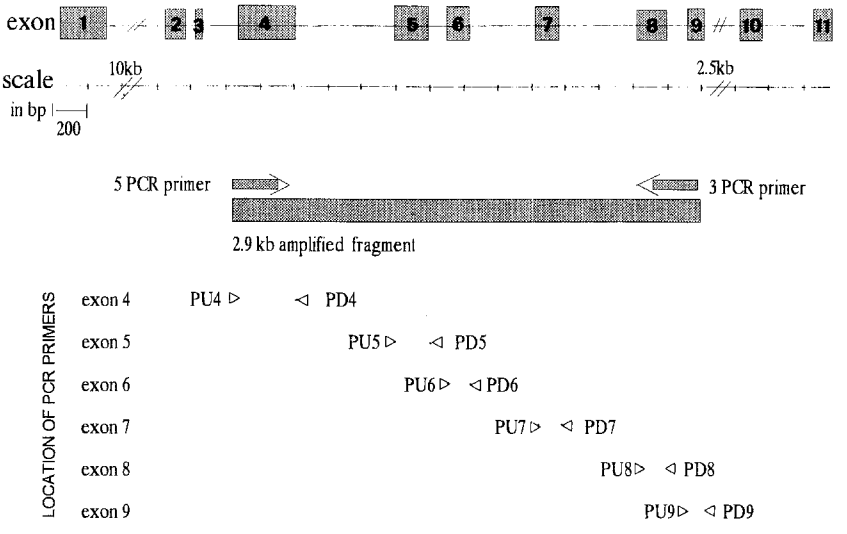


Fig. 1 Genomic organization of *p53* gene. The 2.9 kb *p53* gene fragment generated by polymerase chain reaction (PCR) amplification, and the *p53* gene exon fragments generated by nested PCR amplification. Exon 9 was not examined

Table 2 Primers for nested polymerase chain reaction amplification of *p53* gene

Exon	Primer sequence	
4–9	5′-GTAGGAATTCGTCCAAGCAATGGATGAT-3′	(sense)
	5′-CATCGAATTCTGGAAACTTTCCACCTTGAT-3	(antisense)
4	PU4: 5′-AATGGATGATTGATGCTGTCC-3′	(sense)
	PD4: 5′-CTCAGGGCAACTGACCGTGC-3′	(antisense)
5	PU5: 5′-GACTTTCAACTGTCTC-3′	(sense)
	PD5: 5′-CTGGGACCCTGGGCAAC-3′	(antisense)
6	PU6: 5′-GAGACGACAGGGCTGGTT-3′	(sense)
	PD6: 5′-CCACTGACAACCACCCTT-3′	(antisense)
7	PU7: 5′-GTGTTGTCTCCTAGGTTGGC-3′	(sense)
	PD7: 5′-AAGTGGCTCCTGACCTGGAG-3′	(antisense)
8	PU8: 5′-TGGTAATCTACTGGGACG-3′′	(sense)
	PD8: 5′-CTCGCTTAGTGCTCCCTGG-3′	(antisense)

tion (50 µl) contained 250 ng of genomic DNA, 0.4 µM of each respective primer, 2.5 units of *Taq* polymerase, 5 µl of 10×PCR buffer and 1 µl dNTP mixture (0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP and 0.2 mM dTTP). The reaction was initially denaturated at 98° C. The *Taq* polymerase was then added to the samples and were placed in a thermocycler. The DNA was amplified for 30 cycles consisting of 95° C for 1 min, 58° C for 1 min and 70° C for 4 min. Five microlitres PCR samples were analysed by electrophoresis on a 0.8% ultrapure agarose gel (BRL, USA) and the amplified products visualized under UV light after staining with ethidium bromide. One microlitre of each sample's first PCR reaction mixture (amplified DNA size 2.9 kb) was combined with 5 µl of 10×PCR buffer, 1 µl [α-^{32}P] dCTP (3000 Ci/mmol; Amersham, UK), 1 µl dNTP mixture and 0.4 mM of both primers for the specific exon in a final volume of 50 µl (Table 2). After an initial denaturation step at 98° C for 5 min, 2.5 units of *Taq* polymerase was added to each reaction tube which was then placed in the thermocycler for 30 cycles. Temperature and time for the reaction cycles of the examined exons was 95° C (denaturation) for 25 s, 62° C (annealing) for 25 s and 72° C (extension) for 30 s, respectively. Five microlitres of the PCR – amplified product was then diluted with 50 µl of denaturation buffer [0.1% sodium dodecyl sulphate in 10 mM ethylenediaminetetracetic acid (EDTA)] and 5 µl of this mixture 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, USA). Samples were denaturated at 98° C for 7 min and placed immediately on ice to prevent renaturation before being run on 0.1×20×24 cm, 0.6× hydrolink polyacrylamide non-denaturing gel. The DNA was electrophoresed in TBE (0.09 M TRIS base, 0.09 M boric acid and 2.5 mM EDTA) running buffer at 12–15 V at room temperature. Gels were dried and exposed to XAR-5 film (Kodak) for 12–24 h. In addition, several SSCP analyses were performed without radioactive dNTP and results were visualized with the silver staining technique [5] in order to compare the sensitivity of both methods.

Immunohistochemical analysis for PCNA was undertaken using the monoclonal antibody PC-10 (Oncogene Science). After dewaxing and dehydration through gradient ethanols, slides were immersed for 10 min in 3% hydrogen peroxide to block endogenous peroxidase and then washed with phosphate-buffered saline (pH 7.6). Immunostaining was performed using the PAP method (DAKO, Denmark). A dilution of 1:10 with 1 h incubation was found to be optimal. Sections were developed with diaminobenzidine for 10 min, counterstained with haematoxylin and mounted in resin. PCNA staining (PC-10) was scored by V.G. and G.R. One thousand tumour cells were counted at ×400 magnification from ten representative fields. Positive non-neoplastic cells or cells showing only cytoplasmic staining were not taken into account. Labelling index (LI) was calculated as the percentage of positive nuclei.

All statistical analysis was based on chi square test with Yates' correction. Additional double tailed Fisher's exact test was used when the number of samples in any cell of a given statistical table was less than or equal to five.

Results

Results of the SSCP analysis for exons 4–8 are summarised in Table 3. *p53* mutations in exons 5 or 6 were found in 9 of 12 immunohistochemically p53(+) cases (75%). Seven specimens (six carcinomas and one dysplasia) had mutations in exon 5 (Fig. 2) and two (one carcinoma and one dysplasia) in exon 6 (Fig. 3). The immunohistochemically p53(–) specimens included in the molecular study, were also negative for *p53* mutations in exons 4–8. Although there was no statistical correlation between mutation frequency and differentiation grade of the lesions, the former tended to be seen more often in grade III LSCCs. Comparisons between the electrophoretic patterns of the lesions and adjacent normal tissue DNA did not reveal loss of heterozygosity (LOH) at the 2.9 Kb region which encodes exons 4–8 the *p53* gene (Fig. 4).

PCNA immunostaining was exclusively nuclear with variation in the staining intensity (Fig. 5). Mean PCNA LI in relation to p53 expression was statistically increased in group 1 compared with groups 2 and 3, as shown in Table 4. PCNA LI was significantly higher in carcinomas than dysplasias [74% vs 38%, $P<10^{-6}$ in p53(+) cases and 65% vs 31%, $P<10^{-5}$ in p53(–) cases] and was also correlated with differentiation grade of the carcinomas [67% (grade I) vs 80% (grade III), $P<0.05$ in p53(+) cases and 55% (grade I) vs 75% (grade III), $P<0.005$ in p53(–) cases]. No significant correlation was observed between of dysplasias grade I and III [34% vs 42, $P>0.1$ in p53(+) cases and 25% vs 37%, $P=0.1$ in p53(–) cases].

Table 4 Relationship between PCNA labelling index (*LI*) and p53 expression in high and low grade LSCCs and dysplasias (*group 1* p53 immunireactivity >10%, of the tumour cells, *group 2* p53 immunoreactivity in <10% of the tumour cells, *group 3* no p53 immunoreactivity)

p53 expression	PCNA LI (%)				
	Dys I (10)	Dys III (10)	Ca I (13)	Ca III (9)	Mean
Group 1	45	43	79	82	68* **
Group 2	21	38	51	77	46*
Group 3	25	37	55	75	41**

* $P<0.005$; ** $P<0.0005$

Table 3 SSCP analysis of the *p53* mutations and correlation between molecular analysis and immunohistochemical findings in laryngeal squamous cell carcinomas (LSCCs) and dysplasias (*Ca* carcinoma, *Dys* dysplasia, *N* normal, *I–III* grade of differentiation, *ND* not done)

p53 immunostaining	–	–	+	+	+	+	+	+	+	+	+	–	+	+	+
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
p53 exon mutation	N	Ca I	Ca I	Ca I	Ca II	Ca II	Ca II	Ca III	Ca III	Ca III	Ca III	Dys II	Dys III	Dys III	Dys III
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	+	–	+	+	–	+	–	+	+	–	–	–	+
6	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–
7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND

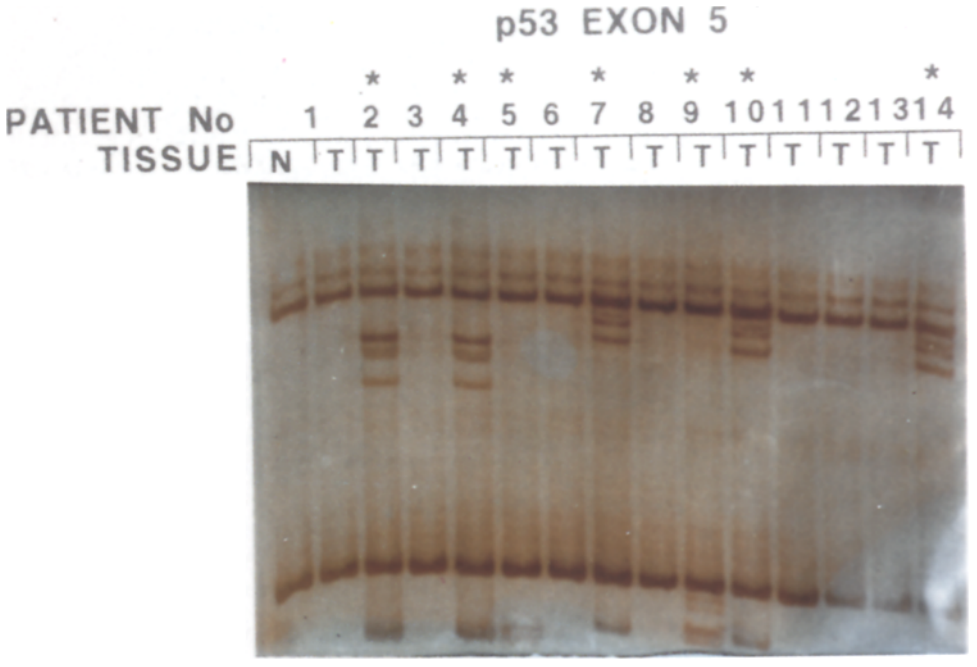


Fig. 2 Single strand conformation polymorphism (SSCP) analysis of *p53* mutations in exon 5. *p53* mutations lead to mobility shifts of the single strand DNA in cases T2, T4,T5, T7, T9, T10 and T14 (*asterisks*) as evidenced by additional banding patterns in comparison with non-mutated, adjacent normal tissue DNA (*N*). Since the electrophoretic pattern of the normal adjacent tissue DNA was identical in all cases, we included in the figure only one normal specimen. *T1–T3* grade I carcinoma, *T4–T6* grade II carcinoma, *T7–T10* grade III carcinoma, *T11* grade II dysplasia, *T12–T14* grade III dysplasia. There is no indication of loss of heterozygosity (LOH) in exon 5. The results are visualized with the silver staining technique (*T* tumour)

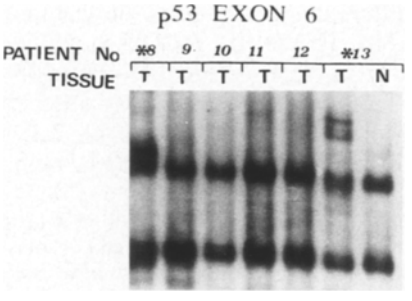


Fig. 3 SSCP analysis of *p53* mutations in exon 6. Mutations are observed in specimens T8 and T13 (*asterisks*)

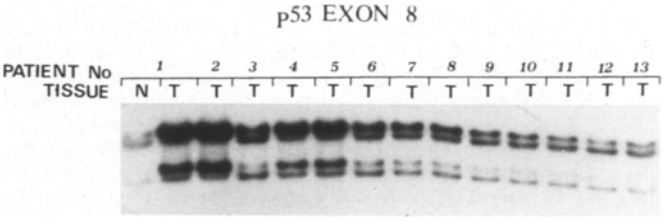
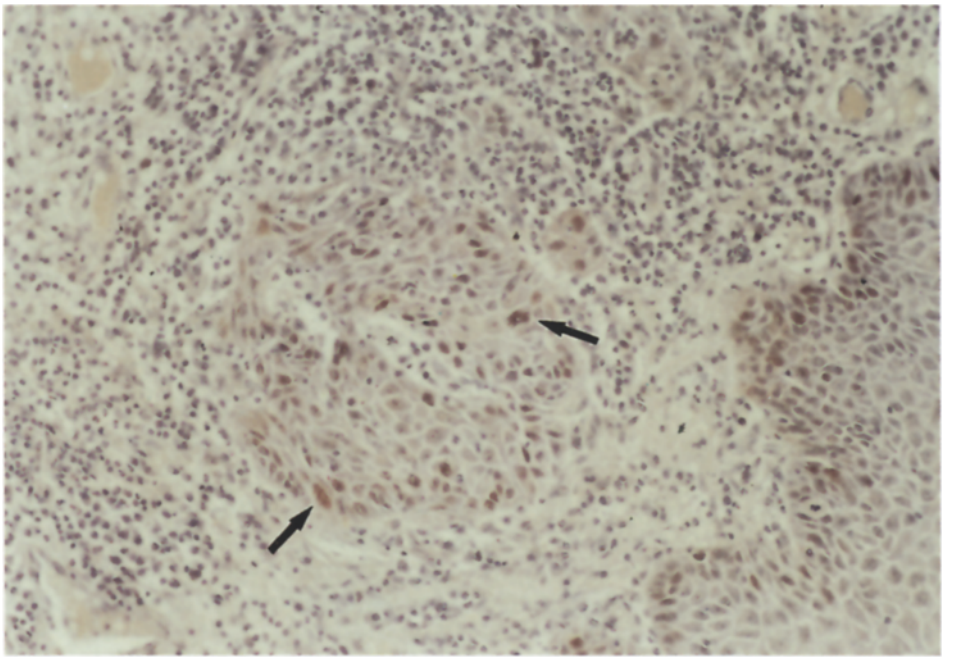


Fig. 4 SSCP analysis of exon 8 of the *p53* gene: there is no indication of mutations and LOH as compared with adjacent normal laryngeal tissue DNA

Fig. 5 Proliferating cell nuclear antigen (PCNA; PC-10) nuclear immunostaining in a p53 positive grade III laryngeal squamous cell carcinoma. *Arrows* demonstrate the characteristic PCNA nuclear staining pattern. Peroxidase/antiperoxidase immunohistochemical method. Diaminobenzidine as chromogen (×200)



Discussion

In this study, which extends our previous observations [10], we investigated the molecular basis of p53 overexpression in 19 LSCCs and dysplasias and the possible relationship between p53 protein expression and PCNA LI in 42 laryngeal premalignant and malignant lesions, including 14 of 19 specimens examined in the molecular analysis.

The molecular study was performed with the SSCP technique in exons 4–8, as the majority of previous reports have shown that the mutations in human tumours are found in this phylogenetically conserved sequence domain [11]. In order to enhance the sensitivity of the method we analysed only the cases which gave p53 immunohistochemical signal in more than 50% of the cells and we also included a pair of p53(–) specimens. The analysis revealed mobility shifts of the single strand DNA, indicating mutations, in exons 5 or 6 in 9 of 12 immunohistochemically p53(+) cases. More specifically, seven and two of nine mutations concerned exons 5 and 6 respectively. The remaining three immunohistochemically p53(+) specimens, as well as the two p53(–) ones, had no indication of *p53* gene alterations. The former finding could be due either to mutations outside the examined region of the *p53* gene or the fact that the detected p53 protein is the wild type which is stabilized via interaction with cellular regulators such as the *mdm-2* gene products. The latter are nuclear phosphoproteins which could feedback wild type p53 activity a negative mode in an autoregulatory loop pathway [12]; altered mdm-2 expression has been found to promote tumorigenesis [21]. In our study, there was no indication of deletions in the chromosomal region 17p13.1 (LOH) which encodes exons 4–8 of the *p53* gene, while Maestro et al. [18] using two probes which correspond to the larger part of the *p53* gene, found LOH in four of eight (50%) p53 immunohistochemically positive cases and 1 of 12 (8%) p53(–) ones.

To our knowledge, molecular analysis of *p53* gene in LSCCs has also been carried out by four research groups in USA and Italy [1, 2, 18, 25] who have confined their analysis of the *p53* gene to exons 2–11. Although no specific mutation pattern emerges from these studies, as shown in Table 5, in our communication *p53* gene alterations were more frequent in exon 5. Interestingly exon 5 encodes residue 175 (arginine-175) which is involved in stabilizing interactions between loops 2 and 3 of the p53 protein. Mutations concerning residue 175 are considered to be among the most disruptive of the p53 DNA-binding function [23, 24]. All the mutations detected in the published work from USA and Italy were missense, with the exception of two frameshift, one splice acceptor site and two termination signal sequence alterations. The majority of the p53 missense mutations concerned amino acid substitutions from different classes. Such vital changes probably alter the configuration of the p53 molecule, removing not only its suppressor activity but also adding stimulatory properties. Thus, the altered p53 protein affects cellular promoters which were inhibited by the wild type p53 [4, 8].

The expression of PCNA, a molecule which is directly implicated in the DNA replication machinery [18], may be modulated by wild or mutated p53 protein [6, 20, 26]. Recenty Subler et al. [26] and Deb et al. [6] reported on the effect of wild and mutant p53 protein on the activity of PCNA promoter using chloramphenicol acetylotransferase (CAT) assays in mammalian cell lines. Subler et al. found that wild type p53 expression correlated with a significant and consistent six- to sevenfold inhibition of reporter enzyme activity (CAT) in HeLa cells, while Deb et al. observed that p53 with a mutation at anyone of the four amino acid positions 175, 248, 273 and 281 correlated with a significant 2- to 11-fold increase in the PCNA promoter activity. Moreover Mercer et al. [20], using a cell line which contained wild type p53 cDNA driven by a hormone-inducible promoter, showed a selective PCNA mRNA down-regulation and decrease PCNA protein level after dexamethasone treatment. However Lechner et al. [16] reported that PCNA promoter is nonresponsive to the wild type p53. Given this line of reasoning, we decided to examine p53 protein detection status in relationship to PCNA labelling score. We found that PCNA LI progressively increased from 41% in group 3 to 46% and 68% in groups 2 and 1, respectively. The difference between the latter group and the others was highly significant. This finding could be explained in part if PCNA expression is affected directly or indirectly by wild or mutated p53 protein, as suggested by the former authors [6, 20, 26]. More specifically, in the p53 immunohistochemically negative cases (group 3) which have the wild type p53, this may exert either direct and specific inhibition of the PCNA promoter or affect common generalized transcription factors, inhibiting cell cycle related genes. In the p53 immunoreactive cases (group 1) which harbour the mutated p53 in a large proportion of cases, the altered from the protein could interact either directly or indirectly with growth related genes

Table 5 *p53* mutations in LSCCs and dysplasias by exon and geographical region (*NM* no mutation, *NS* not studied)

Geographica region [reference]	Number of specimens	*p53* exon									
		2	3	4	5	6	7	8	9	10	11
USA [1]	Unknown	NS	NS	NS	2	3	3	4	NS	NS	Ns
Italy [18]	20	NS	NS	NS	1	2	2	1	NS	NS	NS
USA [25]	4	NS	NS	NS	1	NM	3	NM	NS	NS	NS
USA [2]	4	NM	NM	NM	2	NM	1	NM	NM	NM	NM
Greece (present study)	14	NS	NS	NM	7	2	NM	NM	NS	NS	NS

activating their expression. However, the conformational change of the protein could remove its ability to bind the PCNA promoter releasing its repressor affect and inducing PCNA transcription. In the cases where detectable p53 is represented by the wild type, its supressor activity could be abrogated by the overexpression of other endogenic and/or exogenic factors [28]. Finally the cases which overexpress p53 in less or equal to 10% of the tumour cells (group 2), have a PCNA LI comparable with that of p53 (–) cases (46% vs 41%). Even though the nature of the protein in these tumours is uncertain, elevated p53 levels may represent an appropriate response of the wild type to some external or internal cellular stimuli [13]. Furthermore, since the PCNA gene is regulated in a complex manner [3], ist overexpression may be a result of increased PCNA mRNA stability induced by growth factors, regardless of p53 status [15]. Though our finding is only indicative of a possible association between p53 and PCNA, further studies providing molecular evidence are required so as to prove the interaction between the two molecules.

In addition, as we expected, PCNA expression was increased in carcinomas when compared with dysplasias and was also correlated with differentiation grade of the carcinomas, both comparisons being independent form p53 immunoreactivity.

In conclusion, our study shows that a large proportion of LSCCs and dysplasias express the mutant p53 protein. Interestingly, the immunohistochemical detection status of the p53 protein is proportionally correlated with PCNA LI, a finding which accompanies tumour progression.

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