Virchows Archiv (1994) 425:481–489

ORIGINAL ARTICLE

Vassilis Gorgoulis · George Rassidakis
Andreas Karameris · Alexandra Giatromanolaki
Calypso Barbatis · Christos Kittas

Expression of p53 protein in laryngeal squamous cell carcinoma and dysplasia: possible correlation with human papillomavirus infection and clinicopathological findings

Received: 7 April 1994 / Accepted: 28 July 1994

Abstract In order to evaluate the expression of p53 protein in 28 premalignant and 40 malignant squamous cell proliferations of the larynx and its relationship to tobacco consumption, human papillomavirus infection and differentiation grade of the lesions, p53 expression was examined by means of a microwave post-fixation immunohistochemical method using the PAb 240 and PAb 1801 monoclonal antibodies. HPV infection was assessed by non-isotopic in situ hybridization (NISH) and polymerase chain reaction (PCR). A large proportion of carcinomas (77.5%) and dysplasias (61%) expressed p53. No difference was found between differentiation grades of the lesions regarding p53 detection ($P>0.1$), but moderate or intense p53 expression was more frequent in the carcinomas ($P<0.05$). A statistical correlation was found between cigarette consumption and both p53 detection and p53 staining intensity ($P<0.05$ in each case). HPV study revealed HPV 16 and 18 infection only in carcinomas. The frequency was 28% and the physical state of the virus as demonstrated by NISH was integration into the genome. We observed an inverse relationship between HPV infection and p53 expression ($P=0.006$). Our findings suggest that p53 overexpression is a common and early event which increases in frequency with progression of laryngeal squamous cell carcinoma. The expression of p53 is influenced by tobacco and high-risk types of HPV.

V. Gorgoulis · G. Rassidakis · C. Kittas
Laboratory of Histology and Embryology,
University of Athens School of Medicine, Athens,
Greece

V. Gorgoulis · A. Giatromanolaki · C. Barbatis
Department of Pathology, "Red Cross" Hospital, Athens,
Greece

A. Karameris
Department of Pathology, 401 General Army Hospital, Athens,
Greece

V. Gorgoulis (✉)
Antaiou 53, Lamprini, Ano Patisia, GR-11146 Athens,
Greece

Key words p53 · Human papillomavirus
Polymerase chain reaction · In situ hybridization
Laryngeal squamous cell carcinoma

Introduction

Squamous cell carcinoma is by far the commonest malignant tumour of the larynx [4]. Although there is strong evidence that tobacco and alcohol abuse are correlated with its oncogenesis, there is little genetic information concerning the linkage between these xenobiotic agents and squamous cell carcinoma of the larynx (LSCC) [43, 56, 64, 67]. Because epidemiological evidence suggests that both alcohol and tobacco use are related to the development of lung and oesophageal cancer, in which mutations of the p53 gene are frequent events [32, 36], it is tempting to speculate that the p53 gene represents a particularly susceptible cellular target for these agents in LSCC. The p53 gene is localized to 17q13 chromosome and encodes a 393-amino-acid (aa) protein which structurally resembles a transcriptional activator factor. This p53 protein functions as a negative cell growth regulator and may play an important part in genomic stability and DNA repair [38, 39]. Loss of wild-type p53 function by mutation, by complex formation with viral products or with cellular negative regulator(s) such as the mdm2 gene products, or by alteration in subcellular localization removes an important tumour suppressor mechanism and promotes tumorigenesis [70].

Epidemiological studies indicate that loss of p53 activity by mutation represents the most frequent genetic abnormality in human cancer [39]. In addition, p53 can be degraded by complexing with high-risk human papillomavirus (HPV)-E6 protein via the ubiquitin pathway [53]. The role of HPV has been well documented in preneoplastic and neoplastic squamous cell proliferations in a number of body sites such as the skin, female lower genital tract, and oesophagus [18]. In the larynx, HPV infection has been known to cause juvenile and some cases of adult papillomatosis [50, 63]. Information con-

Table 1 Summary of results of human papillomavirus (*HPV*) positivity in laryngeal squamous cell carcinomas (*LSCCs*) and dysplasias

Study	Country	Year	LSCCs	Dys-plasias	HPV subtype positive								Method			
					6	11	16	18	30	31	33	35	S/B[f]	PCR	D/B[f]	In situ hybrid-ization
Kahn et al. [37]	Germany	1986	42	–					1				+			
Scheurlen et al. [55]	Germany	1986	36	–			1						+			
Brandsma et al. [8]	USA	1986	6[a]	–			6						+		+	
Dekmezian et al. [16]	USA	1987	1	–		1										+
Syrjanen et al. [62]	Finland	1987	116	–	4	9	6									+
Morgan et al. [49]	UK	1990	10	–			4[b]				4[b]			+		
Perez-Ayala et al. [51]	Spain	1990	48	–			26							+		
Perez-Ayala et al. [51]	Spain	1990	3[a]	–			3							+		
Hoshikawa et al. [35]	Japan	1990	34	–	1		6							+		
Watts et al. [66]	USA	1991	9	–	3[c]	3[c]	1[d]	1[d]					+	+		
Hong et al. [34]	China	1991	6	–			3						+			
Anwar et al. [1]	Japan	1993	43	–			3[e]	14[e]			1[e]			+	+	
Our study	Greece	1994	40				9	2						+		+
				28												

[a] Verrucous LSCCs
[b] HPV subtypes 16 and 33 were detected in the same samples
[c] HPV subtypes 6 and 11 were detected in the same three samples
[d] HPV subtypes 6, 11, 16 and 18 were detected in the same sample
[e] Of two of the samples that were HPV 18 positive, one was also positive for HPV 33 and one for HPV 16
[f] S/B and D/B denote Southern blot and dot blot, respectively

cerning the involvement of HPV in LSCC is limited and conflicting (Tabel 1).

This study was undertaken to investigate the expression of p53 protein in premalignant and malignant squamous cell proliferations of the larynx and its relationship to possible carcinogenic factors (tobacco, high-risk HPV infection) and to pathological features of the lesions. The expression of p53 protein was examined with the microwave post-fixation streptavidin horseradish peroxidase (HRP) immunohistochemical method, and HPV detection was assessed by non-isotopic in situ hybridization (NISH) and polymerase chain reaction (PCR).

Materials and methods

Sixty-eight buffered formalin-fixed, paraffin-embedded laryngeal specimens of dysplasia or SCC were obtained from the pathology files of the "Red Cross" Hospital, Athens. All specimens were removed endoscopically and selected on the basis that tumour or dysplastic cells comprised more than 70% of the tissue block. Slides from all cases were reviewed and classified into six broad categories according to the grading system proposed by Hellquist et al. [30] for dysplasias and Broder's method for carcinomas [20, 46]: 1, 2 and 3, grade I, II and III (in situ carcinoma) dysplasias; 4,5 and 6, grade I, II and III LSCCs. In the first three groups 10, 8 and 10 laryngeal dysplasias were placed, while in the three other groups 13, 18 and 9 LSCCs were placed, respectively. The mean age of patients with dysplasia was 55, 53 and 63 years, respectively, of those with LSCC 64, 65 and 60 years. No form of radiation or other preoperative therapy was mentioned in the patients' clinical history. The patients were classified according to smoking pattern into group A (20 cigarettes or more per day) group B (fewer than 20 cigarettes per day) and group C (non-smokers). In group A the mean number of cigarettes consumed daily was 33.04 (standard deviation, SD 10.21) while in group B it was 7.76 (SD 2.3) ($P<0.05$). A group of specimens of normal laryngeal tissue was also included.

Immunohistochemical analysis of p53 protein was undertaken using the mouse monoclonal antibodies PAb 240 [23] and PAb 1801 [3] (both antibodies from Oncogene Science). PAb 240 recognizes an epitope between aa 156 and aa 335, and PAb 1801 recognizes an epitope between aa 32 and aa 79. Serial sections 5 µm thick were cut from all paraffin blocks and mounted on Vectabond (Vector Labs, Burlingame, Calif., USA) -treated glass slides. The slides were air-dried at room temperature (RT) for at least 24 h. Then sections were dewaxed, rehydrated and endogenous peroxidase activity was blocked with 0.6% H_2O_2 in 80% methanol for 20 min. Next the sections were washed with double distilled water (ddH_2O) and placed in a plastic container (Sigma) filled with the following microwave oven (MWO) solution: 0.01 M sodium citrate $Na_3C_6H_5O_7 \cdot 2H_2O$, 0.01 M sodium bicarbonate $NaHCO_3$ and 0.01 M HCl/sodium citrate buffer pH 6.0. The jar was irradiated in a Polaron H2500 microwave processor (BioRad, Watford, UK) for 10 min (2×5 min) at a power output equivalent to 600 W, ensuring no loss of buffer during the first cycle. It has been shown that MWO treatment is superior to convetional enzymatic treatment in unmasking p53 protein in routine material [10].

The sections were then rinsed in ddH_2O water and TRIS-buffered saline (TBS; 0.05 mol/l TRIS-HCl, 0.10 mol/l NaCl, pH 7.2). The second incubation was in biotinylated rabbit anti-mouse [F(ab') 2 fragment; Dako, Denmark] diluted 1:200 in TBS for 25 min and the third, after a wash with ddH_2O and TBS, and in streptavidin-HRP (Dako, Denmark) diluted 1:300 in TBS for 25 min. Signal was developed using diaminobenzidine and hydrogen peroxide as chromogen. Slides were then counterstained in haematoxylin and mounted. Lung carcinomas expressing p53 were used as positive controls, and mouse IgG1 mAb of unrelated specificity and the IgG fraction of normal rabbit serum were used as negative controls. All p53-positive nuclei were counted and classified using the following semiquantitative method: (+)=<10% positive cells, (++)=10–50% positivity and (+++)=>50% positivity.

For NISH of HPV, probes for subtypes 6, 11, 16, 18, 31, 33 and 35 were used. The HPV 6 probe consisted of the amp2 fragment of the viral genome cloned into pBR322 [59]; the HPV 11 probe, the whole viral genome in pBR322 [14]; the HPV 16 probe, the whole viral genome in pAT153 [60]; the HPV 18 probe, the whole viral genome in pBR322 [6]; the HPV 31 probe, the whole viral genome in pT713 [40]; the HPV 33 probe, the whole viral genome in pBR322 [11]; and the HPV 35 probe, the whole viral genome in pT713 [41]. Digoxigenin was incorporated into plasmid DNA by nick translation [31]. The nick translation fragments ranged in size from 50 to 1000 bp with a median of 200–400 bp.

Table 2 Sequences of oligonucleotide primers

HPV type	Sequence (5′→3′)	Genomic location	Size of amplified product (base pairs)
6	A GCTAATTCGGTGCTACCTGT	401–420	140
	B CTGGACAACATGCATGGAAG	521–540	
11	A CGCAGAGATATATGCATATG	221–240	90
	B AGTTCTAAGCAACAGGCACA	291–301	
16	A TCAAAAGCCACTGTGTCCTG	421–440	120
	B CGTGTTCTTGATGATCTGCA	521–540	
18	A ACCTTAATGAAAAACCACGA	371–390	100
	B CGTCGTTGGAGTCGTTCCTG	451–470	

Paraffin wax sections 5 μm thick were cut and attached to glass covered with aminopropyltriethoxysilane, which allows the use of higher concentrations of enzyme without loss of cellular material. Briefly, the sections were deparaffinized in xylene and then rehydrated in gradient ethanol washes. Nucleic acid unmasking was assessed by incubating the sections in proteinase K (Boehringer, Germany) at a concentration of 500 μg/ml in phosphate-buffered saline (PBS; 10 μM phosphate, 100 μM NaCl, pH 7.2) for 15 min, washing them thoroughly in ddH_2O followed by PBS and drying them at 75° C. Aliquots of hybridization mix (10 ml) containing 10–20 ng of the appropriate labelled probe were added to each well on multispot slides, covered with a 14-mm glass coverslip and the slides placed in a moist Terasaki plate. Hybridization mix consisted of 50% formimide, 5% dextran sulphate (BDH, UK), 2×SSC, and 0.05 mol/l TRIS-HCl (pH 7.3); 1×SSC=0.15 mol/l sodium chloride, 0.015 mol/l sodium citrate. Sections and probes were simultaneously denatured at 95° C for 15 min on a solid stainless steel plate in a hot air oven and then hybridized at 42° C for 2 h.

Detection was performed using a three-step method. Briefly, slides were washed in three changes of 4×SSC at 20° C (5 min each change), soaked in blocking agent TBT [0.05 mol/l TRIS-HCl, 0.10 mol/l sodium chloride (pH 7.2) containing 3% (w/v) bovine serum albumin and 0.05% (v/v) Triton X100] at RT for 10 min. Sections were incubated in monoclonal antidigoxin (Sigma, UK) diluted 1:10000 in TBT for 30 min at RT. A second incubation was in biotinylated rabbit anti-mouse F(ab')2 fragment (Dako, Denmark) diluted 1:200 in TBT and the third in avidin-peroxidase (Dako, Denmark) diluted 1:75 in TBT containing 5% (w/v) non-fat milk.

The signal was developed using aminoethylcarbazole and hydrogen peroxide (Zymed, USA). After probe detection, slides were air-dried at 42° C, counterstained progressively in haematoxylin, and mounted in glycerol jelly. As positive controls, CasKi and SiHa cell lines were used for HPV 16, HeLa cells for HPV 18, positive cervical warts for HPV 6 and 11, and positive cervical SCC for HPV 31, 33, and 35.

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, in order to ensure that only neoplastic cells were included. The remaining neoplastic tissue was scraped with a sterile blade and collected in a 1.5-ml microcentrifuge tube. DNA was extracted as previously described [25].

HPV sequences were detected by PCR using oligonucleotide primers complementary to sequences in the E6 region of HPV types 6, 11, 16, and 18 (Table 2).

Five-microlitre quantities of the DNA solutions were used for PCR in 50 μl reaction mixture containing 10 μM TRIS-HCl, pH 8.8, 50 μM KCl, 1.5 μM $MgCl_2$, 0.1% Triton X100, 80 μM of each dNTP (dATP, dCTP, dGTP and dTTP), 1 μM of each primer and 1.5 units of the Taq DNA polymerase (Promega, Madison, Wis.). Template DNA was first denatured at 95° C for 10 min before addition of Taq polymerase at 72° C. This was followed by 35 cycles of PCR with incubations of 2 min at 37° C (annealing), 2 min at 72° C (polymerization) and 1 min at 95° C (denaturation). Appropriate negative and positive controls were used in each set of reagents. After amplification, 10 μl of the reaction mixture was taken for electrophoresis on 10% polyacrilamide gels. The reaction products were visualized by ethidium bromide staining.

All statistical correlations were based on the chi-square (χ^2) test with Yates' correction. An additional two-tailed Fisher's exact test was used only when the number of samples in any cell of a given statistical table was 5 or fewer.

Results

Sixty-eight laryngeal dysplasias and LSCCs were assessed for levels of p53 expression using a combination of two antibodies (PAb 240 and PAb 1801). Seventeen of 28 (61%) of the dysplasias and 31 of 40 (77.5%) of the carcinomas showed immunoreactivity for p53 protein. Immunostaining for both antibodies gave identical results with the exception of one grade I dysplasia and two grade I LSCCs which were positive for PAb 240 and negative for PAb 1801. The pattern of immunoreactivity was exclusively nuclear (Figs. 1, 2). There was no reaction in normal laryngeal epithelium or stromal cells. Variations were observed in the percentage of reactive cells, ranging from 10% to 90%, as well as in the intensity of staining between nuclei of the same tumour.

The relationships of p53 staining pattern with histological grade and smoking are summarized in Tables 3 and 4. No significant difference was found in p53 immunoreactivity between dysplasias and carcinomas [17/28 (61%) vs 71/40 (77.5%), χ^2=2.24, P=0.135], but moderate or intense p53 expression (with positivity ranging from ~50% to >75% of the cells) was statistically more frequent in carcinomas than dysplasias [24/40 (60%) vs 7/28 (25%), χ^2=5.22, P=0.04]. Detection of p53 was significantly more frequent in group A than group B smokers [33/39 (84.6%) vs 14/26 (54%), χ^2=5.92, P=0.015]. We also observed a strong correlation between moderate (++) or intense (+++) p53 staining and smoking in group A compared with group B [25/33 (75.8%) vs 6/14 (42.9%), χ^2=4.74, P=4.74, P=0.029 Fisher's exact test P=0.034].

NISH revealed HPV 16 DNA in 7 of 40 LSCCs (17.5%). Of the HPV 16-positive cases 4 of 7 and 3 of 7 were grade II and grade III LSCCs respectively. The remaining carcinomas were negative for HPV 6, 11, 18, 31, 33, and 35. Dysplasias were also negative for HPV DNA. The hybridization pattern was random in distribution and took the form of a distinct red punctate signal (dots) on a clean background of haematoxylin-stained nuclei. This type of hybridization signal, contrasting with the diffuse and granular one, is indicative of HPV integration (Fig. 3). The number of cells that showed a positive NISH signal in each tumour ranged from a few isolated cells to almost all cells in some carcinomas. PCR analysis confirmed the NISH results (7/40 LSCCs

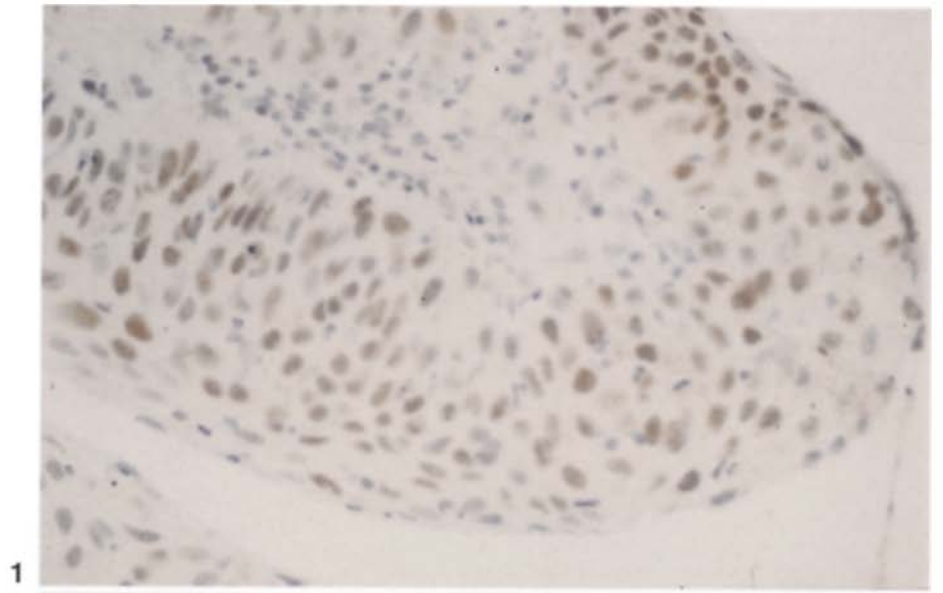

Fig. 1 Immunohistochemical detection of p53 protein stained with PAb 1801 in a grade II laryngeal dysplasia with haematoxylin-stained background. Diaminobenzidine, ×400

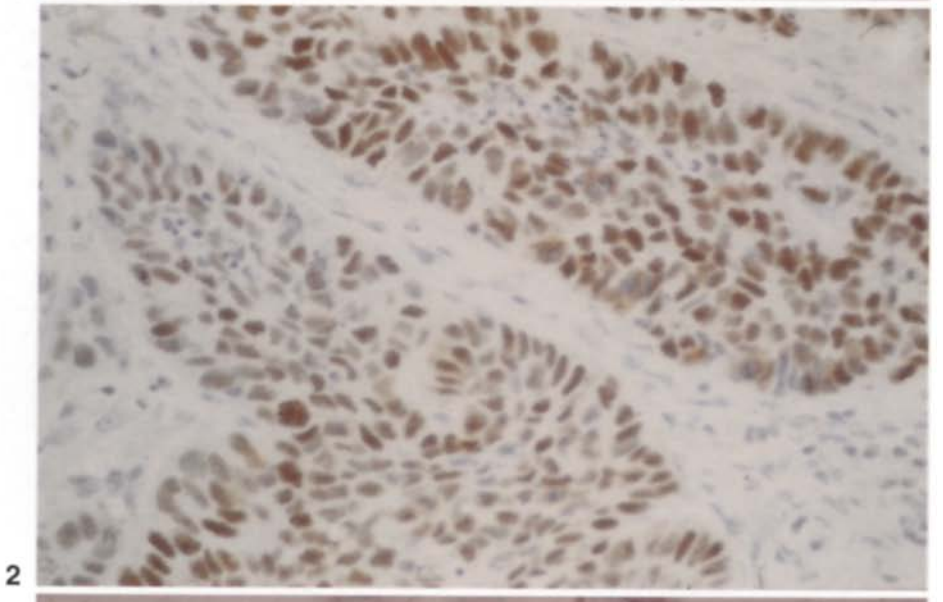

Fig. 2 Immunohistochemical detection of p53 protein stained with PAb 240 in a grade II laryngeal squamous cell carcinoma (LSCC). ×400

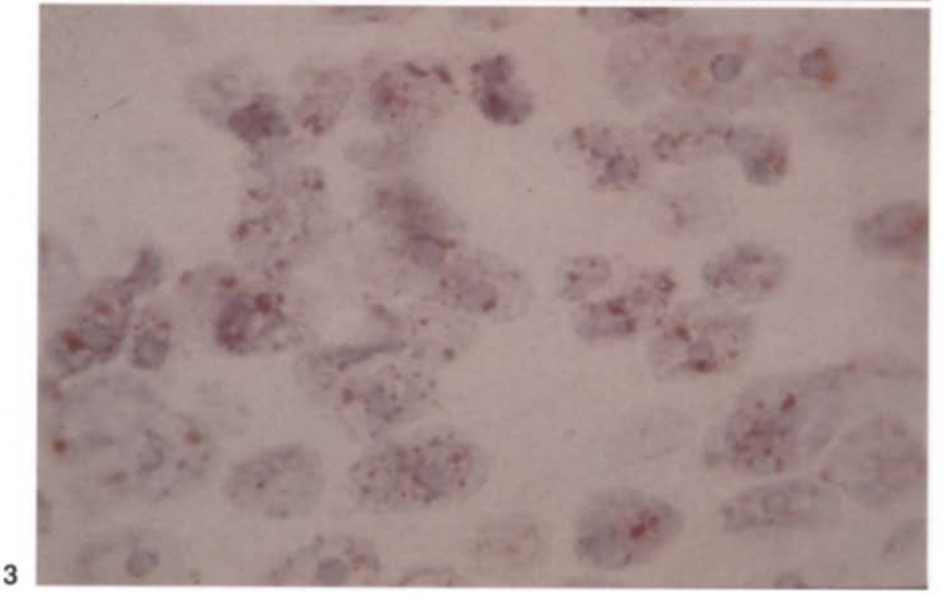

Fig. 3 Punctate non-isotopic in situ hybridization signal representing integration of human papillomavirus (HPV) 16 in tumour cells of a LSCC. Aminoethylcarbazol/peroxidase, ×1000

Table 3 Relationship between p53 expression and histological differentiation

Type of lesion	Expression of p53					
	No of lesions	(−) *n* (%)	(+) ~10% *n* (%)	(++) ~50% *n* (%)	(+++) ~75% *n* (%)	Total p53 expression *n* (%)
Dysplasias						
1. Grade I	10	6 (60)	2 (20)	2 (20)	0 (0)	4 (40)
2. Grade II	8	2 (25)	5 (62.5)	0 (0)	1 (12.5)	6 (75)
3. Grade III (in situ)	10	3 (30)	3 (30)	1 (10)	3 (30)	7 (70)
Carcinomas						
4. Grade I	13	3 (23.1)	3 (23.1)	2 (15.4)	5 (38.5)	10 (77)
5. Grade II	18	4 (22.2)	2 (11.1)	6 (33.3)	6 (33.3)	14 (78)
6. Grade III	9	2 (22.2)	2 (22.2)	3 (33.3)	2 (22.2)	7 (78)

Table 4 Relationship between p53 expression and smoking

Smoking pattern	Expression of p53 protein				
	n	(−) *n* (%)	(+) ~10% *n* (%)	(++) ~50% *n* (%)	(+++) >75% *n* (%)
Group A smokers	39	6 (15.4)	8 (20.5)	10 (25.6)	15 (38.5)
Group B smokers	26	12 (46.1)	8 (30.8)	4 (15.4)	2 (7.7)
Non-smokers	3	2 (66.7)	1 (33.3)	0 (0)	0 (0)

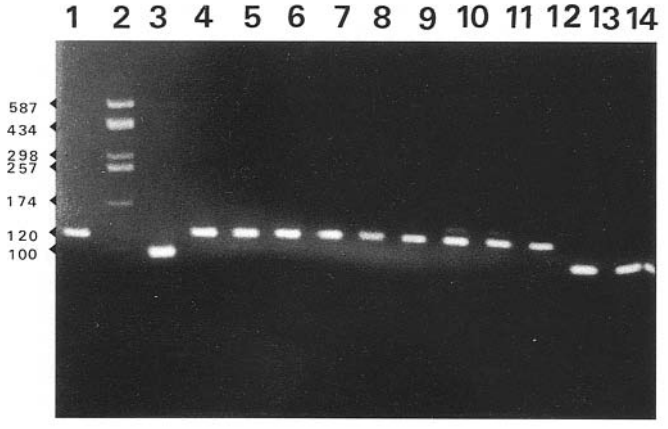

Fig. 4 Amplification products of HPV 16 and 18 obtained by polymerase chain reaction. Polyacrylamide gel electrophoresis and ethidium bromide staining of products obtained with specific oligonucleotide primers. *Lane 1* Positive control for HPV 16 (CaSki cells). *Lane 2* Marker pUC 18 digested with *Hae*III. *Lane 3* Positive control for HPV 18 (HeLa cells). *Lanes 4–12* Specimens positive for HPV 16 (120 bp). *Lanes 13, 14* Specimens positive for HPV 18 (90 bp)

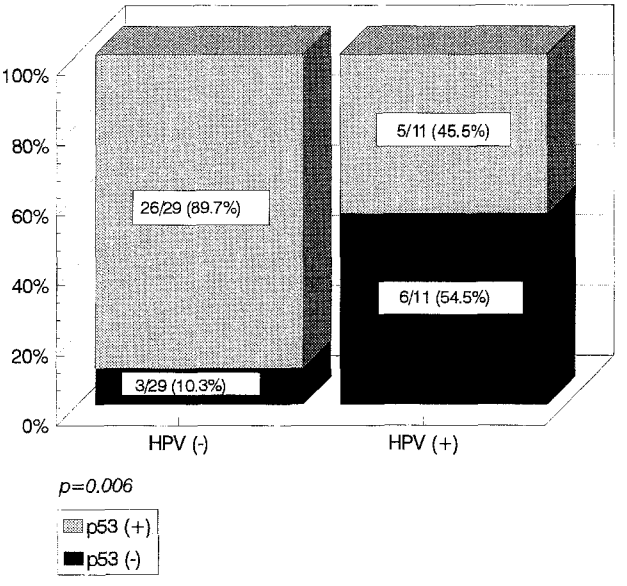

Fig. 5 Diagram showing the inverse relationship between p53 expression and HPV infection in LSCC

HPV 16-positive). Additionally, PCR analysis revealed four more HPV-positive lesions, two for HPV 16 and two for HPV 18 (Fig. 4). The HPV 16- and HPV 18-positive cases were grade I and grade II LSCCs respectively. No significant relationship between HPV detection and histological grade of carcinomas was observed.

Statistical analysis revealed that absence of p53 staining was significantly more frequent in HPV-positive than HPV-negative cases (Fig. 5), while p53 immunoexpression was present in the majority of HPV-negative specimens.

Discussion

This study is the largest reported to date assessing p53 expression and HPV infection in laryngeal carcinomas. Our analysis revealed that p53 nuclear phosphoprotein is overexpressed in a large proportion of laryngeal dysplasias and SSCs. In fact, 61% of the dysplasias and 77.5% of the carcinomas displayed increased nuclear levels of the protein. These results are slightly higher than those found by Maestro et al. [42] and Anwar et al. [2], who observed p53 positivity in 60% and 65% of primary

LSCCs respectively. Although no significant difference was found in p53 overexpression between the different grades of dysplasias and carcinomas ($P>0.1$), moderate or intense p53 expression (>50% of the cells positive) was statistically more frequent in the latter ($P<0.05$). These findings suggest that p53 alterations probably represent an early event in LSCC development, an observation also made by Dolcetti et al. [19]. These alterations seems to increase with its progression. Assuming that the immunohistochemical demonstration of p53, particularly by PAb 240, indicates the mutant protein [23], p53 mutations in early lesions suggest that direct damage of DNA may be important in LSCC progression because of the role of wild-type p53 in inducing G1-S arrest following DNA damage [38]. It appears that continuous DNA damage exerts selective pressure on p53 to mutate early, allowing clonal outgrowth and progression. Moreover, it has been shown that certain mutated versions of p53 acquire novel tumorigenic properties [27]. However, there are several studies which show inconsistencies between immunodetectable p53 protein and direct sequencing of the gene [68]. These findings suggest that PAb 240 positivity does not always equate with p53 mutation. In this case p53 would be represented by the wild-type p53 protein. Previously it has been shown that in rapidly proliferating cells the wild-type protein can be expressed at levels that are immunohistochemically detectable [44]. In addition to this, according to Milner's conformational hypothesis, PAb 240 can recognize the promoter form of the wild-type p53 which shares the conformation of the mutated p53 protein [47].

In our study, p53 detection was carried out with the use of two monoclonal antibodies: PAb 240, which is generally assumed to recognize the mutant form of p53 [23] and PAb 1801, which detects both wild and mutant p53 oncoprotein [3]. Wild-type p53 protein has a very short half-life and is thus present in minute amounts in normal tissues. It cannot, therefore, be detected by immunohistochemical methods. Mutations of the p53 tumour suppressor gene in the highly conserved domains and binding of p53 with certain cellular proteins extends the half-life of the protein from 6–20 min to many hours, resulting in its accumulation to levels detectable by immunohistochemistry [26, 28, 48].

We avoided a cell proliferation index study using antibodies directed against proliferating cell nuclear antigen (PCNA), since published data show that p53 protein has a regulatory effect on the PCNA gene [15, 45, 61].

In order to investigate the nature of immunohistochemically detected p53 protein, we proceeded in the p53 gene analysis of our specimens using the single strand conformation polymorphism technique. Preliminary results showed p53 mutations in 75% of LSCCs and dysplasias. Exon 5 was the most frequently mutated (unpublished data). The immunostaining pattern was identical with both antibodies with the exception of three lesions that were positive for PAb 240 and negative for PAb 1801. This discrepancy could be due either to overexpression of a truncated version of the protein [65], which would not be detected by PAb 1801, or to fixation procedures which would alter the configuration of the protein, masking the epitope recognized by PAb 1801.

Our investigation presents additional evidence of correlation between p53 expression in premalignant and malignant squamous proliferations of the larynx and lifetime cigarette consumption [21]. More specifically, p53 expression was higher in group A smokers (84.6%) than group B smokers [54%], and moderate or intense p53 staining (50% of the cells positive) was also significantly more frequent in group A (76%) than in group B (43%). It is well known that tobacco-associated mutagens form DNA adducts inducing G→T transversions and G→A transitions [29]. Such nucleotide substitutions have been found in the highly conserved region of the p53 gene in tobacco-associated tumours [29, 33]. Similarly, Maestro et al. [42] and Boyle et al. [7] showed that more than 50% of p53 mutations in LSCCs concerned G→T and G→A substitutions.

Next in this study, we examined possible HPV infection in our specimens, using the NISH technique and PCR analysis. The reason for using these two methods was that NISH has the ability to detect the virus at the single cell level, revealing its physical state (episomal or integrated), while PCR, due to its high sensitivity, can theoretically detect a single HPV copy against a background of 10^5 cells [69]. The NISH showed HPV 16 positivity in 4 of 40 and 3 of 40 grade II and grade III LSCCs respectively (total 7/40 or 18%) and PCR analysis revealed four more HPV-positive lesions, two HPV 16 and two HPV 18 (total HPV positivity 11/40 or 28%). There was no correlation between histological grades of the carcinomas and virus infection and, notably, no HPV DNA was detected in normal and dysplastic laryngeal specimens. NISH demonstrated a distinct punctate pattern, which is characteristic of viral DNA integration into host genome [12]. The HPV-specific mode of integration is important in permitting the persistent expression of specific viral genes [E6 and E7) which are necessary for the development of malignant growth [72].

To our knowledge, there are several reports which address the concept of HPV infection in laryngeal carcinomas (Table 1), but none has expanded HPV investigation in dysplastic laryngeal lesions. The frequency of HPV infection in LSCCs (28%) compared with dysplasias (0%) suggests a possible late role of HPV in laryngeal oncogenesis. Unlike in cervical cancer, where HPV-16 and HPV-18 have been preferentially associated with malignancy, our data and other findings so far reported do not reveal a specific HPV genotyping pattern in LSCCs [52]. Therefore, although the presence of HPV, especially the high risk-types 16 and 18, suggests a possible role in this malignancy, it is clear from the incidence of the infection that HPV may represent a contributory but not always necessary step in a multistep progression to laryngeal neoplasia.

The most important point to emerge from our investigation is the inverse relationship between HPV infection and p53 expression. Particularly, 55% of the HPV-positive carcinomas were p53-negative while 90% of the HPV-negative carcinomas gave a positive signal for p53 protein (Fig. 5).

It is possible that in a subset of LSCCs [HPV(+), p53(−)], HPV E6 oncoprotein complexes selectively with wild-type p53, stimulating its degradation via the ubiquitin-dependent protease system [54]. The degree to which normal p53 function is inhibited may depend in part upon the relative amounts of normal p53 and E6. Thus, it seems that in certain LSCCs, HPV infection produces an alternative, p53-altered "pathway" which contributes to malignant progression. A parallel situation exists, in certain HPV-positive cervical carcinoma cell lines and tumours, which had no mutations in the conserved domains of p53 gene [13], while p53 mutations were found where tumours were HPV-negative [54]. However, this kind of HPV/p53 relationship is not the rule, since other investigations have generally shown the presence of p53 gene alterations in HPV-positive tumours [2, 5, 22].

We also observed the simultaneous presence of p53 and HPV in 45% of the carcinomas. In this group, p53 probably represents the altered product of one allele which has lost the capacity to bind viral oncoproteins, while the remaining allele product would be degraded via the E6-ubiquitin pathway [54]. Additional possible explanations have been given by Anwar et al. [2]. Moreover Demers et al. [17] have shown increased p53 protein levels in HPV E7-immortalized epithelial cell lines. The suppressive effect of certain cellular interfering factors, such as transforming growth factor beta, on the viral long regulatory region which governs E6–E7 gene expression cannot be excluded [9, 72]. Such an effect, according to the Zur Hausen theory, would activate the intercellular surveillance system, preventing specific E6 and E7 viral transcription [71]. Finally, since laryngeal epithelium is hormone-dependent [58], hormonal factors may contribute to increased HPV production and enhanced genetic activity of the persisting viral DNA [24, 57]. The final result would depend on the shift of the equilibrium between the inhibitory and stimulatory factors.

In conclusion, our study provides evidence that p53 overexpression is an early and critical event in laryngeal oncogenesis and its altered expression is influenced by tobacco chemicals and HPV. Further investigation is underway to cast light on the molecular nature of p53 overexpression in LSCCs and the role of certain regulatory factors in its development.

References

1. Anwar K, Nakakuki K, Naiki H, Inuzuka M (1993) *ras* gene mutations and HPV infection are common in human laryngeal carcinoma. Int J Cancer 53:22–28
2. Anwar K, Nakakuki K, Imai H, Naiki H, Inuzuka M (1993) Over-expression of p53 protein in human laryngeal carcinoma. Int J Cancer 53:952–956
3. Banks L, Matlashewski G, Crawford L (1986) Isolation of human-p53-specific antibodies and their use in studies of human p53 expression. Eur J Biochem 159:529–534
4. Batsakis JG (1979) Tumors of the head and neck: clinical and pathological considerations, 2nd edn. Williams and Wilkins, Baltimore, p 574
5. Borresen AL, Helland A, Nesland J, Holm R, Trope C, Kaern J (1992) Papillomaviruses, p53 and cervical cancer. Lancet 339:1350–1351
6. Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, Zur Hausen H (1984) A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J 3:1151–1157
7. Boyle JO, Hakim J, Koch W, Riet P van der, Hruban RH, Roa RA, Correo R, Eby YJ, Ruppert JM, Sidransky D (1993) The incidence of p53 mutations increases with progression of head and neck cancer. Cancer Res 53:4477–4480
8. Brandsma JL, Steinberg BM, Abramson AL, Winkler B (1986) Presence of human papilloma virus type 16 related sequences in verrucous carcinoma of the larynx. Cancer Res 46:2185–2188
9. Braun L, Durst M, Mimuko R, Grupposo P (1990) Differential response of nontumorigenic and tumorigenic human papillomavirus type 16-positive epithelial cells to transforming growth factor β. Cancer Res 50:7324–7332
10. Cattoretti G, Pileri S, Parravicini C, Becker MHG, Poggi S, Bifulco C, Key G, D'amato L, Sabattini E, Feudale E, Reynolds F, Gerdes J, Rilke F (1993) Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. J Pathol 171:83–98
11. Cole ST, Streeck RE (1986) Genome organization and nucleotide sequence of human papillomavirus type 33, which is associated with cervical cancer. J Virol 58:991–995
12. Cooper K, Herrington CS, Graham AK, Evans MF, McGee JO'D (1991) In situ evidence for HPV 16, 18, 33 integration in cervical squamous cell cancer in Britain and South Africa. J Clin Pathol 44:406–409
13. Crook T, Wrede D, Tidy JA, Mason WP, Evans DJ, Vousden KH (1992) Clonal p53 mutation in primary cervical cancer: association with human-papillomavirus-negative tumours. Lancet 339:1070–1073
14. Dartmann K, Schwarz E, Gissmann L, Zur Hausen H (1989) The nucleotide sequence and genome organization of human papillomavirus 11. Virology 151:124–130
15. Deb S, Jackson CT, Subler MA, Martin DW (1992) Modulation of cellular and viral promoters by mutant human p53 proteins found in tumor cells. J Virol 66:6164–6170
16. Dekmezian RH, Batsakis JG, Goepfert H (1987) In situ hybridization of papillomavirus DNA in head and neck squamous cell carcinomas. Arch Otolaryngol Head Neck Surg 113:819–821
17. Demers GW, Halbert CL, Galloway DA (1994) Elevated wild-type p53 protein levels in human epithelial cell lines immortalized by the human papillomavirus type 16 E6 gene. Virology 198:169–174
18. De Villiers EM (1989) Heterogeneity of the human papillomavirus group. J Virol 63:4898–4903
19. Dolcetti R, Doglioni C, Maestro R, Gasparotto T, Barzan L, Pastore A, Romanelli M, Boiocchi M (1992) p53 overexpression is an early event in the development of human squamous cell carcinoma of the larynx; genetic and prognostic implications. Int J Cancer 52:178–182
20. Edmundson WF (1948) Microscopic grading of cancer and its practical implication. Arch Dermatol Syphilol 57:141–150
21. Field JK, Spandidos DA, Malliri A, Gosney JR, Yiagnisis M, Stell PM (1991) Elevated p53 expression correlates with a history of heavy smoking in squamous cell carcinoma of the head and neck. Br J Cancer 64:573–577

22. Fuzita M, Inoue M, Tanizawa O, Iwamoto S, Enomoto T (1992) Alterations of the p53 gene in human primary cervical carcinoma with and without human papillomavirus infection. Cancer Res 52:5223–5328
23. Gannon JV, Greaves R, Iggo R, Lane DP (1990) Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J 9:1595–1602
24. Gloss B, Bernard HV, Seedorf K, Klock G (1987) The upstream regulatory region of human papilloma virus 16 contains an E2 protein-independent enhancer with is specific for cervical cells and regulated by glucocorticoid hormones. EMBO J 6:3735–3743
25. Gorgoulis V, Giatromanolaki A, Karameris A, Tsatsanis C, Aninos D, Ozanne B, Velsemes M, Jordanoglou J, Trigidou R, Papastamatiou H, Spandidos DA (1993) Epidermal growth factor receptor expression in squamous cell lung carcinomas: an immunohistochemical and gene analysis in formalin-fixed, paraffin-embedded material. Virchows Arch [A] 423:295–302
26. Hainaut P, Milner J (1992) Interaction of heat-shock protein 70 with p53 translated in vitro: evidence for interaction with dimeric p53 and for a role in the regulation of p53 conformation. EMBO J 11:3513–3520
27. Halevy O, Michalowitz D, Oren M (1990) Different tumor-derived p53 mutants exhibit distinct biological activities. Science 250:113–116
28. Harris AL (1990) Mutant p53 – the commonest genetic abnormally in human cancer; (editorial) J Pathol 162:5–6
29. Harris CC (1993) p53: At the crossroads of molecular carcinogenesis and risk assessment. Science 262:1980–1981
30. Hellquist H, Lundgren J, Olofsson J (1982) Hyperplasia, keratosis, dysplasia and carcinoma in situ of the vocal cords: a follow-up study. Clin Otolaryngol 7:11–27
31. Herrington CS, Burns J, Graham AK, Bhatt B, McGee JO'D (1989) Interphase cytogenetic using biotin and digoxigenin labelled probes. I. Relative sensitivity of both reporter molecules for the detection of HPV 16 in CaSki cells. J Clin Pathol 42:591–600
32. Hollstein MC, Metcalf RA, Welsh A, Montesano R, Harris CC (1990) Frequent mutation of the p53 gene in human esophageal cancer. Proc Natl Acad Sci USA 887:9958–9961
33. Hollstein MC, Peri L, Mandard AM, Wellsh JA, Montesano R, Metcalf RA, Bak M, Harris CC (1991) Genetic analysis of human esophageal tumors from two high incidence geographic areas: frequent p53 base substitutions and absence of *ras* mutations. CancerRes 51:4102–4106
34. Hong W, Yu-chun L, Xiu-shui K, Gang-zhong L, Jie L, Baoyin L, Li-juan Q, Ke-ring M (1991) Detection of human papillomarivus (HPV) in laryngeal carcinoma tissue. Chin Med J 104:523–525
35. Hoshikawa T, Nakajima T, Uhara H, Gotoh M, Shimosato Y, Tsutsumi K, Ono I, Ebihara S (1990) Detection of human papillomavirus DNA in laryngeal squamous cell carcinomas by polymerase chain reaction. Laryngoscope 100:450–647
36. Iggo R, Gatter K, Bartek J, Lane D, Harris AL (1990) Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet 335:675–679
37. Kahn T, Schwarz E, Zur Hausen H (1986) Molecular cloning and characterization of the DNA of a new human papillomavirus (HPV 30) from a laryngeal carcinoma. Int J Cancer 37:61–65
38. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig-RW (1991) Participation of p53 protein in the cellular response to DNA damage. Cancer Res 51:6304–6311
39. Levin AJ, Momand J, Finlay CA (1991) The p53 tumour suppressor gene. Nature 351:453–456
40. Lorincz AT, Lancaster WD, Temple GF (1986) Cloning and characterization of the DNA of a new human papillomavirus from a woman with dysplasia of the uterine cervix. J Virol 58:225–229
41. Lorincz AT, Quinn AP, Lancaster WD, Temple GP (1987) A new papillomavirus associated with cancer of the uterine cervix. Virology 159:187–190
42. Maestro R, Dolcetti R, Gasparotto D, Doglioni C, Pelucchi S, Barzan L, Grandi E, Boiocchi M (1992) High frequency of p53 gene alterations associated with protein overexpression in human squamous cell carcinoma of the larynx. Oncogene 7:1159–1166
43. McCoy DG, Wynder EL (1979) Etiological and preventive implications in alcohol carcinogenesis. Cancer Res 39:2844–2850
44. Mercer WE, Baserga R (1985) Expression of the p53 protein during the cell cycle of human peripheral blood lymphocytes. Exp Cell Res 160:31–46
45. Mercer WE, Shields MT, Lin D, Appela E, Ullrich SJ (1991) Growth suppression induced by wild-type p53 protein is accompanied by selective down-regulation of proliferating cell nuclear antigen expression. Proc Natl Acad Sci USA 88:1958–1962
46. Michaels L (1984) Pathology of the larynx, chapter 34. Squamous cell carcinoma: epidemilogy, early lesions, biopsy diagnosis. Springer, Berlin Heidelberg NewYork, pp 199–204
47. Milner J (1991) A conformational hypothesis for the suppressor and promoter functions of p53 in cell growth control and in cancer. Proc R Soc Lond [B] 245:139–145
48. Momand J, Zambetti GP, Olson DC, George D, Levine AJ (1992) The mdm-2 oncogene product forms a complex with p53 protein and inhibits p53-mediated transactivation. Cell 69: 1237–1245
49. Morgan DW, Abdullah V, Quiney R, Myint S (1991) Human papilloma virus and carcinoma of the laryngopharynx. J Laryngol Otol 105:288–290
50. Mounts P, Shah K, Kashima H (1982) Viral etiology of juvenile- and adult-onset squamous papilloma of the larynx. Proc Natl Acad Sci USA 79:5425–5429
51. Perez-Ayala M, Ruiz-Cabello F, Esteban F, Concha A, Redondo M, Oliva MR, Cabrera T, Carrido F (1990) Presence of HPV 16 sequences in laryngeal carcinomas. Int J Cancer 46:8–11
52. Pfister H (1987) Human papillomaviruses and genital cancer. Adv Cancer Res 48:113–117
53. Rodrigues NR, Rowan A, Smith M et al. (1990) p53 mutations in colorectal cancer. Proc Natl Acad Sci USA 87:7555–7559
54. Scheffner M, Werness BA, Huibregste SM, Levine AJ, Howley PM (1990) The E6 oncoprotein encoded by HPV types 16 and 18 promotes the degradation of p53. Cell 63:1129–1136
55. Scheurlen W, Stremlau A, Gissmann L, Hohn D, Zenner H-P, Zur Hausen H (1986) Rearranged HPV 16 molecules in an anal and in a laryngeal carcinoma. Int J Cancer 38:671–676
56. Schmidt W, Popham RE (1981) The role of drinking and smoking in mortality from cancer and other causes in male alcoholics. Cancer 47:1030-1041
57. Schneider A, Hotz M, Gissman L (1987) Increased prevalence of human papillomaviruses in the lower genital tract of pregnant women. Int J Cancer 40:198–201
58. Schuller DE, Abou-Issa H, Parrish R (1984) Estrogen and progesterone receptors in head and neck cancer. Arch Otolaryngol 110:725–727
59. Schwarz E, Durst M, Demankowski C et al. (1983) DNA sequence and genome organization of genital human papillomavirus type 6b. EMBO J 2:2341–2348
60. Seedorf K, Krammer G, Durst M, Suhai S, Rowekamp WG (1985) Human papillomavirus type 16 DNA sequence. Virology 145:181–185
61. Suler MA, Martin DW, Deb S (1992) Inhibition of viral and cellular promoters by human wild-type p53. J Virol 66:4757–4762
62. Syrjanen S, Syrajanen K, Mantyjarvi R, et al. (1987) Human papillomavirus DNA in squamous cell carcinomas of larynx demonstrated by in situ hybridization. ORL J Otorhinolaryngol Relat Spec 49:175–186

63. Tsutsumi K, Nakajima T, Gotoh M, et al. (1989) In situ hybridization and immunohistochemical study of human papillomavirus infection in adult laryngeal papillomas. Laryngoscope 99:80–85
64. Tuyns AJ (1991) Alcohol and cancer. An instructive association. Br J Cancer 64:415–416
65. Varley GM, Brammar W, Lane DP, Swallow JE, Dolan C, Walker RA (1991) Loss of chromosome 17p13 sequences and mutaion of p53 in human breast carcinomas. Oncogene 6:413–421
66. Watts S, Brewer EE, Fry TL (1991) HPV DNA in head and neck carcinomas. Oral Surg Oral Med Oral Pathol 71:701–707
67. Wynder EL, Stellman SD (1977) Comparative epidemiology of tobacco-related cancers. Cancer Res 37:4608–4622
68. Wynford-Thomas D (1992) p53 in tumour pathology: can we trust immunochemistry? (editorial) J Pathol 166:329–330
69. Young LS, Bevan IS, Johnson MA, Blomfield PI, Maitland NJ, Woodman CBJ (1989). The polymerase chain reaction: a new epidemiological tool for investigating cervical human papillomavirus infection. BMJ 298:14–18
70. Zambetti GP, Levine AJ (1993) A comparison of the biological activities of wild-type and mutant p53. FASEB J 7:855–865
71. Zur Hausen H (1989) Papillomaviruses in anogenital cancer as a model to understand the role of viruses in human cancers. Cancer Res 49:4677–4681
72. Zur Hausen H (1991) Human papillomaviruses in the pathogenesis of anogenital cancer. Virology 184:9–13