Expression of p53, PCNA, Ki-67 and bcl-2 in relation to risk factors in oral cancer - a molecular epidemiological study

E.B. SCHILDT[1,2], K. NYLANDER[3], M. ERIKSSON[1,2], L. HARDELL[4], A. MAGNUSSON[5] and G. ROOS[3]

[1]Department of Oncology, University Hospital, S-221 85 Lund; Departments of [2]Oncology, [3]Medical Biosciences/Pathology, Umeå University, S-901 87 Umeå; [4]Department of Oncology, Örebro Medical Center, S-701 85 Örebro; [5]Department of Occupational and Environmental Medicine, University Hospital, S-581 85 Linköping, Sweden

Received November 18, 2002; Accepted December 23, 2002

Abstract. A group of 133 primary oral squamous cell carcinomas were studied concerning a relationship between exposure factors and tumour biological parameters with a focus on the *TP53* gene and p53 protein status. Tumours were evaluated using immunohistochemistry (IHC) for expression of p53, PCNA, Ki-67 and bcl-2 proteins. The *TP53* gene was studied for mutations using PCR amplification of exons 5-9 and single strand conformation polymorphism (SSCP) analysis. The collected data were correlated to the exposure factors smoking, oral snuff, liquor, oral infections, dental factors, dental X-ray and iron deficiency. When compared with matched controls only oral infections, and reported HSV-infections in particular, gave statistically significant ORs (odds ratio) for all tumours (OR 8.0) as well as for the group of IHC p53 positive tumours (OR 12). No association between smoking and p53 positivity was found (OR 1.0).

Introduction

Tobacco and alcohol are regarded as the two most important risk factors for oral cancer worldwide. The incidence and mortality of this disease is increasing in most West European countries (1). This is also true in Sweden where the incidence is slowly increasing (2,3). In Sweden the etiological role of oral snuff has been much discussed and we performed a case-control study to elucidate a possible association between oral moist snuff as well as other potential risk factors and oral cancer (4). Neither our study nor another recent Swedish epidemiological study (5) could, however, verify any association between oral snuff and oral cancer. Regarding smoking tobacco, our study found a much less pronounced association with oral cancer compared to most other studies in this field.

Molecular epidemiology is a bridge between molecular carcinogenesis and cancer risk assessment. The finding of biological markers associated with risk factors for different tumour diseases will hopefully both be helpful in the interpretation of data from conventional epidemiological studies and tell us more about the carcinogenic process. Most studies in the field of molecular cancer epidemiology have used p53 as a biological marker, since mutations in this gene have been demonstrated in about half of the cases of human cancers studied, and this mutation is thought to stimulate carcinogenesis in different ways (6,7). Squamous cell carcinoma of the head and neck (SSCHN) has been increasingly studied with regard to expression of p53, both in relation to clinicopathological parameters (8-11), patient survival (9,12), and exposure to tobacco and alcohol (9,12-15), but rarely with regard to other potential exposure factors.

Furthermore, numerous studies have investigated the pattern of specific mutations in SCCHN (16). Mutation in the *TP53* gene is the most common genetic alteration in SCCHN (17), and overexpression of the p53 protein has also been demonstrated in 34-80% of the tumours in different studies (13,18-22). The normal p53 protein functions as 'guardian of the genome' with the ability to stop cells with a DNA-damage in late G1 phase (23). Wild-type p53 protein can also induce apoptosis, a process that can be blocked by the bcl-2 protein by diversion of p53 activity from induction of apoptosis to induction of growth arrest (24). The bcl-2 protein, a product of the bcl-2 oncogene on chromosome 18, is a key factor to hamper cell loss by apoptosis and can inhibit most types of apoptotic cell death. The protein can be found in lymphomas bearing the 14;18 translocation as well as in certain normal tissues.

A mutation in the *TP53* gene leads to an increase in protein half-life facilitating immunohistochemical detection, but an increase in half-life can also be achieved by binding of wild-type p53 protein to other proteins or by disturbance in the degradation pathway (25). In our previous studies of SSCHN we have not found any relationship between either p53 expression or *TP53* mutation and cell proliferation judged by *in vivo* incorporation of IdUrd (22,26), and no strict correlation between IHC detected p53 protein and mutation in the *TP53* gene (26,27). It should, however, be

Correspondence to: Dr Mikael Eriksson, Department of Oncology, University Hospital, S-221 85 Lund, Sweden
E-mail: mikael.eriksson@onk.lu.se

Key words: oral cancer, TP53 gene, PCNA, Ki-67, oral infection, molecular epidemiology

kept in mind that in studies using immunohistochemistry on paraffin-embedded samples no distinction between wild-type and mutant protein can be made.

Occurrence of *TP53* mutations seems to be dependent on epidemiological factors in the population, e.g., geographic origin in hepatocellular carcinoma has been shown to affect the mutational spectrum of the *TP53* gene (6). The mutational spectrum in SCCHN is known to vary between different countries and races and may in this tumour type also be influenced by exogenous carcinogens such as mutagens in cigarette smoke (17,28-30). As for a potential association between *TP53* gene status and dental health, including factors like infections and oral hygiene, nothing is so far known.

The proliferating cell nuclear antigen (PCNA) exists in two forms in the cell. One bound to the sites of DNA replication and another soluble in the nucleoplasm. Formaldehyde fixation allows detection of both forms, consequently PCNA labelling in formaldehyde fixed tissue is not S-phase specific. PCNA has a dual function in that it is involved in DNA replication as well as DNA repair. Its role in DNA replication is as a processivity factor for the enzyme DNA polymerase δ which is responsible for replication of chromosomal DNA. In *in vitro* repair assays PCNA seems to be involved in DNA excision and mismatch repair processes (31). Wild-type p53 protein can indirectly and selectively inhibit the activity of PCNA in DNA replication, whereas PCNA function in nucleotide excision-repair is unaffected (32).

The Ki-67 protein is a non-histone protein present in G1, S, G2 and M-phases of the cell cycle but absent in G0 cells (33,34). Expression of Ki-67 thus provides information whether cells are in the cell cycle or not, whereas no information regarding the length of the cell cycle or whether cells are actively cycling is achieved. Several studies have shown a good correlation between Ki-67 immunoreactivity and indices of cell proliferation, whereas variable results concerning its prognostic impact have been presented.

In a previous study we analysed the prognostic significance of p53, PCNA, Ki-67 and bcl-2. None of these cell proliferation and apoptosis associated parameters showed any clear influence on prognosis (35). In the present study we analysed exposure data from an extended group of tumours in correlation to these proteins as well as mutation analysis of TP53 on a subset of cases. Specific interest was also focused on infections, particulary of herpes simplex virus (HSV)-type.

Materials and methods

Material and exposure data. The exposure data in this study were derived from a subset of a population-based case-control study including all histopathologically verified squamous cell oral cancer cases (ICD-7 codes 140 = lip, 141 = tounge, 143 = floor of mouth, 144 = gingiva, 145 = tonsill/mesopharynx) diagnosed in the four most northern counties in Sweden during 1980-1989 and reported to the Cancer Registry. The material was thus limited to tumours located in the oral cavity and the lip.

The case-control study comprised 410 cases and the same number of controls and the main results from this study have been published earlier (4,36). Controls were drawn from the National Population Registry. The matching criteria were age, sex and county, and deceased controls were matched on year of death. For methodological details, see original study (4). To obtain exposure information a questionnaire was sent to the study subjects, and if necessary, the answers given were completed over the phone by a specially trained interviewer. The questions concerned among other things smoking, use of moist snuff, and alcohol exposure. An ex-smoker or ex-snuff user was defined as a person having quit the habit at least one year before the diagnosis; for controls the corresponding year was the year of diagnosis for the respective case. Alcohol use was classified as admitting liquor consumption but the exposure was in this study not divided according to quantity because of the rather small groups to analyse. The subjects were also asked if they had had infections in the oral cavity or on the lips, and if so, they were asked to describe the localization and type of infection, and whether the infection was transitory or of chronic or recurrent character. If necessary, the interviewer completed the description regarding e.g., occurrence of vesicles. However, it must be stressed that no serological tests or other investigations were performed to verify type of infection. Thus, exposure assessments regarding infections were, as for all other exposures, based on information given by the subjects themselves or their relatives.

The most common type of infection seemed to be caused by recurrent HSV, with a history given more or less convincing of this diagnosis. Subjects who stated 'herpes infection' or who described recurrent infections with groups of vesicles were classified as having 'certain HSV infections', whereas subjects who described single infections with vesicles or recurrent infections of unspecified type were classified as 'possible HSV infections'. Infections related to oral cancer were disregarded. Details regarding other exposure factors have been published elsewhere (36).

The present investigation was performed on a subset of cases from the case-control study, i.e., all 133 primary oral squamous cell carcinomas diagnosed at the Department of Pathology in Umeå, Sweden, with representative formalin-fixed and paraffin-embedded samples.

Antibodies and staining. Five μm sections were cut from each block and left at room temperature overnight. For detection of PCNA, Ki-67, p53 and bcl-2 respectively, the following antibodies were used: PC10, a mouse monoclonal antibody (Novacastra Laboratories Ltd., Newcastle, UK), recognising an epitope within aa 111-125 of the PCNA protein (37); MIB-1, a monoclonal antibody against Ki-67 (BioGenex, San Ramon, CA, USA) (38); DO7, a monoclonal antibody (Novacastra) recognising a denaturation resistant epitope between amino acids 1 and 45 in wild-type as well as mutant p53 protein (39); and M0887, a monoclonal antibody (Dakopatts A/S, Denmark) against bcl-2. The PCNA and p53 antibodies were diluted 1:50, the Ki-67 antibody 1:25 and the bcl-2 antibody 1:70. As secondary antibody for PCNA, p53 and bcl-2 an alkaline phosphatase conjugated rabbit anti-mouse antibody (D 314; Dakopatts) was used, and for detection of the Ki-67 antibody a Super Sensitive Multilink Kit (BioGenex) was applied. For visualisation of the staining

reactions fast red (Sigma Chemicals Co., St. Louis, MO, USA) was used. Preincubation of slides in methanol was performed before staining with PC10 antibody, and when staining with the Ki-67 and bcl-2 antibodies slides were pretreated in 10 mM citrate-buffer pH 6.0 using a microwave oven at full effect (900 W) for 2.5 min and at 350 W for 9 min. Slides were left to cool for 20-30 min and then rinsed in distilled water. Incubation with the primary antibodies was carried out at 4°C overnight. After rinsing in Tris-buffer slides were incubated with the secondary antibody at room temperature for 45 min, and with the Multi Link Kit according to the supplier's recommendation. Sections of oral squamous cell carcinoma known to express p53 protein were used as positive controls. Negative controls were prepared as described above but the primary antibody was exchanged for Tris-buffer.

Immunohistochemical evaluation. For PCNA and Ki-67 stained slides a labelling index (LI) was calculated as earlier described in detail (40). In brief a 10x10 square grid comprising 121 cross points was fitted into the eyepiece of the microscope using an objective lens of x40, and cells showing a distinct nuclear staining were counted in 5-10 randomly chosen fields, in most cases covering the whole tumour. The average LI was calculated as the percentage of positively stained nuclei falling in the crossing between two lines of the grid. Only areas with epithelial tumour tissue were examined, and a good concordance was found between all tumour cells inside the grid and those falling in the cross points. The immunohistochemical evaluation and calculation of LI was performed by one of the authors (K. Nylander). Control calculations of LI showed an intra-observer variation that was not statistically significant. For p53 and bcl-2, tumours were graded positive or negative, where tumours with only occasional p53 positive cells were considered negative. For bcl-2 the group of positive tumours was so small, that a proper evaluation of different LIs was not considered relevant.

PCR and SSCP (single strand conformation polymorphism) analysis. DNA was extracted from the paraffin blocks, and PCR/SSCP analysis of exons 5-9 of the *TP53* gene performed the same way as earlier described in detail (26). The results were then correlated to the exposure factors smoking, oral snuff, liquor, oral infections, dental factors, dental X-ray, and iron supplement.

Statistical methods. In the univariate analysis conditional logistic regression was used. The calculations were performed using the EGRET program (Epidemiological Graphics Estimation and Testing package, SERC, Seattle, USA). The variables were expressed in categoric forms and the results are presented as the odds ratio (OR) and 95% confidence interval (CI).

Regarding the IHC and PCR/SSCP analyses of p53, five different ORs were calculated for every exposure factor: all cases, p53 IHC positive, p53 IHC negative, TP53 mutation positive, and TP53 mutation negative cases respectively.

Regarding PCNA the subjects were subdivided into three equally sized groups according to the labelling index (LI) of this proliferation marker: PCNA (group 1): 14-47%, PCNA (group 2): 48-65%, PCNA (group 3): 66-93%. As a consequence, regarding PCNA, four ORs were calculated for every exposure factor; one including all matched pairs in the PCNA analysis (n=106), and one for each category as stated above. As for PCNA, a subdivision into three equally sized groups was also done for the LI of the proliferation marker Ki-67: Ki-67 (group 1): 11-24%, Ki-67 (group 2): 25-38%, Ki-67 (group 3): 39-60%.

Results

Out of the 133 matched pairs of cases and controls 19 were excluded due to lack of exposure data, i.e., at least one of the subjects within a pair refused to participate and answer the questions about exposure history. Thus, the analyses were based on 114 cases and their matched controls. In several subjects, however, there were one or more missing values, i.e., no information was given on a certain exposure factor.

In the following, results of exposure factors of specific interest are presented. Several factors in relation to dental health have been analysed, but none of these were associated with any of the biological parameters studied, and therfore results on these factors are not shown.

p53 analysis. Regarding p53 analysis two different methods were used (IHC and PCR/SSCP) each including 114 cases. 72 out of 114 tumours (63%) were IHC positive, and 41 out of 114 tumours (36%) had mutations within exons 5-9. Results from the IHC analysis regarding selected exposure factors are presented in Table I demonstrating that infections, and HSV-infections as defined above in particular, gave statistically significant ORs for the whole group of tumours as well as for p53 positive tumours only. Results from the PCR analysis, Table II, showed statistically significant OR for HSV-infections in non-mutated tumours. However, no clear difference was detected regarding exposure pattern for p53 positive versus p53 negative cases with either method.

Since there was a discordance between p53 protein expression and presence of mutation in the *TP53* gene, we also compared the exposure patterns between subjects with any sign of p53 aberration, i.e. IHC and/or PCR positivity, and subjects lacking both p53 expression and mutation, Table III. In this analysis infections and specifically HSV infections gave statistically significant ORs for the group of tumours with p53 aberration shown by IHC and/or PCR. Only very few subjects with exposure to oral infection were, however, negative with both methods.

The most common single exon mutated was exon 8, in total 34 cases. We also made an analysis restricted to these cases and their controls. Because of the rather small numbers in different exposure categories analysed only few ORs could be calculated. In these cases ORs were not significantly different (data not shown) from the total mutation analysis as presented in Tables I-III.

PCNA analysis. Due to technical problems only 106 cases were included in the analysis of PCNA expression. Oral infection turned out to be a risk factor for cases with cancers

Table I. Odds ratios (OR) and 95% confidence interval (CI) for different exposure factors in relation to p53 expression [immunohistochemical (IHC) analysis].[a]

	All tumours			p53-positive tumours (IHC)			p53-negative tumours (IHC)		
Exposure factor	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co
Smoking									
Active smoker[b]	1.3	(0.5-2.8)	33/27	1.0	(0.3-2.7)	17/15	1.8	(0.4-6.6)	16/12
Ex-smoker[b]	0.8	(0.3-1.8)	26/32	0.7	(0.3-1.9)	16/20	1.0	(0.2-4.0)	10/12
Snuff user									
Active snuff user[b]	0.5	(0.2-1.3)	12/20	0.7	(0.2-1.9)	9/12	0.3	(0.0-1.4)	3/8
Ex-snuff user[b]	1.2	(0.4-3.6)	8/6	0.9	(0.2-3.3)	5/5	3.0	(0.3-29)	3/1
Alcohol	1.6	(0.8-3.0)	66/55	1.4	(0.6-2.9)	42/37	2.2	(0.7-6.4)	24/18
Infections									
Total	5.2	(1.8-16)	23/6	8.0	(1.8-35)	17/3	2.5	(0.4-13)	6/3
HSV-1 certain	3.5	(0.7-17)	7/3	5.0	(0.5-43)	5/2	2.0	(0.1-23)	2/1
HSV-1 certain + probable	8.0	(1.8-35)	17/3	12	(1.5-93)	13/2	4.0	(0.4-36)	4/1
Infections NUD	2.5	(0.4-13)	6/3	4.0	(0.4-36)	4/1	1.0	(0.0-16)	2/2
Iron supplement	0.4	(0.2-1.0)	15/26	0.4	(0.1-1.0)	9/18	0.7	(0.1-2.4)	6/8

[a]Univariate analysis. Ca, cases; Co, controls; [b]never smokers/never snuff users used as reference. ORs are calculated only on the complete matched pairs, not on the total number of cases and controls given in the table.

Table II. Odds ratios (OR) and 95% confidence interval (CI) for different exposure factors in relation to TP53 mutation (PCR/SSCP analysis), all localizations (ICD 140, 141, 143-145).[a]

	All tumours			TP53 mutation			No TP53 mutation		
Exposure factor	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co
Smoking									
Active smoker[c]	1.3	(0.5-2.8)	33/27	1.0	(0.2-3.5)	9/8	1.5	(0.5-3.9)	24/19
Ex-smoker[c]	0.8	(0.3-1.8)	26/32	0.6	(0.1-2.9)	6/8	0.9	(0.3-2.2)	20/24
Snuff user									
Active snuff user[c]	0.5	(0.2-1.3)	12/20	[b]		2/7	0.7	(0.2-1.9)	10/13
Ex-snuff user[c]	1.2	(0.4-3.6)	8/6	[b]		2/0	0.9	(0.3-3.0)	6/6
Alcohol	1.6	(0.8-3.0)	66/55	1.9	(0.7-4.7)	22/16	1.5	(0.6-3.2)	44/39
Infections									
Total	5.2	(1.8-16)	23/6	3.5	(0.7-17)	8/3	7.0	(1.5-31)	15/3
HSV-1 certain	3.5	(0.7-17)	7/3	2.0	(0.1-23)	2/1	5.0	(0.5-43)	5/2
HSV-1 certain + probable	8.0	(1.8-35)	17/3	5.0	(0.5-43)	5/1	11	(1.4-86)	12/2
Infections NUD	2.5	(0.4-13)	6/3	2.0	(0.1-23)	3/2	3.0	(0.3-29)	3/1
Iron supplement	0.4	(0.2-1.0)	15/26	0.4	(0.1-1.5)	8/13	0.5	(0.1-1.4)	7/13

[a]Univariate analysis. Ca, cases; Co, controls; [b]missing value; [c]never smokers/never snuff users used as reference. ORs are calculated only on the complete matched pairs, not on the total number of cases and controls given in the table.

Table III. Odds ratios (OR) and 95% confidence interval (CI) for different exposure factors in relation to positive mutation (PCR/SSCP analysis) and/or positive staining IHC, and negative mutation and negative staining for p53, all localizations (ICD 140, 141, 143-145).[a]

	Any p53 aberration (IHC and/or PCR)			No p53 aberration (IHC or PCR)		
Exposure factor	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co
Smoking						
Active smoker[c]	1.1	(0.4-2.7)	23/19	2.0	(0.3-11)	10/8
Ex-smoker[c]	0.7	(0.2-1.7)	20/26	1.4	(0.2-7.3)	6/6
Snuff user						
Active snuff user[c]	0.5	(0.2-1.4)	8/14	0.5	(0.0-2.8)	4/6
Ex-snuff user[c]	1.1	(0.3-3.6)	6/5	2.0	(0.1-23)	2/1
Alcohol	1.6	(0.8-3.1)	53/44	1.7	(0.3-7.0)	13/11
Infections						
Total	9.5	(2.2-41)	21/4	1.0	(0.1-7.1)	2/2
HSV-1 certain	6.0	(0.7-50)	6/2	1.0	(0.0-16)	1/1
HSV-1 certain + probable	14	(1.8-107)	15/2	2.0	(0.1-23)	2/1
Infections NUD	5.0	(0.5-43)	6/2	[b]		0/1
Iron supplement	0.4	(0.1-1.0)	12/22	0.7	(0.1-4.0)	3/4

[a]Univariate analysis. Ca, cases; Co, controls; [b]missing value; [c]never smokers/never snuff users used as reference. ORs are calculated only on the complete matched pairs, not on the total number of cases and controls given in the table.

Table IV. Odds ratios (OR) and 95% confidence interval (CI) for different exposure factors in relation to expression of PCNA.[a]

	PCNA group 1-3			PCNA group 1			PCNA group 2			PCNA group 3		
Exposure factor	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co
Smoking												
Active smoker[c]	1.3	(0.6-3.0)	32/26	3.2	(0.8-13)	17/10	1.7	(0.4-6.7)	11/8	0.1	(0.0-1.5)	4/8
Ex-smoker[c]	0.9	(0.4-1.9)	24/29	1.6	(0.4-5.9)	12/14	0.9	(0.3-2.9)	10/12	0.3	(0.0-2.8)	2/3
Snuff user												
Active snuff user[c]	0.6	(0.2-1.5)	11/17	0.5	(0.1-1.7)	5/9	0.5	(0.1-1.9)	4/7	[b]		
Ex-snuff user[c]	1.3	(0.4-4.2)	7/5	4.0	(0.4-36)	4/1	0.6	(0.1-3.0)	3/4	[b]		
Alcohol	1.6	(0.9-3.1)	61/50	2.2	(0.8-5.7)	28/21	2.5	(0.7-8.0)	21/15	0.7	(0.2-2.3)	12/14
Infections	5.0	(1.7-15)	22/6	7.0	(0.8-57)	7/1	9.0	(1.1-72)	10/2	2.0	(0.3-11)	5/3
Iron supplement	0.4	(0.1-1.0)	14/25	0.9	(0.3-2.5)	8/9	0.2	(0.0-1.8)	4/8	[b]		

[a]Univariate analysis. Group 1, labelling index (LI) 14-47%; group 2, LI 48-65%; group 3, LI 66-93%. Ca, cases; Co, controls; [b]missing value; [c]never smokers/never snuff users used as reference. ORs are calculated only on the complete matched pairs, not on the total number of cases and controls given in the table.

belonging to PCNA group 1 and 2, as shown in Table IV. Regarding smoking, it was observed that ORs decreased with increasing PCNA category, both for active smokers and ex-smokers.

Ki-67 analysis. In this IHC analysis 111 cases could be used. Oral infections were significantly associated only in the combined analysis, not in the subgroups, as demonstrated in Table V. Notably, there was rather an inverse trend regarding

Table V. Odds ratios (OR) and 95% confidence interval (CI) for different exposure factors in relation to expression of Ki-67.[a]

	Ki-67 group 1-3			Ki-67 group 1			Ki-67 group 2			Ki-67 group 3		
Exposure factor	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co	OR	(CI)	Ca/Co
Smoking												
Active smoker[c]	1.2	(0.5-2.7)	33/27	1.1	(0.2-4.5)	13/9	1.1	(0.3-3.4)	14/12	1.4	(0.1-13)	6/6
Ex-smoker[c]	0.7	(0.3-1.7)	25/32	0.4	(0.0-2.2)	8/13	0.7	(0.2-2.1)	10/13	1.5	(0.2-9.4)	7/6
Snuff user												
Active snuff user[c]	0.5	(0.2-1.3)	12/20	0.5	(0.0-2.8)	4/6	0.3	(0.0-1.2)	4/11	1.3	(0.2-6.2)	4/3
Ex-snuff user[c]	1.2	(0.4-3.6)	8/6	2.0	(0.1-23)	2/1	1.2	(0.2-5.3)	4/3	1.1	(0.1-8.0)	2/2
Alcohol	1.5	(0.8-2.8)	64/55	1.7	(0.6-4.4)	24/19	1.2	(0.5-3.0)	24/22	2.0	(0.3-11)	16/14
Infections	5.2	(1.8-16)	23/6	[b]			3.5	(0.7-17)	9/4	3.5	(0.7-17)	7/2
Iron supplement	0.4	(0.1-1.1)	14/24	0.4	(0.1-1.7)	6/10	0.5	(0.1-2.0)	3/6	0.4	(0.0-2.1)	5/8

[a]Univariate analysis. Group 1, labelling index (LI) 11-24%; group 2, LI 25-38%; group 3, LI 39-60%. Ca, cases; Co, controls; [b]missing value; [c]never smokers/never snuff users used as reference. ORs are calculated only on the complete matched pairs, not on the total number of cases and controls given in the table.

smoking between the subgroups as compared with PCNA, with increasing OR with increasing Ki-67 category.

bcl-2 analysis. Only 9 cases expressed bcl-2 and this group was therefore not further analysed.

Since cancer of the lip might have a different etiological background, e.g., exposure to UV-light, a separate analysis was performed. The material was divided into two groups, lip carcinoma and other oral cancer, but the risk patterns were shown to be quite comparable.

Discussion

In the present study we have used data from a case-control study on different exposure factors and oral cancer with the purpose to elucidate any probable connection between expression of p53 and different exposure factors, not only the generally studied smoking and alcohol habits but also reported oral infections, dental factors and iron deficiency. One factor of specific interest is the use of oral snuff, which is a common habit in Sweden. We also investigated the relation between the same exposure factors and PCNA, Ki-67 and bcl-2, factors not earlier studied in this respect.

For studying p53 we applied two techniques, IHC and mutation analysis. We found 63% of the tumours to express p53 protein whereas 36% showed mutations. To explain this discrepancy one must remember that the antibody applicable on paraffin-embedded material cannot distinguish between wild-type and mutant protein. p53 positivity in a tumour could therefore be explained in 3 ways: i) the *TP53* gene was mutated but the mutation was located outside the exons studied here (exons 5-9), and accordingly the PCR/SSCP result was negative. The vast majority of p53 mutations in SCCHN are, however, found within exons 5-8 (6); ii) a defect in the degradation pathway increased the half-life for p53; iii) p53 was bound to another protein and retained in the tissue, but in an inactive state.

This study indicates that oral infection increased the risk for all tumours and for the group of IHC p53 positive tumours. The main part of the infections responsible for this association seemed to be HSV-infections judged by the patients/relatives description of the infection. Even if the clinical picture of HSV-infection is very typical and differential diagnoses less likely, it is important to point out that no serological verification was done. While oral infection clearly is a strong risk factor in this study, HSV must be regarded as the most probable candidate infection.

No clear evidence for herpes simplex virus (HSV) as a carcinogenic factor in head and neck carcinomas has been found but data so far available indicate a positive correlation between HSV and human oral cancer (41). Four herpes viruses are known to infect man and one of them, the herpes simplex virus (HSV), has in studies of the oral cavity in mouse and hamster shown co-carcinogenicity with viral inoculation significantly enhancing the oncogenic capacity of chemical carcinogens. HSV has also shown ability to promote the inactivation of p53 (42).

The fact that the p53 protein can bind to other proteins could also explain the statistically significant OR found for HSV-infections in patients with p53 positive tumours, indicating that p53 could in some way interact with the HSV viral protein. It has been shown in *in vitro* studies that HSV can redistribute p53 into its replication compartments (43).

Smoking is regarded as the most important risk factor for oral cancer, but the ORs in our study (4) are not in the same magnitude as in some other reports, mainly from North America and southern Europe. Furthermore, in this molecular

epidemiologic study based on a sample of 114 cases from the whole investigation, we did not see any significantly increased OR for smoking. Regarding correlation to p53 expression it seems that smoking rather was inversely related with an OR of 1.8 and 1.5 for patients with p53 negative tumours using IHC and PCR technique, respectively, compared with 1.0 for patients with p53 positive tumours. This is in accordance with a British-Dutch study on lingual carcinoma (14), but in contrast to several other studies finding a correlation between smoking and p53 expression for head and neck cancer (12,13,15). An earlier Swedish study did not find either any correlation to smoking (9). Concerning PCNA we found decreasing ORs for smoking with increasing LI, whereas the opposite was found in the analysis of Ki-67. Accordingly there does not seem to be any correlation between the expression of these two factors among the tumours in the present study.

The use of oral snuff did not enhance the risk for oral cancer in our case-control study (4). There was in this subgroup no clear pattern in relation to the biological markers. However, the snuff users were very few in this subgroup.

Liquor consumption turned out to be a risk factor for oral cancer in most analyses, i.e., for the different categories of the biological markers studied. However, no significant ORs were found in the rather small material. There was no significant difference between p53 positive and negative cases regarding liquor consumption. In an earlier study on lingual cancer a higher OR for p53 negative tumours was found (14), in contrast to the results of some other investigations (12,15). There might be a problem of confounding between tobacco and alcohol, since we noted a certain co-variation between these two factors in our large case-control study (4). Regarding expression of PCNA and Ki-67 no pattern was seen.

In this study the percentage of tumours expressing bcl-2 was considerably lower (9/114) than in other studies. There are however, a few obvious differences between these investigations and our study. Jin *et al* (44) who showed bcl-2 positivity in 19/82 tumours (23.1%) studied laryngeal carcinomas which are known to differ from oral carcinomas in many ways. In another study Gasparini *et al* (45), found 37.4% bcl-2 positivity but they investigated tumours of clinical stage II-IV that were unresectable in contrast to our group of tumours comprising clinical stages I-IV.

In conclusion, almost all cases in this study showed an aberration in p53 status demonstrated by at least one of two methods used (Table III). Interestingly, oral infections, and probably HSV-infection in particular, was associated with an increased risk for oral cancer in the p53 IHC positive group, but not in the group with TP53 mutation. This could imply that HSV infection, directly or indirectly, can inactivate p53 function by binding of the wild-type protein.

Acknowledgments

We thank Mrs. Monica Sandström for secretarial assistance and Britta Lindgren for technical assistance. This investigation was supported by grants from The Research Foundation of the Department of Oncology, Umeå University, the Mrs. Berta Kamprad Foundation, the Gunnar, Arvid and Elisabeth Nilssons Foundation, Swedish Match, Orebro County Council Research Committee and Swedish Dental Society.

References

1. Boyle P, Macfarlane GJ, Blot WJ, Chiesa F, Lefebre JL, Azul AM, De Vries N and Scully C: European School of Oncology Advisory Report to the Europe Against Cancer Programme: Oral Carcinogenesis in Europe. Eur J Cancer B Oral Oncol 31: 75-78, 1995.
2. Hakulinen T, Andersen AA, Malker B, Pukkala E, Schou G and Tulinius H: Trends in cancer incidence in the nordic countries. Acta Pathol Scand 94 (Suppl. 288): 1-186, 1986.
3. The National Board of Health and Welfare (Socialstyrelsen). Cancer incidence in Sweden 1997.
4. Schildt EB, Eriksson M, Hardell L and Magnuson A: Oral snuff, smoking habits and alcohol consumption in relation to oral cancer evaluated in a Swedish case-control study. Int J Cancer 77: 341-346, 1998.
5. Lewin F, Norell SE, Johansson H, Gustavsson P, Wennerberg J, Björklund A and Rutqvist LE: Smoking tobacco, oral snuff and alcohol in the etiology of squamous cell carcinoma of the head and neck. A population based case-referent study in Sweden. Cancer 82: 1367-1375, 1998.
6. Greenblatt MS, Bennett WP, Hollstein M and Harris CC: Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 54: 4855-4878, 1994.
7. Soussi T: The p53 tumour suppressor gene: a model for molecular epidemiology of human cancer. Mol Med Today 1: 32-37, 1996.
8. Frank JL, Bur ME, Garb JL, Kay S, Ware JL, Sismanis A and Neifeld JP: p53 tumour suppressor oncogene expression in squamous cell carcinoma of the hypopharynx. Cancer 73: 181-186, 1994.
9. Högmo A, Munck-Wikland E, Kuylenstierna R, Lindholm J and Auer G: Nuclear DNA content and p53 immunostaining in oral squamous cell carcinoma - an analysis of a consecutive 10-year material. Int J Oncol 5: 915-920, 1994.
10. Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, Goodman SN and Sidransky D: Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. N Engl J Med 332: 429-435, 1995.
11. Gallo O and Bianchi S: p53 expression: a potential biomarker for risk of multiple primary malignancies in the upper aero-digestive tract. Eur J Cancer B Oral Oncol 31: 53-57, 1995.
12. Awwad S, Jaros E, Somes J and Lunec J: p53 overexpression in head and neck carcinoma and radiotherapy results. Int J Radiat Oncol 34: 323-332, 1996.
13. Field JK, Spandidos DA, Malliri A, Gosney JR, Yiagnisis M and Stell PM: 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, 1991.
14. Matthews JB, Scully C, Jovanovic A, van der Waal I, Yeudall WA and Prime SS: Relationship of tobacco/alcohol use to p53 expression in patients with lingual squamous cell carcinomas. Eur J Cancer B Oral Oncol 29: 285-289, 1993.
15. Brennan JA, Boyle JO, Koch WM, Goodman SN, Hruban RH, Eby YJ, Couch MJ, Forastiere AA and Sidransky D: Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck. N Engl J Med 332: 712-717, 1995.
16. Raybaud-Diogène H, Tétu B, Morency R, Fortin A and Monteil RA: p53 overexpression in head and neck squamous cell carcinoma: review of the literature. Eur J Cancer B Oral Oncol 32: 143-149, 1996.
17. Somers KD, Merrick A, Lopez ME, Incognito LS, Schechter GL and Casey G: Frequent p53 mutations in head and neck cancer. Cancer Res 52: 5997-6000, 1992.
18. Gusterson BA, Anbazhagan R, Warren W, Midgely C, Lane DP, O'Hare M, Stamps A, Carter R and Jayatilake H: Expression of p53 in premalignant and malignant squamous epithelium. Oncogene 6: 1785-1789, 1991.
19. Langdon JD and Partridge M: Expression of the tumour suppressor gene p53 in oral cancer. Br J Oral Maxillofac Surg 30: 214-220, 1992.
20. Warnakulasuriya KAAS and Johnson NW: Expression of p53 mutant nuclear phosphoprotein in oral carcinoma and potentially malignant oral lesions. J Oral Pathol Med 21: 404-408, 1992.

21. Caamano J, Zhang SY, Rosvald EA, Bares B and Klein-Szanto AJP: p53 alterations in human squamous cell carcinomas and carcinoma cell lines. Am J Pathol 142: 1131-1139, 1993.
22. Nylander K, Stenling R, Gustafsson H, Zackrisson B and Roos G: p53 expression and cell proliferation in squamous cell carcinomas of the head and neck. Cancer 75: 87-93, 1995.
23. Lane DP: p53, guardian of the genome. Nature 358: 15-16, 1992.
24. Chiou SK, Rao L and White E: Bcl-2 blocks p53-dependent apoptosis. Mol Cell Biol 14: 2556-2563, 1994.
25. Wynford-Thomas D: p53 in tumour pathology: can we trust immunocytochemistry? J Pathol 166: 329-330, 1992.
26. Nylander K, Nilsson P, Mehle C and Roos G: p53 mutations, protein expression and cell proliferation in squamous cell carcinoma of the head and neck. Br J Cancer 71: 826-830, 1995.
27. Nylander K, Dabelsteen E and Hall P: The p53 molecule and its prognostic role in squamous cell carcinomas of the head and neck. J Oral Pathol Med 29: 413-425, 2000.
28. Puisieux A, Lim S, Groopman J and Ozturk M: Selective targeting of p53 gene mutational hotspots in human cancers by etiologically defined carcinogens. Cancer Res 51: 6185-6189, 1991.
29. Sakai E and Tuchida N: Most human squamous cell carcinomas in the oral cavity contain mutated p53 tumour-suppressor genes. Oncogene 7: 927-933, 1992.
30. Field JK, Pavelic ZP, Spandidos DA, Stambrook PJ, Jones AS and Gluckman JL: The role of the p53 tumour suppressor gene in squamous cell carcinoma of the head and neck. Arch Otolaryngol Head and Neck Surg 119: 1118-1122, 1993.
31. Kelman Z: PCNA: structure, functions and interactions. Oncogene 14: 629-640, 1997.
32. Shivji MK, Grey SJ, Strausfeld UP, Wood RD and Blow JJ: Cip 1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair. Curr Biol 4: 1062-1068, 1994.
33. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U and Stein H: Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133: 1710-1715, 1984.
34. Gerdes J, Li L, Schlueter C, Duchrow M, Wohlenberg C, Gerlach C, Stahmer I, Kloth S, Brandt E and Flad HD: Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol 138: 867-873, 1991.
35. Nylander K, Schildt E-B, Eriksson M and Roos G: PCNA, Ki-67, p53, bcl-2 and prognosis in squamous cell carcinoma of the head and neck. Anal Cell Pathol 14: 101-110, 1997.
36. Schildt EB, Eriksson M, Hardell L and Magnuson A: Oral infections and dental factors in relation to oral cancer - a Swedish case-control study. Eur J Cancer Prev 7: 201-206, 1998.
37. Roos G, Landberg G, Huff JP, Houghten R, Takasaki Y and Tan EM: Analysis of the epitopes of proliferating cell nuclear antigen recognized by monoclonal antibodies. Lab Invest 68: 204-210, 1993.
38. McCormick D, Chong H, Hobbs C, Datta C and Hall PA: Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody M1B1. Histopathology 22: 355-360, 1993.
39. Vojtesek B, Bartek J, Midgley CA and Lane DP: An immunochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53. J Immunol Methods 151: 237-244, 1992.
40. Nylander K, Anneroth G, Gustafsson H, Roos G, Stenling R and Zackrisson B: Cell kinetics of head and neck squamous cell carcinomas. Prognostic implications. Acta Oncol 33: 23-28, 1994.
41. Vasudevan DM and Vijayakumar T: Viruses in human oral cancers. J Exp Clin Res 17: 27-31, 1998.
42. Park NH, Li SL, Xie JF and Cherrick HM: *in vitro* and animal studies of the role of viruses in oral carcinogenesis. Eur J Cancer B Oral Oncol 28: 145-152, 1992.
43. Zhong L and Hayward GS: Asssembly of complete, functionally active herpes simples virus DNA replication compartments and recruitment of associated viral and cellular proteins in transient cotransfection assays. J Virol 71: 3146-3160, 1997.
44. Jin YT, Kayser S, Kemp BL, Ordonez NG, Tucker SL, Clayman GL, Goepfert H, Luna MA, Batsakis JG and El-Naggar AK:The prognostic significance of the biomarkers $p21^{WAF1/CIP1}$, p53, and bcl-2 in laryngeal squamous cell carcinoma. Cancer 82: 2159-2165, 1998.
45. Gasparini G, Bevilacqua P, Bonoldi E, Testolin A, Galassi A, Verderio P, Boracchi P, Guglielmi RB and Pezzella F: Predictive and prognostic markers in a series of patients with head and neck squamous cell invasive carcinoma treated with concurrent chemoradiation therapy. Clin Cancer Res 1: 1375-1383, 1995.