Downregulation of p21/WAF1 is related to advanced and dedifferentiated laryngeal squamous cell carcinoma

P Hirvikoski, J K Kellokoski, E J Kumpulainen, J A Virtaniemi, R T Johansson, V-M Kosma

Abstract

Aim—To analyse p21/WAF1 expression and its relation to p53, apoptosis, cell proliferation, clinicopathological characteristics, and patient survival in human laryngeal squamous cell carcinoma.

Methods—Primary tumours for analyses were obtained from 172 patients with complete follow up data. All patients were treated between 1975 and 1995. Immunohistochemistry was used to evaluate the expression of p21/WAF1, bcl-2, and p53 proteins. The proliferative activity was determined using Ki67 and PCNA antibodies as well as volume corrected mitotic count (M/V index). Volume corrected apoptotic count (A/V index) was determined using an enzymatic in situ cell death detection kit based on the TUNEL method.

Results—High p21 expression was significantly related to high p53 and normal bcl-2 expressions as well as low mitotic count. No association was noticed between p21 expression and apoptotic rate. A significant inverse correlation between p21 expression and advanced stage and poor differentiation was observed, but p21 expression showed no correlation with survival.

Conclusions—The expression of p21 was associated with tumour stage, histopathological grade, node status, and mitotic count, which may indicate a role for p21 in the progression of laryngeal squamous cell carcinoma.

(*J Clin Pathol* 1999;52:440-444)

Keywords: p21/WAF1; laryngeal neoplasms; prognosis

Epidemiological data show that smoking and alcohol consumption are risk factors for laryngeal squamous cell carcinoma.¹ However, the exact molecular mechanisms of the pathogenesis and progression of this tumour are not known. One of the genes involved in its early stages is p53,² and it was recently reported that the accumulation of p53 protein detected by the D 07 antibody is associated with a favourable prognosis.³ As contradictory results have also been published,^{4,5} the prognostic significance of p53 in laryngeal squamous cell carcinoma remains obscure.

One way to examine the prognostic effect of p53 is to study the genes activated by this protein. Tumour suppressor p21 (WAF1/cip1) is

the main downstream effector gene mediating p53 induced cell cycle arrest, upregulated by wild-type (wt) p53.⁶ In previous in vivo studies, p21 expression has been correlated with tumour cell proliferation and dedifferentiation either in a p53 dependent⁷⁻⁹ or independent pathway.¹⁰⁻¹³ In addition to p21 mediated growth arrest, wt p53 can also induce apoptosis. However, apoptosis in response to DNA damage seems to require wt p53 but not p21 activation.¹⁴ Thus the relations between p21 expression and p53 as well as apoptosis seem to be complex.

We undertook the current study because cell cycle regulation in laryngeal squamous cell carcinoma is poorly understood. We studied the expression of p21 immunohistochemically—with special reference to tumour stage, differentiation, cell proliferation, and bcl-2 and p53 accumulation—in a large series of patients with laryngeal squamous cell carcinoma in whom detailed clinical data and long term follow up were available.

Methods

PATIENTS AND TUMOUR SAMPLES

The patients (n = 336) diagnosed for laryngeal squamous cell carcinoma in eastern Finland between 1975 and 1995 were identified from the files of nationwide, population based Finnish cancer registry. The current study cohort with paraffin blocks available (n = 172) can be considered as a representative sample of the whole geographical cohort, because there were no differences between the cohorts when estimated by tumour stage (p = 0.23), Karnofsky performance status (p = 0.83), and age (p = 0.98). The histological diagnosis was confirmed by reviewing haematoxylin and eosin (H&E) stained original sections, and the most representative block was selected to be cut into 3 µm thick sections. The tumours were graded as well differentiated (n = 44), moderately differentiated (n = 85), or poorly differentiated (n = 43).

CLINICAL DATA

The patient record data were re-evaluated by the same oncologist (EK) and otorhinolaryngologist (JV) in cooperation. The exact site and stage of the tumour were recorded according to the TNM classification,¹⁵ based on the initial examination including indirect laryngoscopy, microlaryngoscopy, careful inspection and palpation of the neck, and a chest x ray. The preoperative performance status according to

Department of Pathology and Forensic Medicine, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland

P Hirvikoski
J K Kellokoski
V-M Kosma

Oral and Maxillofacial Unit and Department of Otorhinolaryngology, Kuopio University Hospital
J A Virtaniemi

Department of Oncology, Kuopio University Hospital
E J Kumpulainen
R T Johansson

Correspondence to:
Dr V-M Kosma.
email:
velimatti.kosma@uku.fi

Accepted for publication
4 February 1999

Table 1 Clinical characteristics of the patients

Variable	Number of patients (%)
Sex	
Male	165 (96%)
Female	7 (4%)
Stage of the primary tumour	
I	48 (28%)
II	41 (24%)
III	43 (25%)
IV	40 (23%)
Site of the primary tumour	
Glottic	76 (44%)
Supraglottic	83 (49%)
Subglottic	4 (2%)
Transglottic	9 (5%)
Performance status (Karnofsky)	
40	4 (2%)
50	15 (9%)
60	27 (16%)
70	83 (48%)
80	21 (12%)
90	17 (10%)
Not known	5 (3%)
Smoking habits	
Smokers	151 (88%)
Non-smokers	6 (3%)
Not known	15 (9%)
Alcohol consumption	
Heavy drinkers	15 (9%)
Others (moderate users)	52 (30%)
Not known	105 (61%)
Primary treatment	
Surgery	22 (13%)
Radiotherapy	82 (48%)
Surgery and postoperative radiotherapy	48 (28%)
Preoperative radiotherapy and surgery	11 (6%)
Combination treatments	7 (4%)
No treatment	2 (1%)

Karnofsky *et al*¹⁶ was recorded. Consumption of alcohol and smoking habits were recorded in two groups: heavy drinkers *v* others, and smokers (current and ex-smokers) *v* non-smokers. All patients were followed up regularly until death or January 1997. The date of recurrence was defined as the first documented date of symptom or sign of the disease.¹⁷

IMMUNOHISTOCHEMISTRY

The expressions of p53 and bcl-2 proteins were available from the data used in previous articles.^{3 18} The expressions of p21, bcl-2, and p53 proteins were analysed using monoclonal WAF1 (Novocastra Laboratories), anti-bcl-2 (clone 124, Dako), and D 07 (Dako) antibodies, respectively. Sections were deparaffinised, rehydrated, and washed in phosphate buffered saline (PBS; pH = 7.2). Sections were boiled in a microwave oven in 0.01 M citrate buffer (pH = 6.0) for 2 × five minutes, cooled for 15 minutes in 0.05M tris buffered saline (TBS; pH = 7.5), and washed twice in PBS. Endogenous peroxidase was blocked by 5% hydrogen peroxide for five minutes followed by washing for five minutes with PBS.

The tissue sections were incubated overnight at +4°C with the primary antibody (WAF1 diluted 1:20, anti-bcl-2 1:200 and D 07 1:1000) in PBS with 1% bovine serum albumin. Sections were washed twice with PBS and incubated for 30 minutes with biotinylated secondary antibody (ABC mouse Vectastain

Table 2 The association of p21 index with clinicopathological features

Feature	p21 Index (No of cases)	
	≤25	26–100
Tumour		p=0.007
T1	4	35
T2	14	23
T3	15	24
T4	13	16
Nodal status		p=0.01
N-	33	87
pN+	13	11
Distant metastases		p=0.4*
M-	43	95
M+	3	3
Histopathological grade		p<0.0005
1	4	32
2	21	51
3	21	15
p53 Protein accumulation		p=0.03
Normal	19	23
Overexpression	26	73
bcl-2 Protein accumulation		p=0.03
Normal	24	69
Overexpression	22	29
Smoking		p=0.2*
Non-smokers	0	6
Smokers	42	84
Alcohol		p=0.7*
Heavy drinkers	3	8
Others	17	29

p Value: statistical significance using the χ^2 test; *Fischer's exact test.

Elite kit; Vector Laboratories) in PBS. Slides were washed twice in PBS for five minutes and incubated for 40 minutes in preformed avidin-biotinylated peroxidase complex. Sections were washed twice for five minutes with PBS and the colour was developed with diaminobenzidine tetrahydrochloride substrate (Sigma). Finally, the sections were lightly counterstained with Mayer's haematoxylin, dehydrated, cleared, and mounted. In each staining batch, a section of human malignant melanoma with known positivity for p21 was used as a positive control for p21. In the negative control slide the antibody was omitted.

The proportion of p21 positive cells within invasive carcinoma tissue was evaluated semi-quantitatively by two observers (PH and V-MK) with a dual head microscope and was reported as a percentage of positive tumour cells. The intensity of the staining was either weak (+) or strong (++). A p21 index was calculated (percentage of positive cells × intensity) potentially ranging from 0 to 200. For statistical analyses, the index was divided into two groups: (0–25 and 26–200).

CELL PROLIFERATION (M/V INDEX AND MIB-1)

The proliferative indices were available from database used in the previous reports^{3 18} (MIB-1 status was available for 165 patients). For immunodemonstration of Ki-67, a monoclonal antibody MIB-1 (Immunotech) was used, as previously described.³ Briefly, the labelling index for MIB-1 was calculated using a computer assisted image analysis system (CAS-2000® Software, Becton Dickinson).

Table 3 The association of p21 index with cell proliferation and apoptotic indices

Index	Median p21 index (range), number of tumours	
	≤25	26–200
A/V index, p=0.8	14.8 (0 to 61.8), n=36	14.9 (0 to 89.1), n=45
M/V index, p=0.002	19.5 (3.1 to 103.9), n=46	14.5 (0 to 67.5), n=96
MIB-1 PI, p=0.4	42.4 (0 to 62.2), n=44	39.5 (0 to 57.4), n=97

Statistical significance determined using the Mann–Whitney U test.

Table 4 Cumulative disease-free and corrected overall survival rates at 10 years

Feature	DFS (95% CI)	n	OS (95% CI)	n
P21 index				
≤25	58% (39% to 73%)	44	57% (39% to 71%)	46
26 to 200	70% (59% to 79%)	92	61% (48% to 72%)	98
	p=0.3		p=0.5	
Stage (TNM)				
I	83% (67% to 92%)	46	89% (72% to 96%)	48
II	68% (50% to 80%)	40	64% (39% to 81%)	41
III	60% (43% to 74%)	42	52% (35% to 66%)	43
IV	24% (2% to 59%)	32	28% (11% to 48%)	40
	p<0.0005		p<0.00005	
Histopathological grade				
1	81% (64% to 91%)	41	85% (68% to 93%)	44
2	67% (54% to 77%)	79	60% (46% to 72%)	85
3	43% (26% to 59%)	40	36% (20% to 53%)	43
	p=0.003		p<0.00005	
Karnofsky performance status				
≤70	64% (53% to 73%)	117	52% (39% to 62%)	129
>70	65% (47% to 78%)	38	79% (62% to 89%)	38
	p=0.3		p=0.01	
All patients	65% (56% to 72%)	160	60% (51% to 69%)	172

CI, confidence interval; DFS, disease-free survival; OS, overall survival.
The log rank test was used to determine statistical significance.

The highest MIB-1 activity area in the tumour was selected, and a total of 20 fields vertically and horizontally to this point were selected. The proliferation index (PI) expresses the number of proliferative nuclei as percentages.

In addition, volume corrected mitotic (M/V) index (data available for 170 patients) was measured from H&E stained sections by the method and formula originally introduced by Haapasalo *et al.*¹⁹ The M/V index expresses the number of mitotic figures/mm² of neoplastic epithelium. Mitotic figures were counted in the invasion front areas with the highest mitotic frequency, and 10 neighbouring fields were selected.

APOPTOSIS (A/V INDEX)

The data of apoptosis were available for 85 patients from an earlier datafile.¹⁸ For measurement of apoptosis, enzymatic in situ labelling of apoptosis induced DNA strand breaks was applied, using the in situ cell death detection kit, AP (Boehringer-Mannheim)—based on the TUNEL method (TdT mediated dUTP-biotin 3'-OH nick-end labelling)—as previously described.¹⁸ As an adaptation of the M/V index, the volume corrected apoptotic (A/V) index was used to demonstrate apoptotic rate.

STATISTICS

To demonstrate the representativeness of the current study cohort compared with the whole population, a χ^2 goodness of fit test was used. The χ^2 test was used to study the association of p21 with clinicopathological variables. We used the Mann–Whitney U test to study the proliferative indices between the groups of p21.

The patterns of overall and disease-free survival were estimated by means of the product-limit method (Kaplan–Meier). Corrected survival rates were used—that is, only deaths caused by laryngeal squamous cell carcinoma were taken as outcome events and all other deaths as censored events. The statistical significance for difference between Kaplan–Meier estimates was obtained using a log rank test.

ETHICS

The research plan was approved by the research ethics committee of Kuopio University and Kuopio University Hospital. For obtaining data from the Finnish Cancer Registry, the study was approved by the Finnish Social and Health Ministry (permission No 117/07/95).

Results

CLINICAL DATA

The characteristics of the patients are summarised in table 1. The median and mean age of the patients was 64 years (range 31 to 85) and median time of follow up 67 months (range 1 to 241). For patients alive at the end point of follow up (n = 42) the median time of follow up was 128 months (range 72 to 241). Relapses of laryngeal squamous cell carcinoma were detected in 55 patients (33%). The recurrence was local in 21 patients (38%), elsewhere in the neck in 21 patients (38%), and distant in 13 patients (24%). Fifty eight patients (33%) died of laryngeal squamous cell carcinoma. Other causes of death were as follows: second neoplasms in 25 (15%), coronary heart disease in 26 (15%), pulmonary disease in 10 (6%), cerebrovascular disease in three (2%), and other causes (suicide and so on) in eight (5%).

p21 INDEX

Representative samples of invasive laryngeal squamous cell carcinoma were available in 144 sections for immunodemonstration of p21. In all samples at least some cells were positive for p21. The median p21 index was 75.5 (range 2.5 to 180). Forty six tumours (32%) were considered negative (p21 index ≤ 25) and 98 (68%) positive for p21.

THE ASSOCIATION OF p21 INDEX WITH CLINICOPATHOLOGICAL CHARACTERISTICS

A high p21 index (≤25) was often detected in small tumours (p = 0.007), in tumours without nodal (pN-) involvement (p = 0.01), and in well differentiated tumours (p < 0.0005). The expression of p21 was frequently associated with p53 accumulation (p = 0.03) and with normal expression of bcl-2 (p = 0.03) (table 2). Tumours with a high p21 index had low mitotic rates (p = 0.002). The proliferation marker MIB-1 did not correlate with p21 expression (p = 0.4). In addition, apoptotic rates were equal in tumours with high and low indices for p21 (p = 0.8) (table 3).

p21 INDEX AND SURVIVAL

Disease-free survival for 10 years occurred in 58% of patients with a low p21 index and in

70% of patients with a high p21 index ($p = 0.3$). No difference in overall survival was observed (57% *v* 61%, respectively; $p = 0.5$). In univariate analyses (table 4), stage ($p < 0.0005$) and histopathological grade ($p = 0.003$) were significant predictors of disease-free survival. Stage ($p < 0.00005$), histopathological grade ($p < 0.00005$), and Karnofsky performance status ($p = 0.01$) were significant predictors of overall survival. The selection of treatment (surgical *v* radiotherapy) had no effect on survival ($p = 0.1$ for overall survival and $p = 0.8$ for disease-free survival). Furthermore, p21 index had no association with survival in subgroups of tumours with either normal p53 protein expression ($p = 0.68$ for overall survival and $p = 0.41$ for disease-free survival) or p53 accumulation ($p = 0.38$ for overall survival and $p = 0.19$ for disease-free survival).

Discussion

Previous papers have reported p21 regulatory pathways to be either p53 dependent or p53 independent.⁷⁻¹³ In line with the recent observations in laryngeal squamous cell carcinoma,¹³ head and neck carcinomas,²⁰ and breast cancer,²¹ we found a significant association between p21 and p53 expression levels. However, the antibody (D O7) used in our present study recognises both normal and mutated forms of p53 protein. If the immunohistochemically detected overexpression of p53 represents mainly the mutated form,²² the current results favour p53 independent induction of p21 in laryngeal squamous cell carcinoma. However, it is obvious that the p21 status does not directly express the functional status of the p53 gene. Thus p53 dependent induction of p21—at least in some cases of laryngeal squamous cell carcinoma (table 2)—cannot be excluded on the basis of the current study. When using immunohistochemical detection of p53, possibilities such as stabilisation of wt p53²³ or p21 activation by p53 despite underlying p53 mutation^{8, 24} cannot be excluded. Even though the exact mutational status of p53 was not determined, previous observations of the p21/p53 relation in laryngeal squamous cell carcinoma¹³ are not in disagreement with the current study.

Regulation of the cell cycle by p21 is through inhibition of cyclin dependent kinases in both normal tissues²⁵ and tumour cells.⁶ In line with this, low mitotic (M/V index) rates were associated with high p21 levels in the current study. Similarly, Nadal *et al* found that the peripheral layers of laryngeal squamous cell carcinomas with MIB-1 positivity lacked p21 expression.¹³ In addition, the S phase fraction of the tumours did not correlate with p21.¹³ However, we found no correlation between p21 expression and cell proliferation marker MIB-1. The same controversy between p21 expression and cell proliferation has been observed in studies of other tumours.^{21, 26, 27} Possible explanations include p21 induced inhibition of phosphorylation of the retinoblastoma protein (pRb).²⁸ Based on recent observations, it seems that cell proliferation control is primarily accomplished

in the G₁ phase and that both p21 and pRb are critical components of this pathway.²⁹ However, it has also been noticed that p21 can inhibit growth of cells which are devoid of a functional pRb.³⁰ In addition, wt p53 dependent pathways can also be inactivated by other proteins, such as mdm2.³¹ Its overexpression might represent an alternative mechanism by which p53 is inactivated in early stages of laryngeal squamous cell carcinoma.³² Taken together, these observations, as well as our current findings, suggest that the association between p21 and cell proliferation is complex and demands further exploration.

Apoptosis may be dominant over cell cycle arrest, and p21 is not required or may even need to be absent for p53 dependent apoptosis.¹⁴ In the case of p53 independent apoptosis it has been suggested that cell cycle arrest through expression of p21 can also be an early event in the sequence leading to apoptosis.^{33, 34} In addition, the frequency of apoptotic cells has been significantly higher in p21 positive than in p21 negative cases.³⁵ Our current study suggests that the rate of apoptosis in laryngeal squamous cell carcinoma is independent of p21 accumulation. A delay, and in some cases even a prevention, of apoptosis independent of p53 has been shown to be caused by bcl-2.³⁶ On the other hand, p53 transcriptionally activates the expression of the bax gene, which in turn antagonises the inhibitory effect of bcl-2 in apoptosis.³⁶ The present study showed that in some cases with normal bcl-2 expression p21 expression was increased. This again reflects the intricate mechanisms regulating apoptosis in laryngeal squamous cell carcinoma.

It has previously been suggested that the differentiation of head and neck carcinomas is p21 independent.²⁰ However, our present results confirm the observation of p21 associated differentiation in laryngeal squamous cell carcinoma.¹³ In our study the low p21 index was common in advanced tumours and in tumours with nodal metastases. This is in contrast to the data of Nadal *et al*,¹³ who found that the expression of p21 was not associated with stage in laryngeal squamous cell carcinoma.¹³ However, in most other studies the expression of p21 has been associated with stage,^{7, 37-39} though inconsistent results have also been reported.^{21, 40, 41} In the cancers of lung,³⁷ endometrium,⁴² and stomach,⁴¹ the expression of p21 has been associated with a favourable prognosis. On the other hand, high expression of p21 has been linked with poor survival in head and neck neoplasms.²⁰ Our current observation of high p21 expression in low stage, well differentiated tumours suggests a role for p21 in patient survival. The association previously shown between p53 accumulation and favourable survival in this same patient material³ also supports this speculation. In definitive survival analyses, however, p21 had no effect on patient survival. This is in line with a recent observation by Jin *et al*.⁴³ Thus p21 analysis did not further strengthen our earlier finding.³ The lack of significance of p21 as a prognostic factor in laryngeal squamous cell carcinoma

remained unchanged when tested in subgroups of tumours with both normal expression and overexpression of p53. Consequently, our current survival data emphasise the continued importance of carefully determined traditional prognostic factors (stage and grade) in daily clinical practice.

We conclude that enhanced level of p21 was associated with p53 protein accumulation as well as with a low mitotic rate. However, no association with apoptotic rate was noted. Although p21 had no prognostic significance in a survival analyses, the association of p21 with tumour stage, node status, differentiation, and mitotic count suggests that it plays a role in the progression of laryngeal squamous cell carcinoma.

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