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p21$^{WAF1/CIP1}$ expression is a marker of poor prognosis in oral squamous cell carcinoma

Judit A. Nemes[1], Zoltán Nemes[2], Ildikó J. Márton[1]

[1]*Faculty of Dentistry and* [2]*Institute of Pathology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary*

BACKGROUND: Previous research on the prognostic relevance of p21$^{WAF1/CIP1}$ in oral squamous cell carcinomas (OSCC) yielded inconclusive and contradictory data.
OBJECTIVES: To investigate the prognostic significance of p21$^{WAF1/CIP1}$ expression, its relationship to p53 accumulation, proliferation-associated proteins Ki-67 and cyclin D1 in relation to survival and clinicopathological features in OSCC.
METHODS: Surgical specimens taken from 106 randomly selected patients were studied by immunohistochemistry. Expression of the protein of interest was correlated with clinical data.
RESULTS: p21$^{WAF1/CIP1}$ expression was found in 61.3% of OSCCs. Expression of p21$^{WAF1/CIP1}$ significantly correlated with tumor size ($P = 0.005$), lymph node involvement ($P = 0.002$), clinical stage ($P < 0.001$), and tumor site ($P = 0.002$). Patients with tumors showing p21$^{WAF1/CIP1}$ immunopositivity had decreased 2-year survival ($P = 0.018$). Expression of p21$^{WAF1/CIP1}$ was not related to age, gender, risk factors (tobacco, alcohol), dental status, or tumor differentiation grade. The p21$^{WAF1/CIP1}$ expression positively correlated with proliferation-related variables Ki-67 ($P = 0.010$) and cyclin D1 ($P < 0.001$), but not with p53 expression.
CONCLUSIONS: The expression of p21$^{WAF1/CIP1}$ was found to be associated with poorer prognosis and tumor aggressivity in OSCC.
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Keywords: cyclin D1; Ki-67; oral; p21; p53; squamous cell carcinoma

Introduction

The majority of oral cancers are squamous cell carcinomas (SCC). In the oral cavity SCCs are thought to develop from precancerous dysplastic lesions by multi-step carcinogenic processes where oncogene activation and loss of tumor suppressor genes are the key features (1–3). The cell cycle progression is regulated by the cyclin-dependent kinases (4). The cyclin dependent kinase inhibitor p21$^{WAF1/CIP1}$, encoded by the WAF1/CIP1 gene, plays an important role in the regulation of the G1-S transition of the cell cycle. The p21$^{WAF1/CIP1}$ protein works as a main downstream effector of p53 protein (5–7). In response to DNA damage, wild-type p53 accumulates and binds to the promoter region of the WAF1/CIP1 gene and this induces the expression of the p21$^{WAF1/CIP1}$. The expression of functional p21$^{WAF1/CIP1}$ will inhibit the activity of the cyclin/cyclin-dependent kinase complex to promote the cell cycle progression (5). The p21$^{WAF1/CIP1}$ expression can also be induced by p53-independent pathways such as the result of genotoxic drugs and growth factors (8, 9). Mutation of the p21$^{WAF1/CIP1}$ gene, however, frequently occurs in malignant tumor cells and the mutated p21$^{WAF1/CIP1}$ which is unable to block the cell cycle (10, 11) accumulates in the nuclei of tumor cells. This phenomenon is detectable by immunostaining of tumor sections with anti-p21$^{WAF1/CIP1}$ antibodies. The overexpression of mutated and inactive p21$^{WAF1/CIP1}$ implies that tumor cells can express p21$^{WAF1/CIP1}$ protein, without regard to the expression of p53.

The prognostic significance of p21$^{WAF1/CIP1}$ expression has scarcely been reported in oral squamous cell carcinomas (OSCC) and the results presented by the previous studies on the correlation of p21$^{WAF1/CIP1}$ expression and prognosis are controversial. p21$^{WAF1/CIP1}$ overexpression has been reported to be associated with worse prognosis in bladder (12), in ovarian (13), in breast (14), in esophageal carcinoma (15, 16) and in oral SCCs (17) too. No association (18–20) or association with better outcome (21–26) was found by other studies.

In our study, we investigated the expression of p21$^{WAF1/CIP1}$ in OSCC and the possible correlation between this parameter and clinicopathologic features, p53 accumulation and the proliferation-associated markers Ki-67 and cyclin D1.

Correspondence: Dr Judit Nemes, Faculty of Dentistry, Medical and Health Science Center, University of Debrecen, 4012 Debrecen, Nagyerdei krt. 98., PO Box 13, Hungary. Tel./Fax: +36 52 413 545. E-mail: nemesj@jaguar.unideb.hu
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Materials and methods

A total of 106 patients with primary OSCC treated between 1996 and 2001 in the Department of Oral Surgery, Faculty of Dentistry, University of Debrecen, Hungary, were studied. 92 patients were men and 14 women ranging in age from 32 to 87 years (mean age 58.1 years).

The formalin-fixed, paraffin-embedded blocks were retrieved from the surgical pathology archives of the Department of Pathology. The tissue samples were of the following sites: 29 lip, 26 tongue, 35 mouth floor, six gingiva, six retromolar region, three palate and one oropharynx. All tumors were classified according to the International Union Against Cancer (UICC) tumor size nodal metastases distant metastases (TNM) classification (27). Histological grading were done according to World Health Organization (WHO) classification (28). Clinicopathologic information on each case, including age, gender, smoking and alcohol intake history, oral status, tumor size, nodal status, location, treatment and presence or absence of tumoral recurrence was obtained from patient record files. Serial 4 μm thick sections were cut from the tissue blocks and mounted on silanized slides. One section was stained with hematoxylin-eosin and examined to confirm the original diagnosis and tumor grade.

Immunohistochemistry

Immunohistochemical studies were performed on tissue sections mounted using commercially available mouse anti-human monoclonal antibodies; anti-$p21^{WAF1/CIP1}$ (clone 4D10, IgG_1) and anti-cyclin D1 (clone P2D11F11, IgG_{2a}) from Novocastra (Newcastle upon Tyne, UK), anti-p53 (clone DO-7, IgG_{2b}/κ) from Lab Vision-NeoMarkers (Fremont, CA, USA), and anti-Ki-67 (MIB1, $IgG_{1\kappa}$) from BD PharMingen (San Diego, CA, USA). Both antibodies have been previously characterized (29–32). Immunohistochemical staining was performed using DAKO LSAB2 peroxidase system according to the manufacturer's instructions. Briefly, dewaxed sections were treated with 0.3% H_2O_2 in methanol at room temperature for 30 min to block endogenous peroxidase activity. Unmasking of antigens was carried out by heating in a microwave oven for 2×5 min in 10 mM citrate-Na (pH 6.0). After incubation with blocking non-immune swine serum for 20 min, sections were incubated with the primary antibodies for 1 h at room temperature with an antibody dilution of 1:40 for anti-p21, 1:100 for anti-p53, 1:50 for anti-cyclin D1, and 1:50 for MIB1. After further incubations with the biotynylated link antibody and peroxidase-labeled streptavidin according to the manufacturer's instructions, peroxidase activity was visualized with SK 4600 kit (Vector Laboratories, Burlingame, CA, USA). In each experiment, a negative control, in which the primary antibodies were replaced by pre-immune mouse IgG, and positive control slides were included. Nuclear staining was considered positive for both $p21^{WAF1/CIP1}$, p53, cyclin D1 and Ki-67. A tumor was recorded positive if $>10\%$ of the tumor cells showed immunoreactivity. The staining characteristics were compared with adjacent non-neoplastic squamous epithelium.

Statistical analysis

The data were stored and analyzed by means of SPSS 11.0 for Windows software (SPSS Inc., Chicago, IL, USA). Chi-square test was used for univariate analysis of categorical data, whereas a t-test was used for continuous data. Correlation among variables was estimated by Spearman rank correlation coefficient. Survival curves were generated using Kaplan–Meier method and compared using the log-rank test. Tests were considered significant when their P-values were <0.05.

Results

$p21^{WAF1/CIP1}$ nuclear staining was present in 65 (61.3%) of the 106 specimens. The nuclear staining was most frequent in the suprabasal region, less in the upper region, and least in the basal region.

p53 expression could be detected in 63 of 105 (60.0%) cases. The proliferation antigen Ki-67 (MIB1) was present in 78 of 102 (76.5%) OSCC samples. The frequency of cyclin D1 positivity was 48 of 105 (45.7%; Fig. 1).

Expression of $p21^{WAF1/CIP1}$ and clinicopathological parameters

Relationships between the expression of $p21^{WAF1/CIP1}$ in OSCCs and clinicopathological parameters of OSCC patients are summarized in Table 1. The higher expression of $p21^{WAF1/CIP1}$ in OSCCs was significantly associated with the larger tumor size (T3 and T4 tumors, $P = 0.005$), positive lymph node metastasis ($P = 0.002$), advanced cancer stages (stages III and IV, $P < 0.001$), and the location of the tumor at the tongue and the retromolar region ($P = 0.002$). Furthermore, survival of $p21^{WAF1/CIP1}$ immunopositive cancer patients was markedly less immediately after diagnosis and this difference was significant after 2 years ($P = 0.018$; Fig. 2). However, there was no significant correlation between $p21^{WAF1/CIP1}$ expression and age, gender, alcohol and tobacco use, dental status or histological grade of the OSCC.

Co-expression of $p21^{WAF1/CIP1}$, p53, Ki-67 and cyclin D1 in OSCC

The $p21^{WAF1/CIP1}$ protein expression in OSCC cases significantly and positively correlated with the expression of Ki-67 ($P = 0.010$) and cyclin D1 ($P < 0.001$). Expression of $p21^{WAF1/CIP1}$ was not associated with the expression of p53. None of the investigated proteins exhibited significant correlation with p53 immunopositivity. As expectable, Ki-67 expression showed strong association with cyclin D1 immunopositivity ($P < 0.001$; Table 2).

Discussion

In the present study, no correlation was found between the expression of $p21^{WAF1/CIP1}$ and p53 accumulation in

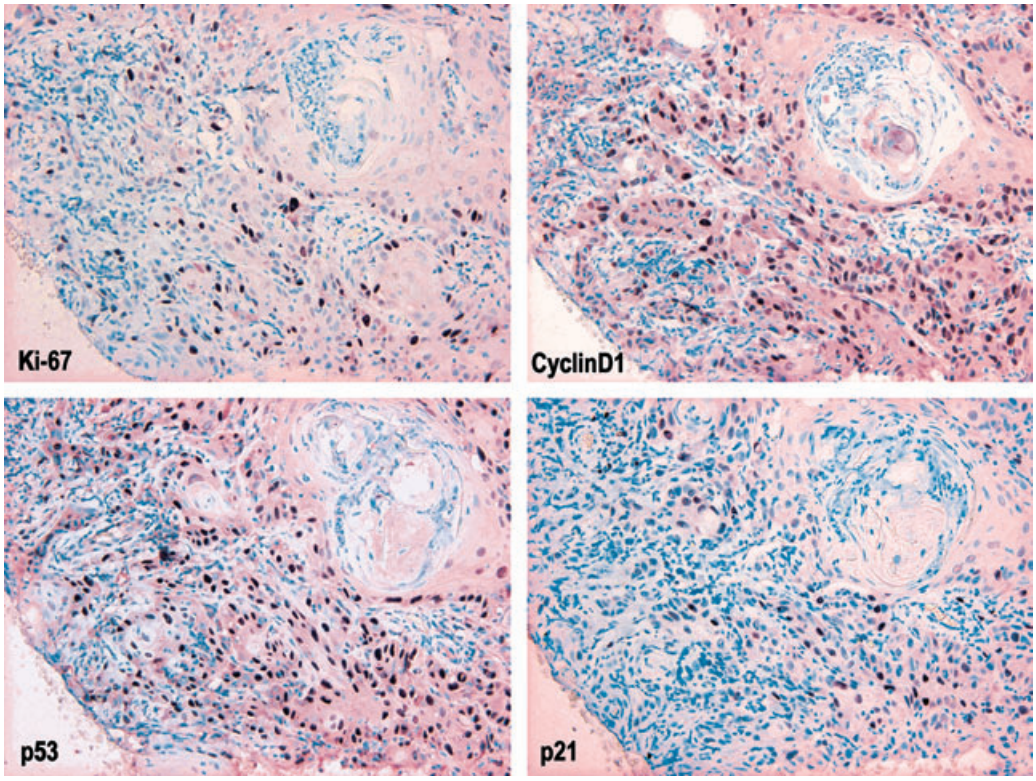

Figure 1 Immunohistochemical demonstration of Ki-67, cyclin D1, p53 and $p21^{WAF1/CIP1}$ in the nuclei of squamous cell carcinoma cells in adjacent sections. Streptavidin-biotin-peroxidase reaction (×200).

OSCCs. This is in agreement with many of the previous reports from oral SCCs (18, 33–36) and other carcinomas (15, 37). According to previous investigations, wild-type p53 is able to upregulate WAF1/CIP1 gene transcription, resulting in enhanced expression of $p21^{WAF1/CIP1}$ protein (6). This observation was supported by studies in non-OSCC cases, where authors found inverse correlation between $p21^{WAF1/CIP1}$ and p53 expression (38–40). This antagonism was not found by other reports (41–43). These findings suggest that the physiological control of $p21^{WAF1/CIP1}$ by p53 may be irrelevant for the immunophenotype of 'common' OSCC, as tumor progression requires the 'individual' inactivation of most of the key antioncogens.

$p21^{WAF1/CIP1}$ expression may be regulated by p53-independent (8, 44) and p53-dependent pathways (45, 46). This implicates that functional $p21^{WAF1/CIP1}$ might inhibit tumor growth even after mutations or deletions inactivate both functional p53 alleles, and tumor progression favors the loss of functional $p21^{WAF1/CIP1}$ expression. In the present study 38 of 63 (60.3%) of the p53 positive tumors overexpressed the $p21^{WAF1/CIP1}$ antigen, which is not different from the frequency of p53 (60%) and $p21^{WAF1/CIP1}$ (61.3%) overexpression in the total sample pool. One can assume that the remaining tumors were immunonegative owing to other ways of antioncogen inactivation than point mutations preserving the antigenicity of the (over)expressed antioncogen proteins, like premature termination or allele loss (47, 48). Our result support the finding that both p53-dependent and p53-independent mechanisms are involved in the expression of $p21^{WAF1/CIP1}$ in OSCCs, and getting rid of active p53 protein does not completely abolish the antiproliferative activity of functional $p21^{WAF1/CIP1}$expression.

In our series, $p21^{WAF1/CIP1}$ overexpression significantly associated with tumor size ($P = 0.005$), lymph node metastasis ($P = 0.002$), clinical stage ($P < 0.001$), and tumor site ($P = 0.002$), whereas p53 did not, the later is in agreement with numerous previous findings (21, 49, 50). Patients with OSCC overexpressing $p21^{WAF1/CIP1}$ had decreased 2-year survival ($P = 0.018$; Fig. 2). Twenty-nine cases (27.4%) of the study population were lip SCC cases, which are typically associated with lower stages and histological grades at the time of diagnosis, and therefore have better outcome than the SCCs of the oral cavity. Also, the importance of sun-induced DNA damage is thought to be more important for the genesis of lip SCCs than the role of mutagenic oral habits (51). We found a lower occurrence of $p21^{WAF1/CIP1}$ immunopositivity in lip SCC (nine of 29) than in other locations, (56 of 77), which is reflected by the association of $p21^{WAF1/CIP1}$ immunopositivity with the inner mouth tumors, and could be attributed to the lower tumor staging of the lip cases. However, the outcome and expression of neither p53, nor proliferation markers were found to be significantly different from the residual population of the same $p21^{WAF1/CIP1}$ status. This might indicate the similar effects of random physical and chemical mutagens on the evolution of malignant epithelial stem cells.

Table 1 Relationship between clinicopathological parameters and $p21^{WAF1/CIP1}$ expression

Factor	*No. of cases*	*p21 positivity* (%)*	*P-value†*
Age			
≥65	31	17 (54.8)	0.378
<65	75	48 (64.0)	
Gender			
Male	92	56 (60.9)	0.807
Female	14	9 (64.3)	
Tobacco			
Yes	74	49 (66.2)	0.116
No	32	16 (50.0)	
Alcohol			
Yes	76	48 (63.2)	0.536
No	30	17 (56.7)	
Dental status			
Toothless	24	13 (54.2)	0.098
1–5 teeth	9	3 (33.3)	
Missing teeth, no replacement	29	19 (65.5)	
Missing teeth with replacement	12	4 (33.3)	
Untreated carious, periodontotic	16	12 (75.0)	
Tumor size			
T1, T2	77	41 (53.2)	0.005
T3, T4	29	24 (82.8)	
Lymph node metastasis			
Node negative	78	41 (52.6)	0.002
Node positive	28	24 (85.7)	
Clinical staging			
I	36	12 (33.3)	<0.001
II	30	19 (63.3)	
III	25	21 (84.0)	
IV	15	13 (86.7)	
Histologic differentiation			
Grade I (well)	45	26 (57.8)	0.800
Grade II (moderate)	52	33 (63.5)	
Grade III (poor)	9	6 (66.7)	
Tumor site			
Lips	29	9 (31.0)	0.002
Tongue	26	21 (80.8)	
Mouth floor	35	23 (65.7)	
Gingiva	6	4 (66.7)	
Retromolar region	6	5 (83.3)	
Palate	3	3 (100.0)	
Oropharynx	1	0	
Outcome after 2 years			
Survivor	61	32 (52.5)	0.018
Non-survivor	41	31 (75.6)	

**P*-value vs. immunonegative cases.
†*P*-values were obtained from chi-square test.

The fact that high $p21^{WAF1/CIP1}$ expression significantly correlates with poorer clinical outcome is in agreement with previous studies conducted on both OSCC (17) and other carcinomas, like prostate carcinoma (52), superficial bladder tumors (53), and gastric carcinoma (39). Opposite results have also been reported from studies of several other types of tumor such as breast carcinoma (25), lung (26), laryngeal (54), and tongue SCCs (33). These contradictory results for oral cancer as well as for tumors of other organs indicate that association between $p21^{WAF1/CIP1}$ expression and prognosis is complex, tumor-type and possibly etiology-related. Some studies reveal that combined p53/

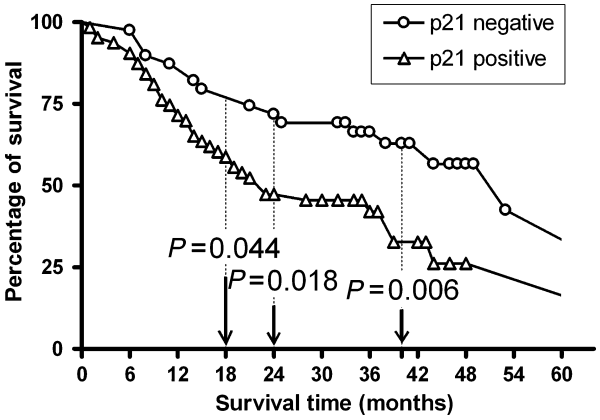

Figure 2 Kaplan–Meier survival curves for patients with (positive) or without (negative) $p21^{WAF1/CIP1}$ expression in oral squamous cell carcinomas.

Table 2 Correlation between $p21^{WAF1/CIP1}$ and p53/Ki-67/cyclin D1 expression

	p21 positive (%)	*p21 negative (%)*	*Total (%)*	*P-value**
p53 positive	38 (36.2)	25 (23.8)	63 (60.0)	0.682
p53 negative	27 (25.7)	15 (14.3)	42 (40.0)	
Total	65 (61.9)	40 (38.1)	105 (100)	
Ki-67 positive	55 (53.9)	23 (22.6)	78 (76.5)	0.010
Ki-67 negative	10 (9.8)	14 (13.7)	24 (23.5)	
Total	65 (63.7)	37 (36.3)	102 (100)	
Cyclin D1 positive	43 (40.9)	5 (4.8)	48 (45.7)	<0.001
Cyclin D1 negative	22 (21.0)	35 (33.3)	57 (54.3)	
Total	65 (61.9)	40 (38.1)	105 (100)	

**P*-values were obtained from chi-square test.

$p21^{WAF1/CIP1}$ expression may be a predictive factor for reduced survival time when $p21^{WAF1/CIP1}$ or p53 alone is not a significant prognostic variable (55). Other studies reported that $p21^{WAF1/CIP1}$ expression in combination with p53 accumulation provides better prognostic information than evaluation of either individual variable alone (33). Our observation is that although p53+/p21+ immunohistochemical phenotype means significantly ($P = 0.046$) worse prognosis than p53−/p21− or p53+/p21− phenotype, of which the prognostic value for $p21^{WAF1/CIP1}$ immunopositivity alone is stronger ($P = 0.018$).

This study found a significant correlation between $p21^{WAF1/CIP1}$ overexpression and the expression of Ki-67 ($P = 0.010$) or cyclin D1 in OSCCs ($P < 0.001$), although the association of $p21^{WAF1/CIP1}$ positivity with histological grading was not significant. This observation argues for the higher diagnostical accuracy of checking possibly more than one proliferation-related antigen expression for the assessment of tumor dignity and suggests that the inactivation of $p21^{WAF1/CIP1}$ might be delayed to later stages of the tumor dedifferentiation, whereas p53 must be neutralized in the early steps of tumor progression, otherwise the tumor could not grow. Alternatively, the genomic mutations leading to the overproduction of both inactive p53 and $p21^{WAF1/CIP1}$ proteins may reflect a higher mutagenesis frequency and

genomic instability, which makes such tumors grow and evolve faster than others. We find it noteworthy to mention that $p21^{WAF1/CIP1}$ positivity was significantly more common in large and metastatic tumors ($P < 0.001$), which association supports the previous assumption and might be reconciled with the lack of association with histological grading. In either case, however, the immunopositivity for $p21^{WAF1/CIP1}$ is a marker of higher tumor aggressivity and worse prognosis.

In conclusion, the overexpression of $p21^{WAF1/CIP1}$ was unrelated to the overexpression of p53, the main regulatory protein of $p21^{WAF1/CIP1}$ in cell cycle control and antitumor surveillance. However, $p21^{WAF1/CIP1}$ immunopositivity was associated with higher cell proliferation as indicated by Ki-67 and cyclin D1 expression and advanced clinical stages. Our results suggest that immunohistochemically detectable overexpression of $p21^{WAF1/CIP1}$ protein has diagnostic value in the pathological assessment of oral SCCs.

References

1. Matsuda H, Konishi N, Hiasa Y, et al. Alterations of p16/CDKN2, p53 and ras genes in oral squamous cell carcinomas and premalignant lesions. *J Oral Pathol Med* 1996; **25**: 232–8.
2. Kashiwazaki H, Tonoki H, Tada M, et al. High frequency of p53 mutations in human oral epithelial dysplasia and primary squamous cell carcinoma detected by yeast functional assay. *Oncogene* 1997; **15**: 2667–74.
3. Field JK, Spandidos DA. Expression of oncogenes in human tumors with special reference to the head and neck region. *J Oral Pathol* 1987; **16**: 97–107.
4. Sherr CJ. Cancer cell cycles. *Science* 1996; **274**: 1672–7.
5. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 1993; **75**: 805–16.
6. el-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993; **75**: 817–25.
7. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature* 1993; **366**: 701–4.
8. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res* 1994; **54**: 3391–5.
9. Fan Z, Shang BY, Lu Y, Chou JL, Mendelsohn J. Reciprocal changes in p27(Kip1) and p21(Cip1) in growth inhibition mediated by blockade or overstimulation of epidermal growth factor receptors. *Clin Cancer Res* 1997; **3**: 1943–8.
10. Lukas J, Groshen S, Saffari B, et al. WAF1/Cip1 gene polymorphism and expression in carcinomas of the breast, ovary, and endometrium. *Am J Pathol* 1997; **150**: 167–75.
11. Lu Y, Yamagishi N, Yagi T, Takebe H. Mutated p21(WAF1/CIP1/SDI1) lacking CDK-inhibitory activity fails to prevent apoptosis in human colorectal carcinoma cells. *Oncogene* 1998; **16**: 705–12.
12. Garcia del Muro X, Condom E, Vigues F, et al. p53 and p21 Expression levels predict organ preservation and survival in invasive bladder carcinoma treated with a combined-modality approach. *Cancer* 2004; **100**: 1859–67.
13. Rose SL, Goodheart MJ, DeYoung BR, Smith BJ, Buller RE. p21 expression predicts outcome in p53-null ovarian carcinoma. *Clin Cancer Res* 2003; **9**: 1028–32.
14. Caffo O, Doglioni C, Veronese S, et al. Prognostic value of p21(WAF1) and p53 expression in breast carcinoma: an immunohistochemical study in 261 patients with long-term follow-up. *Clin Cancer Res* 1996; **2**: 1591–9.
15. Lam KY, Law S, Tin L, Tung PH, Wong J. The clinicopathological significance of p21 and p53 expression in esophageal squamous cell carcinoma: an analysis of 153 patients. *Am J Gastroenterol* 1999; **94**: 2060–8.
16. Sarbia M, Stahl M, zur Hausen A, et al. Expression of p21WAF1 predicts outcome of esophageal cancer patients treated by surgery alone or by combined therapy modalities. *Clin Cancer Res* 1998; **4**: 2615–23.
17. Erber R, Klein W, Andl T, et al. Aberrant p21(CIP1/WAF1) protein accumulation in head-and-neck cancer. *Int J Cancer* 1997; **74**: 383–9.
18. Ibrahim SO, Lillehaug JR, Vasstrand EN. Mutations of the cell cycle regulatory genes p16INK4A and p21WAF1 and the metastasis-inducing gene S100A4 are infrequent and unrelated to p53 tumor suppressor gene status and data on survival in oropharyngeal squamous cell carcinomas. *Anticancer Res* 2003; **23**: 4593–600.
19. Przybojewska B, Rydzynski K, Stepnik M, Jakubiak M, Kozak J, Szymczak W. Immunohistochemical evaluation of P21ras and P53 proteins expression in human non-small-cell lung cancers. *Neoplasma* 2003; **50**: 198–203.
20. Liu CJ, Chang KW, Chao SY, et al. The molecular markers for prognostic evaluation of areca-associated buccal squamous cell carcinoma. *J Oral Pathol Med* 2004; **55**: 327–34.
21. Kapranos N, Stathopoulos GP, Manolopoulos L, et al. p53, p21 and p27 protein expression in head and neck cancer and their prognostic value. *Anticancer Res* 2001; **21**: 521–8.
22. Shoji T, Tanaka F, Takata T, et al. Clinical significance of p21 expression in non-small-cell lung cancer. *J Clin Oncol* 2002; **20**: 3865–71.
23. Chien CY, Huang CC, Cheng JT, Chen CM, Hwang CF, Su CY. The clinicopathological significance of p53 and p21 expression in squamous cell carcinoma of hypopharyngeal cancer. *Cancer Lett* 2003; **201**: 217–23.
24. Gomyo Y, Ikeda M, Osaki M, et al. Expression of p21 (waf1/cip1/sdi1), but not p53 protein, is a factor in the survival of patients with advanced gastric carcinoma. *Cancer* 1997; **79**: 2067–72.
25. Jiang M, Shao ZM, Wu J, et al. p21/waf1/cip1 and mdm-2 expression in breast carcinoma patients as related to prognosis. *Int J Cancer* 1997; **74**: 529–34.
26. Komiya T, Hosono Y, Hirashima T, et al. p21 expression as a predictor for favorable prognosis in squamous cell carcinoma of the lung. *Clin Cancer Res* 1997; **3**: 1831–5.
27. Sobin LH, Wittekind C, eds. *Head and Neck tumors. International Union Against Cancer (Union Internationale Contre le Cancer): TNM classification of malignant tumors*. New York: Wiley-Liss, 1997; 20–4.
28. Pindborg JJ, Reichart PA, Smith CJ, van der Waal I. *Histological typing of cancer and precancer of the oral mucosa*. Berlin: Springer Verlag, 1997.
29. Sorrentino V, Nebreda AR, Alonso T, Santos E. Preparation, characterization and properties of monoclonal antibodies against intact H-ras p21 proteins. *Oncogene* 1989; **4**: 215–22.
30. Vallance SJ, Lee HM, Roussel MF, et al. Monoclonal antibodies to mammalian D-type G1 cyclins. *Hybridoma* 1994; **13**: 37–44.

31. Vojtesek B, Bartek J, Midgley CA, Lane DP. An immunochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53. *J Immunol Methods* 1992; **151**: 237–44.
32. Key G, Becker MH, Baron B, et al. Preparation and immunobiochemical characterization of new Ki-67 equivalent murine monoclonal antibodies (MIB 1–3) generated against recombinant parts of the Ki-67 antigen. *Anal Cell Pathol* 1992; **4**: 181–9.
33. Xie X, Clausen OP, Boysen M. Prognostic significance of p21WAF1/CIP1 expression in tongue squamous cell carcinomas. *Arch Otolaryngol Head Neck Surg* 2002; **128**: 897–902.
34. Kudo Y, Takata T, Ogawa I, Sato S, Nikai H. Expression of p53 and p21CIP1/WAF1 proteins in oral epithelial dysplasias and squamous cell carcinomas. *Oncol Rep* 1999; **6**: 539–45.
35. Ng IO, Lam KY, Ng M, Regezi JA. Expression of p21/waf1 in oral squamous cell carcinomas–correlation with p53 and mdm2 and cellular proliferation index. *Oral Oncol* 1999; **35**: 63–9.
36. van Oijen MGCT, Tilanus MGJ, Medema RH, Slootweg PJ. Expression of p21(Waf1/Cip1) in head and neck cancer in relation to proliferation, differentiation, p53 status and cyclin D1 expression. *J Oral Pathol Med* 1998; **27**: 367–75.
37. Shih HC, Shiozawa T, Kato K, et al. Immunohistochemical expression of cyclins, cyclin-dependent kinases, tumor-suppressor gene products, Ki-67, and sex steroid receptors in endometrial carcinoma: positive staining for cyclin A as a poor prognostic indicator. *Hum Pathol* 2003; **34**: 471–8.
38. Pasz-Walczak G, Kordek R, Faflik M. P21 (WAF1) expression in colorectal cancer: correlation with P53 and cyclin D1 expression, clinicopathological parameters and prognosis. *Pathol Res Pract* 2001; **197**: 683–9.
39. Noda H, Maehara Y, Irie K, Kakeji Y, Yonemura T, Sugimachi K. Growth pattern and expressions of cell cycle regulator proteins p53 and p21WAF1/CIP1 in early gastric carcinoma. *Cancer* 2001; **92**: 1828–35.
40. Lele SM, Pou AM, Ventura K, Gatalica Z, Payne D. Molecular events in the progression of recurrent respiratory papillomatosis to carcinoma. *Arch Pathol Lab Med* 2002; **126**: 1184–8.
41. Giannoudis A, Herrington CS. Differential expression of p53 and p21 in low grade cervical squamous intraepithelial lesions infected with low, intermediate, and high risk human papillomaviruses. *Cancer* 2000; **89**: 1300–7.
42. Xie HL, Su Q, He XS, et al. Expression of p21(WAF1) and p53 and polymorphism of p21(WAF1) gene in gastric carcinoma. *World J Gastroenterol* 2004; **10**: 1125–31.
43. Warnakulasuriya KAAS, Tavassoli M, Johnson NW. Relationship of p53 overexpression to other cell cycle regulatory proteins in oral squamous cell carcinoma. *J Oral Pathol Med* 1998; **27**: 376–81.
44. Sheikh MS, Garcia M, Zhan Q, Liu Y, Fornace AJ Jr. Cell cycle-independent regulation of p21Waf1/Cip1 and retinoblastoma protein during okadaic acid-induced apoptosis is coupled with induction of Bax protein in human breast carcinoma cells. *Cell Growth Differ* 1996; **7**: 1599–607.
45. el-Deiry WS, Harper JW, O'Connor PM, et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 1994; **54**: 1169–74.
46. Dulic V, Kaufmann WK, Wilson SJ, et al. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell* 1994; **76**: 1013–23.
47. Cheng J, Huang H, Pak J, et al. Allelic loss of p53 gene is associated with genesis and maintenance, but not invasion, of mouse carcinoma in situ of the bladder. *Cancer Res* 2003; **63**: 179–85.
48. Sanz-Ortega J, Valor C, Saez MC, et al. 3p21, 5q21, 9p21 and 17p13 allelic deletions accumulate in the dysplastic spectrum of laryngeal carcinogenesis and precede malignant transformation. *Histol Histopathol* 2003; **18**: 1053–7.
49. Gonzalez-Moles MA, Galindo P, Gutierrez-Fernandez J, et al. P53 protein expression in oral squamous cell carcinoma. Survival analysis. *Anticancer Res* 2001; **21**: 2889–94.
50. Kodani I, Osaki M, Shomori K, et al. Minichromosome maintenance 2 expression is correlated with mode of invasion and prognosis in oral squamous cell carcinomas. *J Oral Pathol Med* 2003; **32**: 468–74.
51. Ostwald C, Gogacz P, Hillmann T, et al. p53 mutational spectra are different between squamous-cell carcinomas of the lip and the oral cavity. *Int J Cancer* 2000; **88**: 82–6.
52. Omar EA, Behlouli H, Chevalier S, Aprikian AG. Relationship of p21(WAF-I) protein expression with prognosis in advanced prostate cancer treated by androgen ablation. *Prostate* 2001; **49**: 191–9.
53. Zlotta AR, Noel JC, Fayt I, et al. Correlation and prognostic significance of p53, p21WAF1/CIP1 and Ki-67 expression in patients with superficial bladder tumors treated with bacillus Calmette-Guerin intravesical therapy. *J Urol* 1999; **161**: 792–8.
54. Jeannon JP, Soames J, Lunec J, Awwad S, Ashton V, Wilson JA. Expression of cyclin-dependent kinase inhibitor p21(WAF1) and p53 tumor suppressor gene in laryngeal cancer. *Clin Otolaryngol* 2000; **25**: 23–7.
55. Yanamoto S, Kawasaki G, Yoshitomi I, Mizuno A. p53, mdm2, and p21 expression in oral squamous cell carcinomas: relationship with clinicopathologic factors. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002; **94**: 593–600.