in vivo *18*: 719-724 (2004)

Expression of Cyclin-dependent Kinases Inhibitors p21(WAF1)and p27(KIP1) in Benign, Premalignant and Malignant Laryngeal Lesions. Correlation with Cell Cycle Regulatory Proteins

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Abstract. *Background: Cell cycle progression and transition of cells from the first gap phase (G1) to the DNA replication phase (S) depend on a finely tuned balance between the levels of cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). Materials and Methods: We analyzed 57 squamous cell invasive carcinomas of the larynx, 10 in situ carcinomas, 56 cases of dysplasia, 11 papillomas and 26 keratosis. We investigated: a) the immunohistochemical expression of CDKIs, p21 and p27, b) any possible relation between normal and abnormal immunoprofiles of these proteins and p53 protein and proliferation status as determined by the expression of Ki67 and PCNA, and c) their presence in pre-malignant and malignant laryngeal lesions. Results: Expression of p21 and p27 was observed in 58.9% and 89.5% of the laryngeal carcinomas, respectively. High levels of p21 were significantly correlated with increased cyclin D ($p=0.001$), cyclin E ($p<0.001$) and Ki67 ($p<0.001$), while increased expression levels of p27 were associated with p53 accumulation ($p=0.02$) and with increased proliferation status as expressed by Ki67 ($p=0.05$). Conclusion: Due to the increased expression levels of CDKIs in laryngeal carcinomas, we suggest the existence of a mechanism by which tumor cells tolerate the inhibitory effect of these proteins on cell cycle progression.*

Entry and gradual progression through the cell cycle is controlled by cyclins and cyclin-dependent kinases (CDKs), whose activities are inhibited by cyclin-dependent kinase inhibitors (CDKI) (1,2). All these molecules, termed collectively G1 regulators, play a pivotal role in regulating positively or negatively the cell cycle, thus contributing to the tissues' homeostatic mechanisms. Among the cell-cycle regulators, only the G1 regulators have been found to be altered or mutated during tumorigenesis, leading to uncontrolled cell proliferation (3), which is the hallmark of cancer.

CDKIs inhibit cell cycle progression by inactivating cyclin-CDK complexes (negative regulators). This is accomplished when CDKIs bind to CDKs at the expense of cyclins (4). CDKIs are divided into two groups: the INK4 group includes p16/INK4A (p16), p15/INK4B (p15), p18/INK4C (p18) and p19/INK4D (p19), which form complexes with CDK4 and/or CDK6 and the D-type cyclins. Their function depends on the presence of normal retinoblastoma protein (5).The second group consists of p21WAF1/ CIP 1 (p21), p27/kip1 (p27) and p57/kip2 (p57). These proteins are named universal CDKIs because they interact with various CDK complexes, with cyclins A, E, D1, D2 and D3 and with CDKs (5).

The first publication (6) which analysed p21 expression in neoplastic laryngeal tissues suggested that p21 overexpression may be associated with a malignant phenotype, its levels being significantly higher in cancer than in normal tissues. Previous studies (7) have identified aberrant expression of p21 in laryngeal cancer. It has been reported that high levels of p21 correlate with an adverse clinical course in head and neck cancer (8) and laryngeal cancer (9-10).

Some investigators have provided evidence that down-regulation of the p27 protein plays a pivotal role in the progression of laryngeal cancer. Recently (11) it was reported that p27 immunostaining was observed with decreasing frequency in hyperplastic, dysplastic and malignant laryngeal

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Key Words: p21, p27, laryngeal cancer, proliferation markers, cell cycle regulatory proteins.

Table I. *Expression of p21 and p27 in malignant and premalignant laryngeal lesions.*

	N	Minimum	Maximum	Mean	Std Deviation
p21 cancer	56	0	70	23.67	20.29
p21 *in situ*	9	0	45	13.44	14.47
p21 dysplasia	56	1	65	24.00	16.47
p21 papilloma	10	2	60	25.40	17.64
p21 keratosis	26	0	75	25.80	17.95
p27 cancer	57	8	100	78.47	19.80
p27 *in situ*	10	10	85	47.50	30.75
p27 dysplasia	55	15	100	81.36	20.44
p27 papilloma	11	2	100	70.63	3.48
p27 keratosis	25	70	100	91.20	8.45

Results are expressed as the percentage of positive cells per total number of counted cells

Table II. *Correlation of p21, p27 with cyclins, p53 and Ki67.*

	p21		p27		
	<10	> 10	<50	>50	*p* value
Cyclin D					
<5	21	23	5	40	$p=0.001$
>5	2	9	1	10	NS
Cyclin E					
<5	17	17	5	30	$p<0.001$
5-50	6	12	1	17	NS
>50	0	3	0	3	
p53					
<5	12	9	4	18	NS
>5	5	11	0	16	$p=0.02$
Ki67					
<5	5	4	1	8	$p<0.001$
5-50	15	19	5	29	$p=0.05$
>50	3	8	0	11	

lesions, but its possible role in precancerous lesions remains obscure. Low p27 expression in laryngeal carcinomas has been significantly associated with aggressive tumor behaviour and poor prognosis (12), advanced clinical stage and presence of nodal metastasis (13-14).

It is also essential to underline that little is known regarding changes in these proteins in hyperplastic and pre-malignant lesions. We undertook this study since cell cycle regulation in cells of benign lesions, including papillomas and keratosis, dysplastic laryngeal lesions and laryngeal cancer is poorly understood. We studied the expression of p21 and p27 immunohistochemically, with particular reference to tumor stage, cell proliferation and p53 accumulation in invasive squamous cell carcinomas. We also tried to elucidate the presence and associations of these cell cycle inhibitors through the transformation of non-malignant to malignant lesions.

Materials and Methods

Patients and study design. The study sample consisted of formalin-fixed and paraffin-embedded tissues from 160 laryngeal lesions including 57 squamous cell carcinomas, 10 *in situ* carcinomas, 56 cases of dysplasia, 11 papillomas and 26 cases of keratosis. Detailed clinical and laboratory data, along with follow-up data, were available for 46 patients with laryngeal cancer. All relevant data such as age, clinical stage and number of extranodal disease sites were recorded. The mean age was 58.5 and they were followed up for a period of 4 years(range 1-7).

Immunohistochemistry. Immunohistochemistry was performed on one or two selected paraffin blocks, from each case on 4-μm tissue sections placed on poly-L-lysine-coated glass slides. In brief, tissue sections were deparaffinized in xylene and dehydrated. A step of immersion in citrate buffer (0.1m, pH 0.6) in plastic coplin jars was followed by microwave irradiation twice for 15 min. Monoclonal antibodies directed against p21 (4D10, Novocastra, dilution 1:20), p27 (IB4, Novocastra, dilution 1:20), cyclin D1 (P2D1F11, Novocastra), cyclin E (13a3, Novocastra, dilution 1:10), p53 protein (DO-7, Dako SA, Glostrup, Denmark, dilution 1:50), proliferation-associated nuclear antigen Ki67 (MIB-1, Immunotech, Marseille, France; dilution 1:20), PCNA (PC-10, Dako, 1:50) were applied. Subsequently, all sections were treated for 30 min with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity and then incubated with primary antibodies. The method involving the avidin-biotin-peroxidase complex was used and the chromogen was developed by immersion of the slides in a diaminobenzidine-H_2O_2 substrate for 5 min. The slides were counterstained in Harris' haematoxylin, dehydrated and mounted. To assess the specificity of the reaction, positive control slides were included in all cases.

Immunohistochemical evaluation. For evaluation of immunostaining, a continuous score system was adopted by using the x40 objective lense and counting at least 5 to 10 representative fields, selected on the basis of existence of immunopositive cells. The number of immunopositive cells was divided by the total number of counted cells and the expression was defined as the percentage of positive cells in the total number of the counted cells. When the positive cells were more than 5%, p53 expression was considered increased. The cut-offs for the remaining regulatory proteins were, as described by other authors, assessed as follows: for cyclin D1, p21/WAF1 and p27/kip1 a percentage of <5%, <10% and <50% positive cells was considered negative (15), respectively. For the estimation of cyclin E and Ki67, a 3-point scale system (<5%, 5-50% and >50%) was used (16-17). Non-specific immunostaining was omitted from the study.

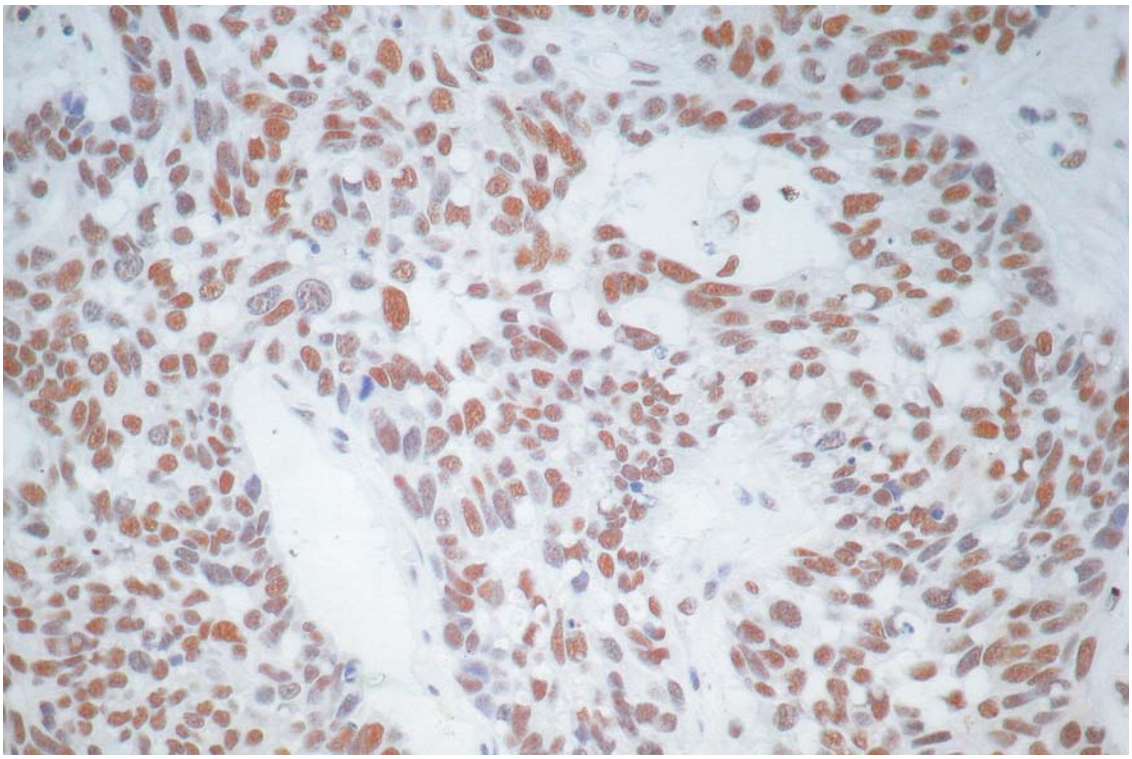

Figure 1. *Strong nuclear immunoreactivity of p21 protein in laryngeal carcinoma.*

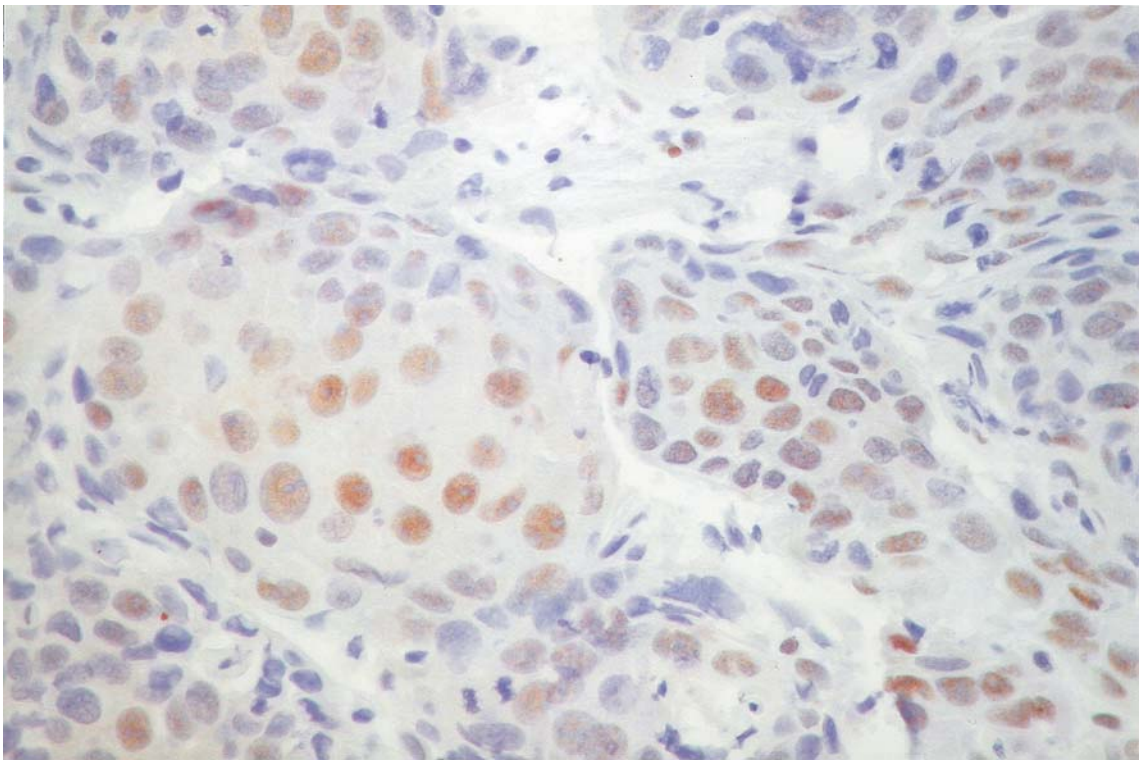

Figure 2. *Nuclear immunolabelling of the carcinoma cells for p27 protein.*

Statistical analysis. The program SPSS for Windows Release 10.0 was used for statistical analysis. Pearson's and Spearman's correlation coefficients were used for the assessment of correlation between continuous variables. The results were considered as statistically significant when $p<0.05$.

Results

Invasive carcinomas. Overexpression of p21 was observed in 33 cases (58.9%) of invasive squamous cell carcinoma, while 23 cases (41.1%) presented with partial or complete loss (Figure 1). Tumors exhibiting extensive p21 immunoreactivity correlated strongly with high cyclin D ($p=0.001$), cyclin E ($p<0.001$) and Ki67 ($p<0.001$) content. In addition, the majority of neoplastic cells exhibited high levels of p27 protein since 51 cases (89.5%) presented with increased immunoreactivity (>50% of tumor cells).Only 6 cases (10.5%) presented with partial loss of p27 (Figure 2). Increased expression levels of p27 were associated with p53 accumulation ($p=0.02$) as well as with increased proliferation status as expressed by Ki67 ($p=0.05$). Results are shown in Tables I and II. No relationship between the two proteins and clinical stage or tumor grade was demonstrated.

In situ carcinomas. A high percentage of *in situ* carcinomas (66.7%) unexpectedly presented with loss of p21 expression, while half of the cases also demonstrated loss of p27 protein. A significantly lower p21 expression level in this group compared with dysplastic cases was observed ($p=0.037$). Unfortunately, our sample was not sufficient to assess the significance of correlations with other proteins.

Dysplastic cases. Nineteen out of 56 dysplastic cases(33.9%) presented with p21 loss of expression, while only 4 cases(73%) exhibited analogous loss of p27 protein. Furthermore, p27 strong immunoreactivity correlated with p53 expression status ($p=0004$), a phenomenon also demonstrated in neoplastic cells.

Papillomas-keratosis. Concerning papillomas, 3 (30%) and 2 (18.2%) cases showed loss of p21 and p27 proteins, respectively. For keratotic lesions, 6 cases (23.1%) presented loss of p21 and all cases (100%) were strongly immunoreactive for p27. No relationship with other regulatory proteins was demonstrated.

Discussion

Disrupted cell cycle regulation plays an important role in the abnormal proliferation of tumor cells. Information related to proteins that exert inhibitory effects on cell cycle progression, like CDKIs, could be useful in understanding the behavior of cancer. It has been reported that alterations of this family of proteins is correlated with aggressive behavior of certain human malignancies (18,19).

Previous studies (20) in the same series of laryngeal lesions documented the presence of altered p53 and Rb proteins, which are correlated with carcinogenesis, probably from an early stage. Concerning p21 protein expression, we found a significant reduction in *in situ* carcinomas, while the other lesions presented a descending frequency of loss from malignant to non-malignant cases. p21 expression was found to be present in 58.9% of laryngeal carcinomas, while in the literature (21) this percentage ranges from 46-82%. We demonstrated a strong positive relationship between p21 and cyclins D and E in cancer cells, thus indicating the co-expression of cyclins and CDKIs in highly proliferative aggressive tumors. It has been shown (22) that p21 protein blocks the interaction between cyclin D1 and the exportin CRM1, leading to increased cyclin D levels in the nucleus. This is probably the reason for the positive association found between cyclin D and p21. It is essential to underline that the balance of the two opposing signals of CDKIs and cyclins/CDKs, rather than the absolute level of the individual signals, should finally determine the proliferation status of tumor cells. Contrary to previous studies (10), we demonstrated a significant positive correlation of this cell cycle inhibitory protein with the Ki67 protein level in cancer cells. This controversy suggests that the association between p21 and cell proliferation may be complex and requires further exploration. Our observation that p21 accumulates in tumor cells, and the lack of correlation with p53 protein, suggests that p53-independent mechanisms may be involved in human laryngeal carcinomas. It also suggests that p21 expression is not linked specifically to p53 functional status, in squamous cell laryngeal cancer, as has been proposed in other cancer types (23).

The main inhibitory action of p27 arises from its binding with the cyclin E-CDK2 complex (24) and its expression level decreases during tumor development (25). We demonstrated that, in accordance with our p21 results, only *in situ* carcinomas presented with a high percentage (50% of the cases) of p27 immunonegativity. On the contrary, all other lesions, including invasive carcinomas, exhibited high levels of p27, with keratotic lesions displaying the strongest positive reaction. It is impressive that only 10.5% of malignant cases had low p27 levels, a percentage that contradicts previous studies (11), which reported that the majority of laryngeal squamous carcinomas (88%) have reduced p27 expression, which is opposed to the strong immunoreactivity of benign (60%) and dysplastic (31%) lesions. Our results do not confirm that loss of p27 reflects the malignant transformation of precancerous lesions and, therefore, its role is not pivotal in laryngeal carcinogenesis. Here, we must emphasise that the phenomenon of *in situ*

carcinomas presenting as the lesions with the maximum degree of p21 and p27 loss needs to be explored in larger samples than the one used in the current study.

In the present study, the percentage of p27-immunostained cancer cells was not correlated with clinical stage and histological differentiation, in contradiction with other studies (26) that demonstrated a significant relationship between p27 and tumor grade. Regulation of cell cycle by p27 is through inhibition of CDKs in cancer cells (27). Since loss of p27 results in an alteration in the balance between proliferating and non-proliferating cells, reduction of p27 expression may lead to tumor growth. In line with this, low mitotic rates should be accompanied with high levels of p27. Although previous studies (11) failed to display any correlation of p27 immunoreactivity and Ki67 expression in laryngeal cancer, we were able to find a significant positive relation, a quite bizarre phenomenon, considering that p27 is a negative regulator of the cell cycle. Specifically in laryngeal cancer cells, aberrant expression of other cell cycle-related molecules, such as CDK2 and cyclin A (28,29), can interfere with DNA synthesis, leading to unbalanced proliferation and thus high proliferation indices. This may indicate that low p27 expression in cancer cells does not simply reflect high replication activity, but may also be related to other factors that influence cell survival or apoptosis (30).

Laryngeal cancer, similar to carcinogenesis models proposed in other sites, may be a multistage process involving a different number of genetic and epigenetic alterations. The accumulation of such changes is thought to underlie the progression of precancerous epithelial dysplasia to invasive carcinomas. We can postulate, based on the current and our previous study (16) concerning cyclins D and E in the same series of patients, that overexpression of both cyclins may act as the first hit in the transformation of pre-malignant to malignant lesions, but the second hit, that could be the down-regulation of CDKIs, does not take place in squamous cell laryngeal carcinomas. Taken together these data support the hypothesis that p21 and p27 are not critically involved in laryngeal carcinogenesis, probably because cancer cells develop complex mechanisms in order to tolerate their negative regulation on the cell cycle.

Acknowledgements

The authors thank Antigoni Christodoulou for her technical assistance.

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Received April 23, 2004
Revised October 5, 2004
Accepted October 11, 2004