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The Cell Cycle Regulators *P16^{INK4a}*, *P15^{INK4b}* and Cyclin D1: Relationship to Clinicopathological Parameters and Disease-free Survival in Laryngeal Carcinoma Patients

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Abstract. *Laryngeal squamous cell carcinoma (LSCC) is a frequent malignancy with a complex and undefined etiology to date. The recently identified cyclin-dependent kinase inhibitor p15^{INK4b} is frequently deleted in human tumors. Previous evidence has pointed to a related gene, p16^{INK4a}, as another target for deletion. Both genes express cyclin D inhibitor proteins. To determine the importance of cell cycle regulators in LSCC relative to more traditional surgical and pathological prognostic factors, p15^{INK4b}, p16^{INK4a} and cyclin D1 analyses were performed. Forty-one malignant tumor tissues and 20 minimal pathological lesions (MPL) of the larynx were examined for deletion of the p16^{INK4a} and p15^{INK4b} genes using polymerase chain reaction. Cyclin D1 expression was studied by Western blotting. Deletions of p16^{INK4a} and p15^{INK4b} were observed in 48.8 % and 51.2% of LSCC patients, respectively. Meanwhile, no deletion was observed in MPL ($p<0.001$). Cyclin D1 was expressed in 43.9% of patients with LSCC versus 30% with MPL ($p=0.29$). Although the frequency of p16^{INK4a} and p15^{INK4b} deletions were higher in advanced than early tumor stages, the difference was statistically insignificant. Ninety percent of patients with deletion of p16^{INK4a} had deletion of the p15^{INK4b} gene. Both cyclin D1 expression and deletion of p15^{INK4b} were found to be independent prognotic predictors of disease recurrence. p16^{INK4a} and p15^{INK4b} gene deletions are exclusively related to malignancy of the larynx. Cyclin D1 expression and p15^{INK4b} gene deletion are potential prognostic indicators of recurrence of LSCC.*

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Despite the lack of precise genetic information, it seems clear that tumors of the head and neck (including squamous cell carcinoma of the larynx) result from the accumulation of changes in genes controlling proliferation as well as apoptosis and invasion (1).

Control of the cell cycle integrates many factors such as cyclins, cyclin-dependent kinases (CDK), viral products and tumor-suppressor gene products. Aberration of normal cell cycle control reflects some of the genetic changes specific to cancer. Cyclin D1 (CCND1) accelerates the G1-phase by binding to CDK4 or 6. The CCND1/CDK4 or 6 complex is activated by the CDK-activating kinase, which phosphorylates a threonine (amino acid 172) in CDK. This activated complex phosphorylates the retinoblastoma (Rb) protein, causing the release of bound transcription factors from the Rb protein. The released E2F transcription factors, mainly E2F1, activate the gene products required for entry into the S-phase (2). Overexpression of CCND1 has been found in 16–64% of head and neck squamous cell carcinomas (3-5).

Both p16 and p15 proteins are inhibitors of the cyclin-dependent kinases that prevent the cell going through the G1/S-phase transition. Inactivation of p16 and p15 are important in cancer development (6). To date, information about *p15^{INK4b}* and *p16^{INK4a}* gene deletion in laryngeal squamous cell carcinoma (LSCC) is very scanty.

This study aimed to analyze the frequency of *p16^{INK4a}* and *p15^{INK4b}* gene deletion and cyclin D1 expression in LSCC. Their relationship to clinicopathological parameters and disease-free survival was also addressed.

Patients and Methods

Patients and tissue samples. The study included a total of 61 subjects divided into two groups: the malignant group, containing 41 patients suffering from laryngeal squamous cell carcinoma (LSCC); the benign control group of 20 patients suffering from minimal pathological lesions (MPL). All patients presented to the Department of Otolaryngology of Ain Shams University Hospital,

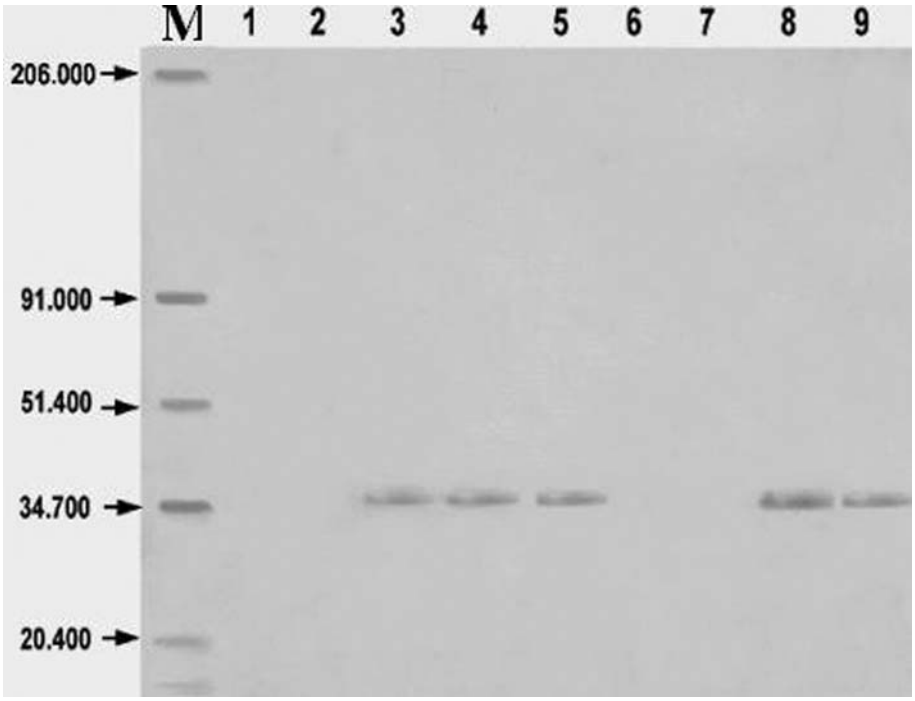

Figure 1. *Western blot analysis of the cyclin D1 in LSCC tissues. M: molecular weight marker. A single band, which corresponds to the molecular weight of cyclin D1, is recognized at 35 kDs in lanes 3, 4, 5, 8 and 9.*

Egypt between May 2002 and July 2004. Written consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All patients were subjected to a full history and clinical examination, focusing on the neck lymph node status. Direct laryngoscopy was performed on all laryngeal carcinoma patients, during which the local extent of the tumor was accurately determined and biopsy specimens were taken from the site of the tumor. Tumors were classified according to the International Union against Cancer Tumor-node-metastasis Classification system (7). All the biopsy materials were excised before any line of treatment was applied. MPL were obtained during micro-laryngosurgury. LSCC patients were followed up for 24 months. Tumor recurrence was detected using direct laryngoscopy and pathological examination of a second biopsy. The surgically excised tissue specimens were divided into two portions: one was processed for histopathological evaluation and the other was frozen at –80˚C for further assay.

Preparation of cell lysates. Tissues were homogenized on ice in an extraction buffer: 10 mM HEPES buffer (pH 7.5 containing 10 mM K_2 EDTA, 50 mM NaCl, 5 mM benzamidine, 10 mg/L Triton X-100, 10 mM 2-mercaptoethanol, 0.39 mM PMSF and 5 mg/L aprotinin) and processed, as previously described (8). After centrifugation at 20,000 x g for 20 min at 4˚C, the protein concentration was estimated in the supernatants (cell lysate) using the Bradford method (9), and was adjusted to 4 μg/ml in all samples. The cell lysates were frozen at –80˚C until Western blot analysis.

Detection of cyclin D1 protein by Western blotting technique (10). Four μl (100 μg total protein) from each sample per lane were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to nitrocellulose membranes. Cyclin D1 was probed with anti-cyclin D1 (Oncogene Science, BO, USA) monoclonal antibodies at a dilution of 1:1000 (in 1% BSA/PBS), respectively, at room temperature (RT) for 1 h. The membranes were then, visualized by incubation with rabbit anti-mouse IgG-alkaline phosphatase conjugate for 90 min at RT. Cyclin D1-positive samples showed a clear band at 35 kDs (Figure 1).

Detection of $p16^{INK4a}$ and $p15^{INK4b}$ gene deletion by polymerase chain reaction (PCR). The biopsy tissues were digested with proteinase K and the high molecular weight genomic DNA was extracted using the phenol-chloroform method (11). Fifty ng each of genomic DNA extracted from the excised tissues and from human placental DNA (the positive control) were subjected to PCR. The primer set for $p16^{INK4a}$ was forward: 5'-GGAAATTGGAAACTGGAAGC-3', and reverse: 5'-CTGCCCATCATGACCTG-3'. It was used to amplify a 167 bp product from an intron-exon boundary of *$p16^{INK4a}$* (Figure 2). The cycling conditions were 40 cycles at 95˚C for 1 min, 62˚C for 30 sec and 72˚C for 30 sec, then final extension for 10 min at 72˚C (12). The primers for $p15^{INK4b}$ were forward: 5'-CCT TAAATGGCTCCACCTGC-3' and reverse: 5'-CGTTGGCAGCC TTCATCG-3'. They were used to amplify a 430 bp product from exon 2 of the *$p15^{INK4b}$* gene (Figure 3). The cycling conditions were initial denaturation at 95˚C for 2 min, followed by 30 cycles at 95˚C for 30 sec, 66˚C for 1 min and 70˚C for 1 min, then final extension for 5 min at 70˚C (13). The PCR products were run on 2% agarose gels and visualized with UV light after staining with ethidium bromide. The quality of genomic DNA for each sample was checked by amplification of the glyceraldehyde-3-phosphate dehydrogenase

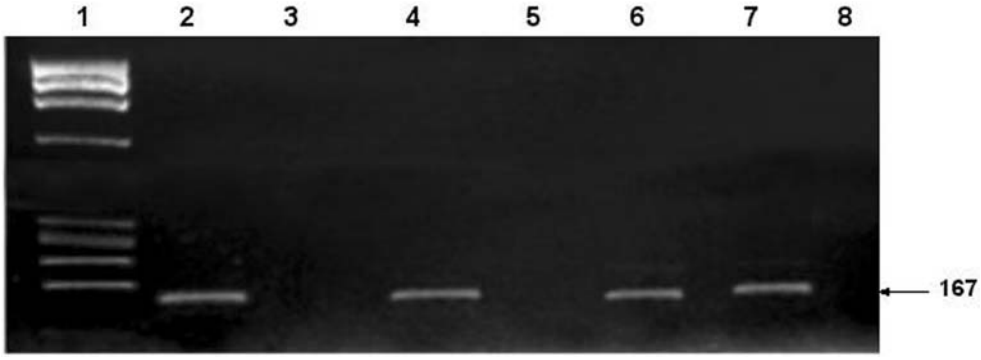

Figure 2. *Analytical gel electrophoresis showing the 167 bp amplification product of the $p16^{INK4a}$ gene. Lane 1: øX174 Hae III DNA molecular weight marker. Lane 2: positive control. Lane 3: negative control. Lanes 4, 6 and 7: LSCC samples having the $p16^{INK4a}$ gene. Lanes 5 and 8: samples with $p16^{INK4a}$ gene deletion.*

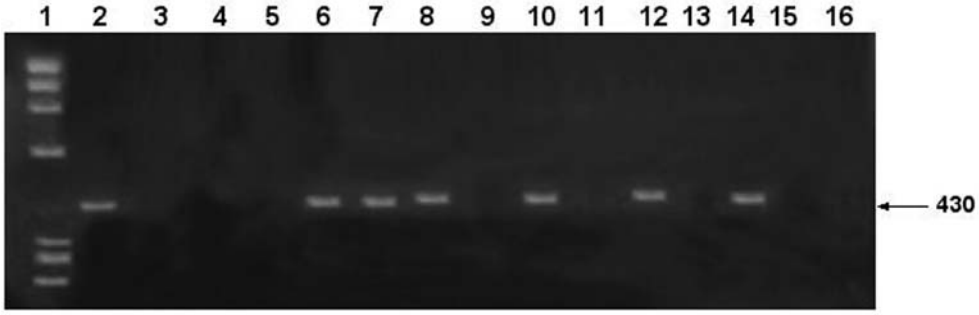

Figure 3. *Analytical gel electrophoresis showing the 430 bp amplification product of the $p15^{INK4b}$ gene. Lane 1: øX174 Hae III DNA molecular weight marker. Lane 2: positive control. Lane 3: negative control. Lanes 6, 7, 8, 10, 12 and 14: LSCC samples having the $p15^{INK4b}$ gene. Lanes 4, 5, 9, 11, 13, 15 and 16: samples with $p15^{INK4b}$ gene deletion.*

(GAPDH) gene using the following primer pair: sense 5'-AAGGCTGGGGC TCATTTGCAG-3', and antisense 5'-CCAAA TTCGTTGTTGTCA TACCAGG-3'. The GAPDH amplification product appeared at 637 bp.

Statistical analysis. The Chi-square and Fisher's exact tests were used for comparison of different variables among various groups. A Kaplan-Meier univariate survival analysis was performed for each single parameter (14). To define independent prognostic parameters for varied follow-up, Cox's proportional hazards model was utilized (15). A p value of less than 0.05 was considered to be statistically significant. The analyses were performed using the Statistical Package for the Social Sciences software, version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

The study included 61 subjects divided into two groups: a malignant group of 41 patients suffering from laryngeal squamous cell carcinoma (age mean ± SD 52.21±8.77), and a control group of 20 patients suffering from minimal pathological lesions (age mean ± SD 43.9±17.03). The male to female ratio for both groups was the same (4:1). Over a total follow-up period of 24 months, 18 out of the 41 patients (43%) had recurrence of the disease, as diagnosed by direct laryngoscopy and pathological examination of the second biopsy.

$p16^{INK4a}$/$p15^{INK4b}$ deletion. $p15^{INK4b}$ was found by PCR to be deleted in 21 patients (51.2%) of the malignant group. On the other hand, no band at the expected size of $p16^{INK4a}$ was detected in 20 patients (48.8%) of the malignant group. No deletion of either $p15^{INK4b}$ or $p16^{INK4a}$ genes was detected in the benign group, with a statistically significant difference from the malignant group ($p<0.001$, Table I). $p15^{INK4b}$ or $p16^{INK4a}$ gene deletion was not related to the stage or pathological grade of the tumor; however, $p15^{INK4b}$ deletion tended to be relatively more frequent than $p16^{INK4a}$ gene deletion in higher stages (57.1% *versus* 51.4%, Tables II and III, respectively). Co-deletion of the two genes was found in 18 patients of the malignant group (43.9%, Table II).

Table I. *$p15^{INK4b}$, $p16^{INK4a}$ gene deletion and cyclin D1 expression in relation to type of tumor using Chi-square analysis.*

	Type		
	Benign (n=20)		Malignant (n=41)
$p15^{INK4b}$			
No deletion	20 (100%)		20 (48.8%)
Deletion	0.0 (0.0%)		21 (51.2%)
X^2		15.622	
p		<0.001*	
$p16^{INK4a}$			
No deletion	20 (100%)		21 (51.2%)
Deletion	0		20 (48.8%)
X^2		14.51	
p		<0.001*	
cyclin D1			
Negative	14 (70%)		23 (56.1%)
Positive	6 (30%)		18 (43.9%)
X^2		1.089	
p		0.297	

*p value is significant.

Table II. *$p15^{INK4b}$ gene deletion in relation to clinicopathological parameters of laryngeal cancer patients using Chi-square analysis.*

	Number	*$p15^{INK4b}$*		X^2	*p*
		No deletion (%)	Deletion (%)		
Stage					
I	1	1(100)	0.0 (0.0)	3.538	0.316
II	5	4 (80)	1 (20)		
III	25	11(44)	14 (56)		
IV	10	4 (40)	6 (60)		
Stage code					
I & II	6	5(83.3)	1(16.7)	3.359	0.067
III & IV	35	15(42.9)	20 (57.1)		
Grade					
1	4	0.0(0.0)	4(100)	4.237	0.12
2	20	11(55)	9 (45)		
3	17	9(52.9)	8 (47.1)		
Recurrence					
No	23	15(65.2)	8(25.6)	5.665	0.017*
Yes	18	5(27.8)	13(72.2)		
$p16^{INK4a}$					
No deletion	21	18(85.7)	3(14.3)	23.5	<0.0001*
Deletion	20	2(10)	18(90)		

*p value is significant.

Table III. *$p16^{INK4a}$ gene deletion in relation to clinicopathological parameters of laryngeal cancer patients using Chi-square analysis.*

	Number	p16^{INK4a}		X^2	*p*
		No deletion (%)	Deletion (%)		
Stage					
I	1	1 (100)	0.0 (0.0)	1.617	0.656
II	5	3 (60)	2 (40)		
III	25	13 (52)	12 (48)		
IV	10	4 (40)	6 (60)		
Stage code					
I & II	6	4 (66.6)	2 (33.3)	0.671	0.413
III & IV	35	17 (48.8)	18 (51.4)		
Grade					
1	4	0.0 (0.0)	4 (100)	5.449	0.06
2	20	10 (50)	10 (50)		
3	17	11(64.7)	6 (35.3)		
Recurrence					
No	23	13 (56.5)	10 (43.5)	0.589	0.443
Yes	18	8 (44.4)	10 (55.6)		

*p value is significant.

Table IV. *Cyclin D positivity in relation to clinicopathological parameters of laryngeal cancer patients using Chi-square analysis.*

	Number	Cyclin D		X^2	*p*
		Negative (%)	Positive (%)		
Stage					
I	1	1(100)	0.0 (0.0)	1.371	0.712
II	5	2 (40)	3 (60)		
III	25	14 (50)	11 (44)		
IV	10	6 (60)	4 (40)		
Grade					
1	4	4 (100)	0.0(0.0)	3.501	0.174
2	20	10 (50)	10 (50)		
3	17	9 (52.9)	8 (47.1)		
Recurrence					
No	23	19 (82.6)	4 (17.4)	14.95	<0.0001*
Yes	18	4 (22.2)	14 (77.8)		
$p15^{INK4b}$					
No deletion	20	13 (65)	7 (35)	1.257	0.262
Deletion	21	10(47.6)	11(52.4)		
$p16^{INK4a}$					
No deletion	21	12 (57.1)	9 (42.9)	0.019	0.89
Deletion	20	11(55)	9 (45)		

*p value is significant.

Table V. *Cox multivariate analysis of risk factors in laryngeal squamous cell carcinoma (LSCC).*

Parameter	Wald Chi-square	*p* value	Risk ratio
Stage	1.016	0.313	-
Histological grade	1.768	0.184	-
$p15^{INK4b}$ deletion	5.799	0.016*	3.69
$p16^{INK4a}$ deletion	3.068	0.08	-
cyclin D1	10.58	0.001*	6.421

*p value is significant

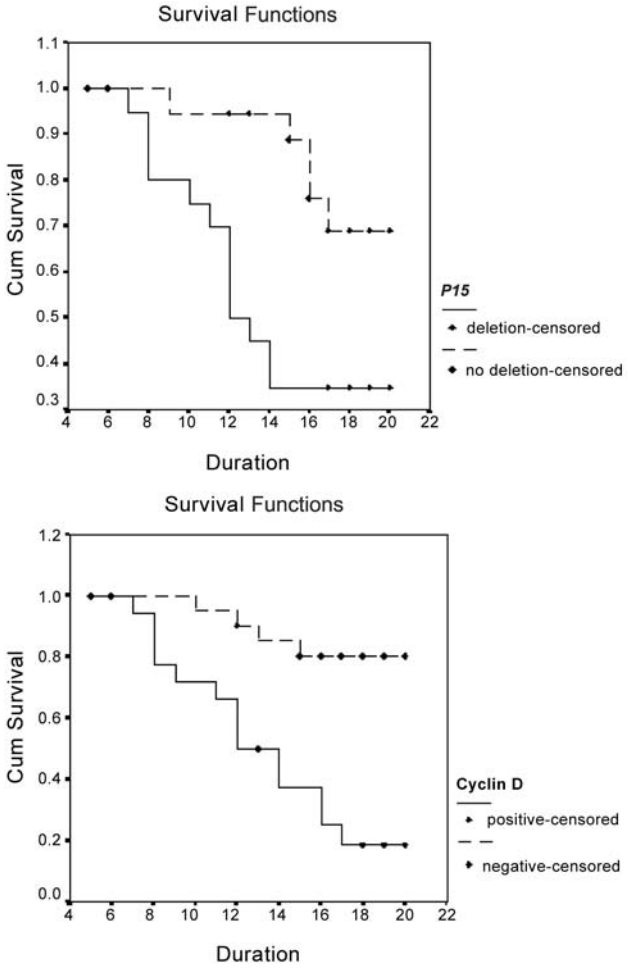

Figure 4. *Kaplan-Meier survival analysis for $p15^{INK4b}$ deletion and cyclin D1 expression in LSCC patients. Log rank test is 7.06 ($p=0.0079$) for $p15^{INK4b}$ and 14.38 ($p=0.0001$) for cyclin D1.*

Cyclin D1 expression. As was revealed by Western blot analysis, there was no statistically significant difference in cyclin D1 expression between the benign and malignant groups (Table I). In the malignant group, cyclin D1 was not related to any of the clinicopathological parameters (Table IV). Although statistically insignificant, this expression of cyclin D1 was more frequent in patients with $p15^{INK4b}$ than those with $p16^{INK4a}$ gene deletion (52.2% and 45%, respectively).

Relationship between $p16^{INK4a}$/ $p15^{INK4b}$ deletion and cyclin D1 to recurrence. Over a follow-up period of 24 months, 18 out of the 41 (43.9%) malignant patients had recurrence of their tumors. A significant relationship was detected between recurrence and $p15^{INK4b}$ deletion (72.2%) and cyclin D1 expression (77.8%) (Tables II and IV). Using the multivariate Cox regression analysis, the latter were found to be independent prognostic factors for recurrence of LSCC (Table V). Univariate Kaplan-Meier analysis showed that the mean and median disease-free survival was significantly higher in patients who demonstrated no $p15^{INK4b}$ deletion (mean 18.41, median 17 *versus* 14.05 and 12 in patients with $p15^{INK4b}$ deletion, log rank = 7.06, $p=0.008$) or cyclin D1 expression (mean 18.54, median 17 *versus* 14.38 and 13.4 in patients with negative cyclin D1 expression, log rank = 14.38, $p=0.0001$, Figure 4). Neither deletion of $p16^{INK4a}$ or the combined deletion of $p16^{INK4a}$ and $p15^{INK4b}$ was found to be related to tumor recurrence.

Discussion

Head and neck cancer is one of the most distressing human cancers causing pain and affecting the basic survival functions of breathing and swallowing. Mortality rates have not changed despite recent advances in radiotherapy and surgical treatment (16). Although considerable information is available on the expression of individual cell cycle-related genes in different tumor types, no consensus on their clinical relevance for many malignancies has been reached (17-20). Chromosome 9p21, the location of the $p16^{INK4a}$/$p15^{INK4b}$ genes, is frequently altered in LSCC and pre-malignant lesions (21, 22). Both the $p16^{INK4a}$ and $p15^{INK4b}$ genes express cyclin D1 inhibitory proteins (23). The current study is the first to address $p16^{INK4a}$/$p15^{INK4b}$ gene deletion and cyclin D1 expression in Egyptian patients with LSCC.

The high frequency of deletion of both $p16^{INK4a}$ (48.8%) and $p15^{INK4b}$ (51.2%) in our patients with LSCC is consistent with these genes being candidate tumor suppressor genes. In addition, the co-deletion of both $p16^{INK4a}$ and $p15^{INK4b}$ genes in 43.9% of LSCC patients suggests that their loss may be mechanistically important during the process of carcinogenesis. The assumption that

both genes are functionally related and that deletion of one of them may affect the expression of the other cannot be excluded. Consistent with our results, Wing Yuen *et al.* (24) and Wen *et al.* (25) demonstrated reduced expression of p15 and p16 proteins (using immunohistochemistry) in LSCC. Also, Koscielny *et al.* (26) concluded that the inactivation of the tumor suppressor gene $p16^{INK4a}$ plays a role in the carcinogenesis of squamous cell carcinomas of the oral cavity, the pharynx and the larynx. We demonstrated a higher frequency of $p15^{INK4b}$ than $p16^{INK4a}$ gene deletion in advanced than early tumor stage (57.1% *versus* 16.7% for $p15^{INK4b}$, and 51.4% *versus* 33.3% for $p16^{INK4a}$). Consequently, LSCC tumors with deletion of the $p15^{INK4b}$ gene may demonstrate more proliferative cancer behavior and would tend to be of a large size.

The most well understood function of the D-type cyclins is activation of the G1 kinases, cdk4 and cdk6, and the target retinoblastoma gene product (pRb) for phosphorylation and inactivation. pRb can suppress S-phase entry, cause a transient G1 arrest following DNA damage, and is critical in establishing terminal cell cycle withdrawal in cells exposed to differentiation or senescence-inducing signals (27). Normally, there is an auto-regulatory feedback loop between unphosphorylated pRb, cyclin D1 and p16. P16 is able to bind to cdk4 and cdk6 directly, thereby preventing cyclin D-cdk4/6 assembly, as well as inhibiting the enzymatic activity of pre-assembled cyclin cdk by forming stable ternary complexes with them (28). In the current study, the presence of a statistically significant high frequency of the $p16^{INK4a}$ gene in LSCC (48.8%) could reveal loss of this auto-regulatory feedback loop with a consequent increase of cyclin D1 and cell proliferation. Meanwhile, the statistically insignificant increase of cyclin D1 expression in the malignant (43.9%) over the control group (30%) may be accompanied by a compensatory increase of its activity, a feature that cannot be detected by Western blot analysis.

Based on the relatively higher frequency of $p15^{INK4b}$ than $p16^{INK4a}$ gene deletion in LSCC (51.2% *versus* 48.8%), the present data confirm the importance of $p15^{INK4b}$ gene deletion in the pathogenesis of LSCC. The higher association between cyclin D1 and $p15^{INK4b}$ deletion (52.4%) as compared to $p16^{INK4a}$ deletion (45%) would suggest the presence of another feedback loop between $p15^{INK4b}$ and cyclin D1, similar to that previously reported between cyclin D1 and $p16^{INK4a}$ (28). Meanwhile, the lack of any statistically significant association between cyclin D1 expression and the deletion of either of the two genes may indicate a contribution of other inhibitory factors to cyclin D1 expression, such as the p21 protein, the product of *WAF1* (29).

Similar to $p16^{INK4a}$ and $p15^{INK4b}$, no statistically significant relationship could be demonstrated between cyclin D1 expression and high histological grade or advanced tumor stage. This supports the findings reported by Wing Yuen *et al.* for $p16^{INK4a}$ (24) and Haas *et al.* for cyclin D1 (30), but were in contrast to Jares *et al.* (31) and Wen *et al.* (25). These differences could be due to variable methodologies, materials and patient populations used in the different studies.

Evaluation of the examined cell cycle regulators in relation to tumor recurrence over a total follow-up period of 2 years revealed that $p15^{INK4b}$ gene deletion and cyclin D1 expression could be considered as potential independent prognostic indicators of tumor recurrence. This is consistent with the findings of Nogueira *et al.* (32) and Menita *et al.* (33), who reported that cyclin D1 overexpression correlates with poor prognosis in head and neck cancers. No previous reports commented on the relationship between $p15^{INK4b}$ gene deletion and disease-free survival in LSCC. On the other hand, deletion of $p16^{INK4b}$ exerted no influence on tumor prognosis, similar to data reported by Koscielny *et al.* (26).

In conclusion, the presented data confirms that dysfunction of cell cycle regulation is a common event and may play a significant role in the development and progression of LSCC. $p15^{INK4b}$ gene deletion and cyclin D1 expression could serve as potential indicators of this disease recurrence.

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