

ORIGINAL ARTICLE

## Clinical significance of Cyclin D1, FGF3 and p21 protein expression in laryngeal squamous cell carcinoma

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### Summary

**Purpose:** Laryngeal squamous cell carcinoma (LSCC) represents one of the most common cancers of the head and neck and the search for molecular markers is required for early diagnosis, prognosis and optimal therapy. The purpose of this study was to investigate the clinical significance of Cyclin D1, FGF3, p16 and p21 protein expression in LSCC and laryngeal dysplasia (LD) and to evaluate the associations between their expression levels and clinicopathological parameters of patients with LSCC.

**Methods:** Immunohistochemistry was employed to detect and quantify the expression levels of Cyclin D1, FGF3, p16 and p21 in the laryngeal tissues of 48 LSCC patients, 32 patients with LD and 28 subjects with healthy laryngeal mucosa (HLM).

**Results:** Significantly higher percentage of LSCC patients had positive Cyclin D1 expression compared with LD patients and HLM subjects (both  $p < 0.01$ ) and positive FGF3 expression than HLM subjects ( $p < 0.05$ ), while no differences in p16 and p21 positive expression were found

among studied groups. The levels of Cyclin D1, FGF3 and p16 expression, as evaluated by immunostaining score, were significantly higher in patients with LSCC compared with LD and HLM groups (all  $p < 0.05$ ). Cyclin D1 proved to be highly sensitive and specific marker in differentiating LSCC from LD (sensitivity 81.2%, specificity 83.9%), while high sensitivity (81.2%) and lower specificity (41.4%) was observed in differentiating from HLM. Cyclin D1 and p21 expression levels were associated with regional lymph node metastases (both  $p < 0.05$ ) and Cyclin D1 expression levels significantly correlated with LSCC lymphatic invasion ( $\chi^2 = 8.862$ ;  $df = 3$ ;  $p = 0.031$ ).

**Conclusions:** Cyclin D1, FGF3 and p16 are overexpressed in patients with LSCC. Cyclin D1 is a highly sensitive marker in differentiating LSCC from LD or HLM. Cyclin D1 and p21 expression levels may be useful as predictive markers of metastases in LSCC.

**Key words:** Cyclin D1, FGF3, healthy laryngeal mucosa, p21, laryngeal dysplasia, laryngeal squamous cell carcinoma

### Introduction

Larynx represents a common site of malignant tumors with an incidence rate of 20% for LSCC of all head and neck cancers [1,2]. LSCCs with analogous clinical and histomorphologic features may have variable course and different clinical outcomes, which indicate that TNM staging and histological grading are not sufficient for the prediction of tumor progression [3]. Precancerous

lesions are referred to as dysplastic when histopathologic evidence of loss of the normal progressive maturation of cells from the basal layer to the superficial epithelium is present in the absence of invasion. Prognosis and prediction for cancer progression from LD will continue to be based on the histopathological features of LD until more definitive molecular biomarkers are discovered.

Multiple cellular events are believed to occur in the development of LD and LSCC, as proposed

in the field cancerisation theory [4]. Cell cycle genes, cell cycle cyclin proteins, cyclin kinases, oncoproteins, tumor suppressor genes mutations, microsatellite loss of heterozygosity (LOH), nuclear image parameters and DNA ploidy are the markers of oncogenesis and have been investigated in oral and laryngeal carcinomas and dysplasia. These investigation have provided insights into the molecular mechanisms of carcinogenesis and further search for clinically relevant biomarkers would enable early diagnosis, prognosis and optimal therapy of patients with LD and LSCC [5].

Proteins coded by oncogenes or tumor suppressor genes can be detected and quantified in tissues by immunohistochemistry. Cyclin D1 regulates progression into the G1 cell cycle phase [6]. The FGF3 protein binds to fibroblast growth factor receptor 3 (FGFR3) with broad mitogenic and cell survival activities including tumor growth and invasion [7]. p16 (INK4A) is upregulated by the CDKN2A protein [8]. Increased levels of p16 lead to the formation of inhibitory cdk4-6/p16 complexes, a loss of stimulatory cdk4-6/cyclin D complexes, which in turn results in the degradation of free Cyclin D by an ubiquitin-dependent proteasome pathway and inhibits cell cycle progression [9]. p21 protein is a general inhibitor of cyclin dependent kinases (CDKs) and is upregulated by wild-type p53 in response to DNA damage and contributes to G1 cell cycle arrest under these circumstances [10]. In the literature available to us no one has investigated the expression of these four markers in patients with LSCC and LD in the same study.

Therefore, the purpose of this study was to investigate the expression patterns of Cyclin D1, FGF3, and the suppressor proteins p16 and p21 in patients with LSCC and LD and to evaluate their diagnostic and prognostic value.

## Methods

### *Study patients*

This retrospective study comprised 108 consecutive patients (18 females and 90 males, mean age 58 years, range 20-87 years) who suffered from hoarseness and were referred to the Clinic of Otorhinolaryngology, Clinical Centre, Kragujevac, Serbia, for microlaryngoscopic examination and biopsy. The diagnosis was based on clinical and histopathological criteria. The diagnosis, clinical staging and identification of the anatomical site of the LSCCs and the degree of laryngeal dysplasia were based on the UICC's TNM classification of malignant tumors [11]. The grade of tumor differentiation was determined according to the WHO criteria

[12]. The type of epithelial morphology was classified as HLM (N=28), LD (N=32) and LSCC (N=48). LD was graded as mild (N=9), moderate (N=13) and severe (N=10).

The clinicopathological characteristics of LSCC patients included age, gender, family and personal history of malignancies, alcohol and tobacco use, anatomic subregion (supraglottis, glottis and subglottis), degree of dysplasia, type of tumor growth, TNM stage, clinical stage, histological grade, nuclear grade, in situ component, invasiveness, vascular, lymph and perineural spreading, necrosis, mitotic index, mononuclear stromal reaction and desmoplastic reaction.

Regional metastases were detected by microlaryngoscopy, 64-slice multidetector computed tomography of the neck (MD-CT) and neck ultrasound, while distant metastases were diagnosed by chest X-rays and abdominal ultrasonography.

The design of the study was approved by the Ethics Committee of the Clinical Centre Kragujevac. All enrolled patients gave written informed consent to participate in the study.

### *Immunohistochemistry (IHC)*

The expression of Cyclin D1, FGF3, p16 and p21 proteins was assessed using IHC. Laryngeal biopsy specimens were routinely fixed in 4% buffered formaldehyde, dehydrated through graded alcohols, cleared in xylene, and subsequently embedded in paraffin. The paraffin-embedded tissue samples were sectioned at 4-5  $\mu\text{m}$  and were deparaffinized by two washings in xylene for 10 min and rehydrated in a series of 100, 96, 70 and 50% alcohol solutions. IHC staining was performed by the streptavidin-biotin method. Briefly, sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity and then microwaved for 20 min in 10 mmol/L sodium citrate (pH 6.0). The sections were incubated with mouse monoclonal antibodies against Cyclin D1 (1:1000 dilution; Clone CD1.1; AbD Serotec, Oxford, UK), FGF3 (1:500 dilution; Clone 254625; R&D Systems, Inc., Minneapolis, USA), p16 (1:100 dilution; Clone D25; Millipore, Maryland, USA) and p21/CIP1/CDKN1A (1:200 dilution; Clone 195720; R&D Systems, Inc., Minneapolis, USA.) for 60 min. After the incubation with primary antibodies, biotinylated secondary antibodies were applied, followed by detection using the ABC (Avidin-Biotin peroxidase Complex) method (Mouse UniTect™ ABC Kit, CalBiochem, USA). Diaminobenzidine was used as chromogen. Counterstaining was performed with haematoxylin. Negative controls were obtained using an irrelevant antibody provided in the staining kits (Anti-trpE Mouse monoclonal antibody, Clone I22-25). IHC staining was evaluated independently by three pathologists in the blinded fashion. Only nuclear staining was considered as positive staining for Cyclin D1, FGF3, p16 and p21. Based on the reported data, staining for Cyclin D1, FGF3, p16 and p21 was defined as positive when >10%

of the cells stained positive and negative when <10% of the cells were stained positive [13-15]. The staining was further quantified using a semi-quantitative scoring system as described: score 0, < 10% cells; score 1, 10-30% positively stained cells; score 2, 30-50% positively stained cells; score 3, >50% positively stained cells [15,16].

### Statistics

SPSS for Windows, Release 10.0, (SPSS Inc, Chicago, Ill) was used for statistical analyses. The statistical significance of positive/negative staining of tumor markers among the studied groups was tested by the non parametric Mann-Whitney Rank Sum test or Student's unpaired t-test, where appropriate. Associations between categorical variables were tested with the Pearson chi-square test. Specificity and sensitivity of each single parameter were examined by ROC curve analysis. Cohen's Kappa ( $\kappa$ ) value was used for statistical evaluation of three independent investigators agreement. A  $\kappa$  value of 1 indicated full agreement, while a  $\kappa$  value of 0 indicates agreement by chance. Intraobserver scores showed good agreement ( $\kappa=0.53-0.73$ ).

## Results

### *Demographic and clinicopathological characteristics of study patients*

Clinical and pathological characteristics of the patients with LSCC are presented in Table 1. The patients with LSCC were older than subjects with HLM and patients with LD, but the difference was not statistically significant. There were no significant correlations between the expression of Cyclin D1, FGF3, p16 or p21 with gender, use of alcohol, use of tobacco, anatomical localization of the tumor, type of carcinoma growth (vegetative/infiltrative), TMN stage, histological grade, nuclear grade, invasion, vascular invasion, lymph node invasion, perineural invasion, necrosis, mitotic index and mononuclear stromal reaction (data not shown).

### *Cyclin D1, FGF3, p16 and p21 expression in LSCC, LD and HLM*

The study subjects were classified on the basis of positive or negative expression of Cyclin D1, FGF3, p16 and p21 in laryngeal tissues. The positive expression of Cyclin D1 was found in significantly higher percentages of LSCC patients (39/48) compared with patients with LD (5/32) and the HLM group (17/28) ( $\chi^2=26$ ,  $p<0.0001$ ;  $\chi^2=10.047$ ,  $p=0.0066$ , respectively) (Figure 1A, Figure 2A, Figure 2B). Positive expression of Cyclin

D1 was detected in significantly higher percentages of HLM subjects in comparison with patients with LD (5/31) ( $\chi^2=4.762$ ,  $p=0.029$ ). The positive expression of FGF3 was significantly more frequently observed in LSCC patients (39/48) than in those with HLM (23/28) ( $\chi^2=7.848$ ,  $p=0.0198$ ) (Figure 1B). No significant differences of patients with positive expression of p21 and p16 were observed among the studied groups.

### *Semiquantitative analysis of Cyclin D1, FGF3, p16 and p21 expression in LSCC, LD and HLM*

The semiquantitative analysis of Cyclin D1, FGF3, p16 and p21 expression in LSCC, LD and HLM was done according to the scoring system described in Methods.

The frequency of LD patients with negative Cyclin D1 expression (score 0) was significantly higher (26/31;  $\chi^2=10.093$ ,  $p=0.0064$ ) compared with HLM (12/28) and LSCC patients (9/48; Figure 3A). The percentage of LSCC patients with staining score 1 for Cyclin D1 (19/48) was significantly higher compared with HLM subjects (8/28) and LD patients (4/31;  $\chi^2=11.241$ ,  $p=0.0036$ ). The frequency of LSCC patients (9/28) and HLM subjects with Cyclin D1 score 2 was significantly higher than LD patients (1/31;  $\chi^2=9.909$ ,  $p=0.0071$ ;  $\chi^2=4$ ,  $p=0.0455$ , respectively). In contrast to patients with LSCC none of HLM subjects or LD patients had score 3 Cyclin D1 expression.

There was no significant difference in the frequency of patients with FGF3 expression score 0, 1, and 2 between patients with HLM, LD or LSCC. The frequency of patients with LSCC (23/48) or LD (13/21) with positive staining for FGF3 score 3 was significantly higher compared with HLM subjects (3/28) ( $\chi^2=13.500$ ,  $p=0.0012$ ;  $\chi^2=4.267$ ,  $p=0.0389$ , respectively) with no significant difference between LSCC and LD patients (Figure 3B).

No significant difference in the frequency of patients with p16 expression score 0, 1, and 2 was observed among the studied groups with different laryngeal epithelial cell types. A higher frequency of LSCC patients (20/36) with p16 expression score 3 was observed when compared with LD patients (3/31;  $\chi^2=13.400$ ,  $p=0.0012$ ), but not with subjects with HLM (8/21; Figure 3C). Similarly, there was no significant difference in the percentage of patients with p21 expression score 0, 1, and 2 between the studied groups. The frequency of patients with score 3 p21 expression was significantly higher in the LSCC group (25/46) compared with HLM (7/28) and LD patients (9/31;  $\chi^2=12.684$ ,  $p=0.0018$ ) (Figure 3D).

**Table 1.** Demographic, clinical, and pathological characteristics of patients with laryngeal squamous cell carcinoma

Variable	Number of patients (%)	Variable	Number of patients (%)
Carcinoma growth		Intraepithelial carcinoma	
Vegetative	31 (64.6)	Positive	19 (39.6)
Infiltrative	6 (12.5)	Negative	29 (60.4)
Ulcerovegetative	8 (16.7)	Invasiveness	
Ulceroinfiltrative	3 (6.2)	Positive	46 (95.8)
Tumor stage		Negative	2 (4.2)
T1	9 (18.8)	Vascular invasion	
T2	10 (20.8)	Positive	3 (6.2)
T3	22 (45.8)	Negative	45 (93.8)
T4	7 (14.6)	Lymph node invasion	
Regional nodal stage		Positive	6 (12.5)
N0	40 (83.3)	Negative	42 (87.5)
N1	1 (2.1)	Perineural invasion	
N2	6 (12.5)	Positive	4 (8.3)
N3	1 (2.1)	Negative	44 (91.7)
Distant/metastatic stage		Necrosis	
M0	46 (95.8)	Negative 0	10 (20.8)
M1	2 (4.2)	Positive 1	21 (43.8)
Clinical stage		Positive 2	11 (22.9)
I	12 (25)	Positive 3	6 (12.5)
II	10 (20.8)	Mitotic index	
III	17 (35.4)	Positive 1	17 (35.4)
IV	9 (18.8)	Positive 2	28 (58.3)
Histological grade		Positive 3	3 (6.3)
1	23 (47.9)	Mononuclear stromal reaction	
2	21 (43.8)	Negative 0	2 (4.2)
3	4 (8.3)	Positive 1	15 (31.2)
Anatomical location		Positive 2	18 (37.5)
Supraglottic	8 (16.7)	Positive 3	13 (27.1)
Glottic	9 (18.7)	Desmoplastic reaction	
Supraglottic and glottic	27 (56.3)	Negative 0	2 (4.2)
Supraglottic, glottic and subglottic	4 (8.3)	Positive 1	30 (62.5)
Nuclear grade		Positive 2	16 (33.3)
1	8 (16.7)		
2	35 (72.9)		
3	5 (10.4)		

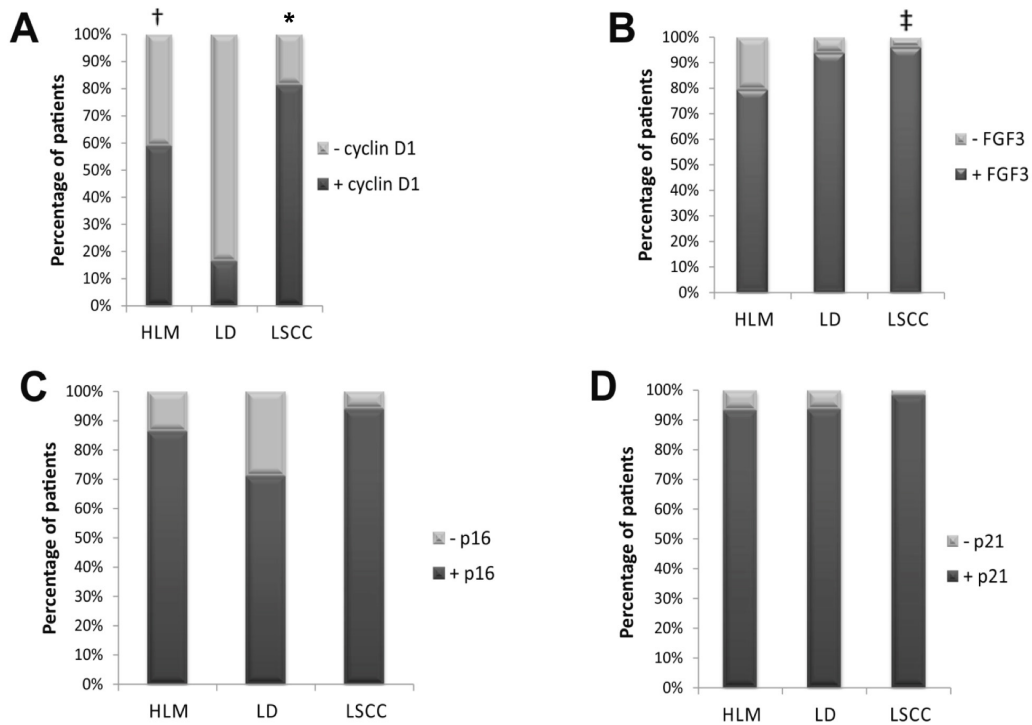
Given are the number and percentage of patients per categorical and ordinal variables

We found significant positive correlation of both Cyclin D1 and p21 expression with the presence of metastasis in regional lymph nodes (both  $p < 0.05$ ), while FGF3 and p16 immunoreactivity in patients with LSCC was not significantly associated with metastasis in lymph nodes of the neck (not shown). Furthermore, the increase of Cyclin D1 expression score significantly correlated with the presence of lymphatic invasion in LSCC

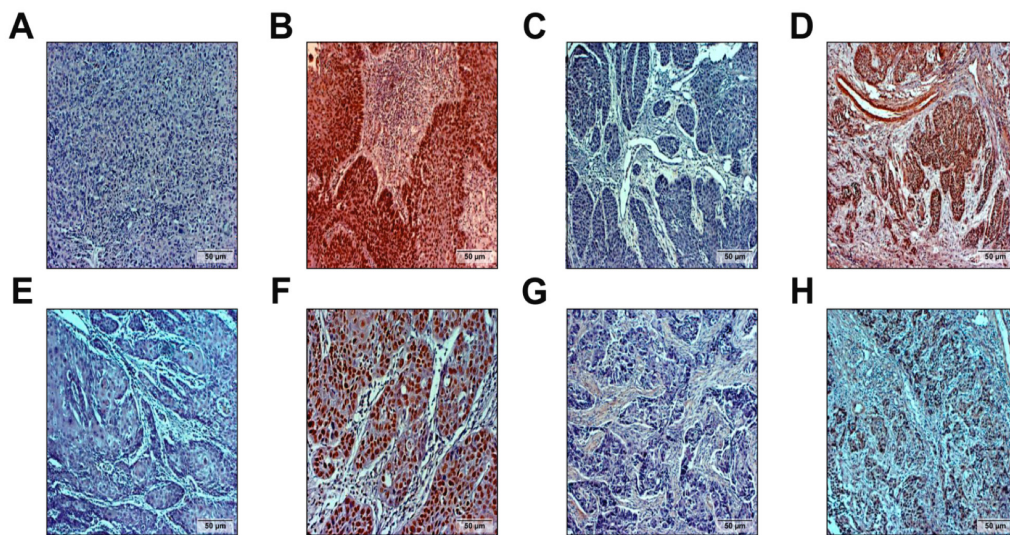
( $\chi^2 = 8.862$ ;  $df = 3$ ;  $p = 0.031$ ).

*Logistic regression analyses of Cyclin D1, FGF3, p16 and p21 expression and laryngeal epithelial tissue types*

In the case of positive expression of Cyclin D1, the odds/likelihood of LSCC increased 22.5-fold ( $p < 0.0001$ , odds ratio/OD = 22.53, 95% CI = 6.78 to 74.86) compared with LD and 3-fold increase com-



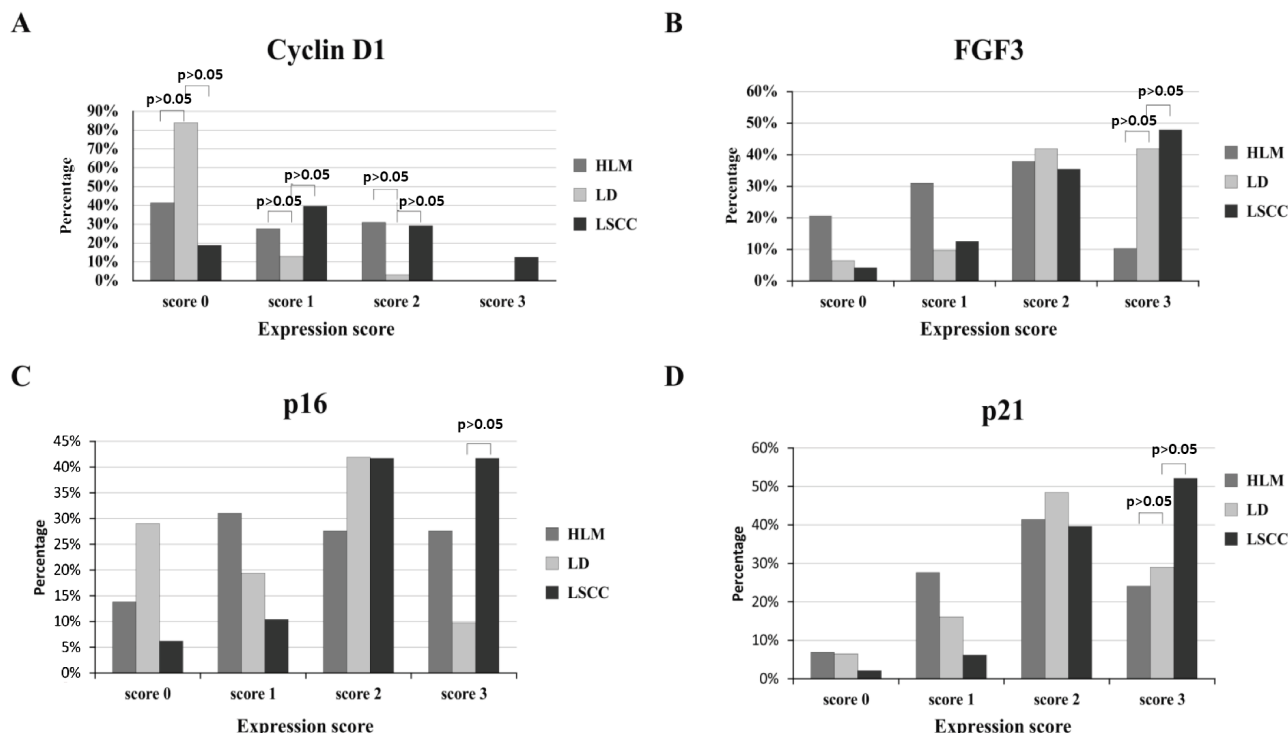
**Figure 1.** The frequency of patients with positive expression of Cyclin D1 (A), FGF3 (B), p16 (C) and p21 (D) within the groups of subjects with healthy laryngeal mucosa (HLM), patients with laryngeal dysplasia (LD) and patients with laryngeal squamous cell carcinoma (LSCC). \* $p < 0.05$ , LSCC vs HLM, LD; † $p < 0.05$ , LSCC vs HLM; ‡ $p < 0.05$ , HLM vs LD ( $\chi^2$ ,  $p$ ).



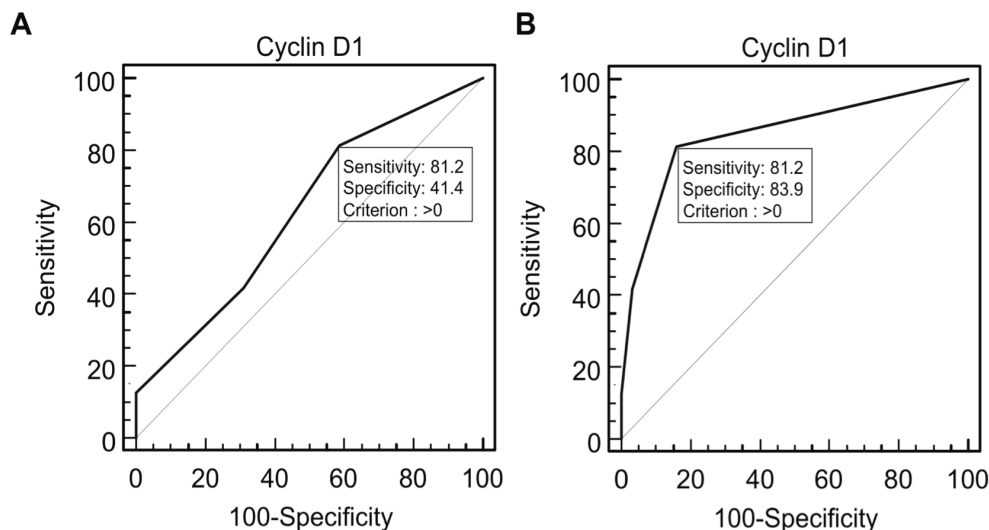
**Figure 2.** Cyclin D1, FGF3, p16 and p21 expression in laryngeal squamous cell carcinoma. A. Negative cyclin D1 staining (40x). B. Positive cyclin D1 staining (40x). C. Negative FGF3 staining (40x). D. Positive FGF3 staining (40x). E. Negative p16 staining (40x). F. Positive p16 staining (40x). G. Negative p21 staining (40x). H. Positive p21 staining (40x).

pared to HLM ( $p=0.0343$ , OR=3.06, 95% CI=1.09 to 8.61). Analysis of positive Cyclin D1 expression in the HLM and LD groups indicated 7.4-fold likelihood of HLM ( $p=0.0012$ , OR=7.37, 95% CI=2.20

to 24.68). The increase of FGF3 expression indicated 6-fold OR/likelihood of LSCC compared to HLM ( $p=0.0362$ , OR 6, 95% CI=1.12 to 32.09). In the case of p16 positive expression 6-fold increase



**Figure 3.** The frequency of patients with graded Cyclin D1, FGF3, p16 and p21 expression (immunostaining scores 0-3) in subjects with healthy laryngeal mucosa (HLM), patients with laryngeal dysplasia (LD) and patients with laryngeal squamous cell carcinoma (LSCC). **A:** Frequency of patients with different cyclin D1 expression scores ( $p < 0.05$ , LSCC vs HLM, LD;  $p < 0.05$ , LSCC vs LD;  $p < 0.05$ , HLM vs LD). **B:** Frequency of patients with different FGF3 expression scores ( $p < 0.05$ , LSCC vs HLM;  $p < 0.05$ , LD vs HLM). **C:** Frequency of patients with different p16 expression scores ( $p < 0.05$ , LSCC vs LD). **D:** Frequency of patients with different p21 expression scores ( $p < 0.05$ , LSCC vs HLM, LD). ( $\chi^2$ ,  $p$ ).



**Figure 4.** Specificity and sensitivity of Cyclin D1 expression. **A:** ROC curve illustrates the specificity and sensitivity of Cyclin D1 expression, comparing LSCC with HLM. **B:** ROC curve illustrates the specificity and sensitivity of Cyclin D1 expression, comparing LSCC with LD.

of likelihood was noticed for LSCC compared to LD ( $p = 0.0112$ ,  $OR = 6.14$ ,  $95\% CI = 1.51$  to  $24.95$ ). The positive p21 expression was not significant-

ly associated with the laryngeal epithelial tissue types studied.

The expression of Cyclin D1 proved to be a

highly sensitive and specific marker in differentiating LSCC from LD (sensitivity 81.2%, specificity 83.9%, cut-off >0; Figure 4B), and showed high sensitivity (81.2%) and lower specificity (41.4%; cut-off >0) in differentiating LSCC from HLM (Figure 4A).

## Discussion

In this study we showed that positive immunostaining for Cyclin D1 and FGF3 was significantly higher in the LSCC group compared with LD and HLM groups. The positive Cyclin D1 expression is a highly sensitive and specific marker in differentiating LSCC from LD, and shows high sensitivity but lower specificity in differentiating LSCC from HLM. In addition, Cyclin D1 and p21 expression levels may be useful as predictive markers of metastases in LSCC. As LSCCs are known to have variable clinical course and clinical outcomes, evidence indicates that TNM staging and histological grading are not sufficient for predicting prognosis of LSCC [17]. Search for specific and sensitive tumor markers is required for improvement of early diagnosis, prognosis and therapeutic interventions in patients with LSCC.

In this study the investigated tumor markers were oncogenes (Cyclin D1, FGF3) and tumor suppressor genes (p16, p21) that are involved in the cell cycle regulatory machinery, deregulation of which is a fundamental hallmark of carcinogenesis [18]. The cell cycle regulatory process is intimately linked to cell proliferation, differentiation, senescence, and apoptosis. Most of the genetic alterations that cause abnormal biologic behaviors in cancer cells are usually aberrations in the cell cycle regulation [19]. The core component of the regulatory cell cycle machinery is a family of enzymes cyclin-dependent kinases (CDKs), which regulate a number of target molecules by phosphorylation. CDKs are activated by a group of positive regulators called cyclins, such as Cyclin D1, and are inhibited by an emerging set of proteins, including p15, p16, p21 and p27 [20]. The reported data indicate various genetic changes and overexpression of tumor markers in a wide range of human tumors, particularly in head and neck squamous cell carcinomas (HNSCC) and LSCC [21]. Overexpression of Cyclin D1 has been observed in primary breast cancers, hereditary colorectal cancers, head and neck carcinomas, thyroid carcinomas, oesophageal cancer, liver carcinomas and in 19-58% of patients with LSCC [22-24].

We found no correlation among clinicopathological parameters and the expression scores of

the investigated tumor markers. The obtained results in this study show that the percentage of patients with positive Cyclin D1 expression was lower in the LD group than in subjects with HLM. Our results were in line with the Ohbu et al. study [25] who found positive Cyclin D1 expression in higher percentages in subjects with normal epithelium compared with patients with dysplastic laryngeal epithelia. Alterations in FGF3 gene to varying degrees have been reported in other studies and FGF3 gene was found to be amplified in 37% of the cases of head and neck cancer [26]. No association was found between p16 expression and conventional clinicopathological factors in HNSCC, which is consistent with our study [27]. Allegra et al. recently showed that expression of BMI-1 marker in the absence p16 expression may identify a subset of patients at higher risk for lymph node metastasis [28]. Previous studies reported detection of p16 protein expression in 58% of LSCC patients [29] and an association between p16 protein expression and epithelial types [30]. This is in agreement with our data which indicate that the expression of Cyclin D1, FGF3 and p16 depends on the laryngeal epithelial cell type. These proteins were expressed in significantly higher percentage of patients with LSCC in comparison with HLM subjects and LD patients. In our study score 3 p16 expression differentiated LSCC patients from those with LD, but not from HLM subjects. It is not clear why the expression of p16 and p21, as products of tumor suppressor genes, were increased in laryngeal carcinoma cells. One possible explanation is that p16 overexpression might indicate a dysfunction in the regulatory complex for G1 arrest in the malignant transformed cells [31].

Significantly higher numbers of LSCC patients had immunostaining score 3 of some of the investigated tumor markers compared with HLM subjects or LD patients. Namely, the Cyclin D1 expression score 3 was found only in LSCC patients and significantly higher numbers of LSCC patients expressed p21 score 3 in comparison with HLM and LD patients. The expression of score 3 FGF3 differentiated patients with healthy laryngeal mucosa from those with pathologic laryngeal mucosa. The association between Cyclin D1 and p16 overexpression and squamous cell carcinoma has also been previously reported [32].

While the expression of the studied tumor markers may be useful in the assessment of advanced LSCC, only Cyclin D1 expression is valuable in early cancer diagnosis. Cyclin D1 showed

a high sensitivity and specificity for LSCC in differentiating from LD and high sensitivity but moderate specificity for LSCC in differentiating from HLM. As previously reported [33], LD led to LSCC 4.4% of the patients, and long-term follow-up studies showed that malignant transformation had occurred in more than half of the patients with severe LD. We demonstrated that the positive expression of Cyclin D1 could increase 22.5-fold the likelihood for LSCC. Huang et al. [34] reported that Cyclin D1 overexpression is an independent prognostic factor for oral cavity squamous cell carcinoma.

We observed significant correlation between higher Cyclin D1 and p21 expression with the presence of regional lymph nodes metastasis. In addition, higher Cyclin D1 expression scores correlated with the presence of lymphatic invasion in LSCC, indicating that Cyclin D1 overexpression may be a useful marker of laryngeal cancer progression. Adenosquamous carcinoma (ADSC), a variant of LSCC often hides human papilloma virus (HPV). p16 is a surrogate marker of HPV, the signals of which activate viral transcription, and may be expressed in ADSC [35]. We did not evaluate LSCC patients for the presence of HPV and this is a limitation of our study. Apart from the high expression of Cyclin D1 and FGF3, p16 and p21

tumor suppressors were also highly expressed in LSCC which might be attributed to the fact that these anti-oncogenes could be mutated and could not function in laryngeal cancer [36].

The molecular biomarkers Cyclin D1, FGF3 and p16 are overexpressed in patients with LSCC. Cyclin D1 expression level shows high sensitivity and specificity for differentiating LSCC from LD. Cyclin D1 and p21 expression scores could serve as predictive markers for metastases in LSCC. The obtained results suggest that Cyclin D1, FGF3 and p16 may play important roles in the development of LSCC and further studies are needed to clarify their roles in laryngeal carcinogenesis.

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