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DIAGNOSTIC ABILITY OF CYCLIN D1 AND PRO APOPTOTIC MARKERS CASPASE 3 AND P21 FOR DETECTION OF REGIONAL METASTASES IN LARYNGEAL CANCER PATIENTS

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Annotation. *Laryngeal squamous cell carcinoma has a high ability to metastasis into regional lymph nodes. The study of molecular markers of cyclin D1 showed its expression at the level of 58.3 (18.5)% (M (SD)) for the group of laryngeal squamous cell carcinoma without metastases - 48.6 (17.7)%, for the group with metastases - 66.0 (15.3)% (P <.001), the investigated level of expression of p21WAF ranged from 18% to 94% and on average amounted to 55.3 (22.1)%, for the group without metastases - 45.2 (26.0)%, for the group with metastases - 63.3 (14.3)% (P <.001). High expression of caspase 3 was detected in 40 (57.1)% and it was significantly lower (P <.001) in the group without metastases 10 (32.3)% as compared with the group of laryngeal cancer patients with regional metastases - 30 (76.9)%. In statistical analysis of the data obtained it was established that cyclin D1 has the highest value of AUC (0.780), caspase-3 - somewhat lower (0.719) and p21WAF has the lowest one (0.694) (P <.01). Investigation of these markers can be used for determining the prognosis of the disease course and the choice of extent of lymph dissection. No reliable association was revealed between the expression of these markers with recurrent laryngeal squamous cell carcinoma and the degree of tumor differentiation. Additional studies of these markers can deepen our knowledge on molecular biology of the tumor and prognosis of the disease course.*

Keywords: *Laryngeal squamous cell carcinoma, metastases, molecular markers, cyclin D1, caspase 3, p21WAF.*

Introduction. Laryngeal cancer is a frequent localization of squamous cell carcinoma of the head and neck [1, 2]. In spite of advances in surgical, radiation and other curative methods of treatment of squamous cell carcinoma of the head and neck, the survival of patients with this disease remains unchanged. The main factor influencing the survival of patients is the presence of regional metastases.

Such fundamental principles of prognosis of tumorous course, specifically metastasis, as TNM classification or the degree of tumor differentiation are being used for a long time. There are two opposite approaches to the management of patients with laryngeal cancer with N0 - the strategy of «wait and watch» and preventive intervention on the lymphatic system of the neck [3-5].

Considering new knowledge, the study of molecular features of the tumor makes it possible to predict aggressive tumor growth and probability of tumor metastasis. Determination of tumor aggression and prognosis of metastasis can be used to decide

the disputable issue of the extent of surgical intervention on the lymphatic system of the neck [6].

Cyclin D1 is a protein responsible for the G1 and S phase of the mitotic cycle in normal and tumor cells, in its expression in tumor cells one can expect shortening of G1 phase and very high proliferative activity[2,7]. Some scientific studies suggest that overexpression of cyclin D1 in squamous cell carcinomas of the head and neck is associated with poor prognosis for patients, continued growth, locoregional recurrences and metastasis [1,2,7].

Some studies point at the relationship of tumor aggression and its resistance to treatment with disorder of apoptosis of cancer cells. Disorder of apoptosis processes leads to uncontrolled and aggressive growth of the tumor. Caspase 3 is closely related to such essential components of apoptosis as TNF- α [8]. The relationship of caspase-3 with a poor prognosis of malignant tumors for other localizations has been proven [9].

Research of Chiappara G. et al. (2014) on effector caspase-3 as a distributor of apoptosis and cytoplasmic expression of inhibitor of cyclin-dependent kinase p21WAF1, involved in the arrest of growth of cellular cycle through such stress factors as cigarette smoke in samples of airways epithelium of smokers, showed interesting results. In the epithelium of smokers there is determined increased expression of p21 and of activated caspase-3, which correlate with each other, and thus they play the role of pro-apoptotic. Besides, change in the expression of p21 may be associated with inhibition of tissue repair in smokers, as evidenced by the low level of expression of such proliferation markers as Ki-67 and PCNA [7].

Materials and methods. The study was approved by the ethics committee of the SE «DMA» and ME «RCHM.» Histological material for research was taken from the archives of ME “Regional pathologicoanatomic office». All patients gave consent to the processing of personal data and participation in the study. To perform tasks of the research under the supervision there were 70 patients with laryngeal cancer III - IV stages (T3-4N0-3M0) and II clinical group who underwent examination, treatment and follow-up in ENT- oncology department of Dnipropetrovsk Regional Clinical Hospital named after I.I. Mechnikov over the period from 2011 to 2014. Of them, 39 patients of the main group had regional metastases of laryngeal cancer, in 31 patients of comparison group regional metastases were not revealed. The age of patients ranged from 33 to 74 years. Male patients were in the absolute majority - 99% No=69. In all patients squamous cell carcinoma was diagnosed histologically. The demographic characteristics of patients is presented in the Table 1.

All patients underwent surgical treatment – laryngectomy and variants of lymphodissection. Histological examination and evaluation of expression of immunohistochemical markers of laryngeal squamous cell carcinoma (LSCC) was performed by two independent pathologists according to WHO recommendations on histological classification of laryngeal tumors [10].

Immunohistochemical study. Histological sections of 4-6 mkm thickness were applied on adhesive subject glasses SuperFrost Plus, after dewaxing and rehydration,

temperature retrieval of antigens was performed - sections were placed in citrate buffer with pH 6.0 and heated in the autoclave at a temperature +121°C during 8 minutes, activity of endogenous peroxidase was inhibited by 3% hydrogen peroxide solution during 20 minutes. Further, incubation of sections with primary antibodies was performed in humid chambers at a temperature of 23-25°C during 30 minutes.

Monoclonal antibodies to cyclin D1, (Clone SP4, ThermoScientific, USA) in dilution 1:100, caspase-3 (Clone 31A893, ThermoScientific, USA) in dilution 1:50 and p21 WAF1 (Clone SR74, ThermoScientific, USA) were used as primary ones. Specimens were stained with Meyer hematoxylin and fixed.

Evaluating IHC reactions with marker cyclin D1, the level of intranuclear expression was calculated as the percentage of cells with nuclear reaction to 1000 keratinocytes in image enlargement ($\times 400$) by 4 gradations: (0) less than 2% of stained cells, (1+) – from 2% to 25%, (2+) - from 26% to 50%, (3+) - from 51% to 75%, (4+) - 76% to 100%. Gradations (0 - 2+) ($<50\%$) were regarded as a low expression and (3+ - 4+) ($> 50\%$) as high, by recommendations of Gülçin Şimsek et al [11] Fig. 1.

Investigating effector caspase 3 by means of monoclonal antibody Caspase 3, intensity score was determined by the following gradation: from 0 - no reaction, 1- weak reaction (a little number of stained cells or pale color staining), 2 - the greatest median staining, 3 - strong staining of almost all cells, by recommendations [12,13]. Fig.1.

In the samples of LSCC marker p21 demonstrated exclusively intranuclear expression, which was calculated as a percentage of stained brown nuclei per 100 of tumor cells. For statistical analysis the obtained data of expression were divided into two groups: high expression ($> 20\%$) and low ($\leq 20\%$)[14,15]. Fig.1.

Statistical data processing. Validation for compliance of distribution of quantitative characteristics to normal law was performed by Shapiro-Wilk criterion (The Shapiro-Wilk test). In terms of normal distribution for comparison of continuous data in different groups, double-sided t-test (t test) was used. For comparison of continuous data which were not distributed normally, two-tailed Mann-Whitney's test (Mann-Whitney U test) was used. Probability of differences of categorical data was evaluated by criterion Pearson's Xi-square (χ^2) (Pearson Chi-square), including Yates' continuity-corrected (Yates Chi-square) for the values close to 0 or 100%.

To assess discriminatory power of different immunohistochemical markers for prediction of regional metastasis of laryngeal cancer (diagnostic value of the test) ROC-analysis was used. (ROC Analysis, ROC - Receiver Operating Characteristic). The result was represented as the value of the area under the ROC-curve (AUC - Area Under Curve), built on indicator values of sensitivity (Se) and specificity test (SP) and with involving of 95% confidence interval (95% CI). ROC-analysis was considered adequate in probably significant (PS) difference of AUC value from 0.5 value. Calculations of standard error of the area under the curve (AUC) and difference between the two AUCs were carried out by the method of DeLong et al. (1988) [16].

By the shape of the ROC curve and the area under it (AUC) comparisons of predictive powers of diagnostic markers were made. The closer is the curve to the upper

left-hand corner, and accordingly, the larger the area under the curve, the better is the discriminatory power of the test. Values of the area under the ROC curve were interpreted in terms of diagnostic accuracy by Šimundić A-M. (2009): 0.9-1.0 - excellent, 0.8-0.9 - very good, 0.7-0.8 – good, 0.6-0.7 - average, 0.5-0.6 - poor; value of 0.5 points at invalidity of the method[17].

Statistical processing of the data was performed using the statistical software R (<https://cran.r-project.org>) and MedCalc Version 17.2 free trial version (<https://www.medcalc.org/download.php>).

Research results. Expression index of cyclin D1 ranged from 18% to 94% and on average amounted to 58.3 (18.5)% (M (SD)), for the group of LSCC without metastases it was 48.6 (17.7)%, for the group with metastases - 66.0 (15.3)% (P <.001). Level of P21WAF expression ranged from 18% to 94% and on average amounted to 55.3 (22.1)%, for the group without metastases - 45.2 (26.0)%, for the group with metastases - 63.3 (14.3)% (P <.001) - Table 1.

The frequency of high expression of caspase-3 in the total sample was 40 (57.1)% and it was significantly lower (P <.001) in the group without metastases 10 (32.3)% as compared to the group of laryngeal cancer patients with regional metastases - 30 (76.9)%.

In comparison with the group without metastasis, in the group with regional metastases the level of expression of cyclin D1 is significantly (P <.001) less by -17.4% (95% CI -25.3 - -9.5), that of p21WAF - by -18.1 (95% CI -27.8 - -8.3)% (P <.001).

The frequency of high expression of caspase-3 for all investigated was reliably (P <.001) higher by 44,6 (95% CI 19.5 - 64.2) in the group with regional metastases as compared with the group without metastases.

In subgroups on the degree of tumor differentiation, significant discrepancies are observed in cyclin D1 and caspase-3 for subgroup of moderate differentiation only, while for p21WAF - in all subgroups, except for the low differentiation of the tumor. Absence of reliability of differences in subgroups according to the degree of tumor differentiation is explained by the small amount of observations in each subgroup separately.

The evaluation of results of diagnostic accuracy of cyclin D1, p21WAF and caspase-3 for predicting metastasis of laryngeal cancer are shown in the Table 2 and Figure 2.

It should be noted that for all markers there is observed dependence in the increase of predictive value of the test at a higher degree of tumor differentiation. So, for p21WAF in a higher degree of differentiation of the tumor, the area under the ROC-curve reaches almost the maximum value (0.927), while for the moderate one it makes up AUR = 0.676. Prognostic value of most markers in subgroups, defined by the degree of tumor differentiation is not statistically reliable (P> .05 for AUR).

By means of ROC-analysis the comparison of predictive power of immunohistochemical markers as for regional metastases of laryngeal cancer was made.

Comparison of all ROC curves against each other showed no significant divergences between them. p21WAF has the greatest sensitivity- 97.4% (95% CI 86.5 - 99.9%), cyclin D1 has less - 87.2% (72.6 - 95.7%) and caspase 3 has the least - 76.9% (60.7 -

88.9%). Levels of specificity are somewhat lower and make up 45.2 % (27.3 - 64.0%); 61.3% (42.2 - 78.2%) and 67.74% (48.6 - 83.3%) respectively.

The areas under the ROC-curve of all studied immunohistochemical markers are more than 0.5 ($P < .01$), thus having predictive value regarding the prediction of laryngeal cancer metastasis. The higher the AUC, the greater is prognostic (diagnostic) value of the test. The greatest value of AUC (0.780) was established for cyclin D1 (Table. 2, Fig. 2), it is somewhat less for caspase-3 (0.719) and the lowest AUC (0.694) is for p21WAF. So, diagnostic accuracy regarding prognosis of metastatic laryngeal cancer may be described as good for cyclin D1 and caspase-3 and as medium - for p21WAF. Pairwise comparison of operating characteristics of immunohistochemical markers against each other was performed by the method of DeLong et al. (1988). The difference between the areas under ROC-curves of caspase-3 and p21WAF was 0.025 (95% CI -0,109 - 0.158); between caspase-3 and cyclin D1 - 0.060 (95% CI -0,041 - 0.162); between p21WAF and cyclin D1 - 0.085 (95% CI -0,040 - 0.210). Significance level of the difference between the areas under the ROC-curves markers is greater than critical level - 5% ($P > .05$ for all comparison pairs), prompting suggestions that two comparable areas are not significantly different, so significant differences in prognostic tests were not found.

So, cyclin D1, p21WAF and caspase-3 can be used for prediction of regional metastatic laryngeal cancer, but cyclin D1 has the best operating characteristics according to ROC analysis, diagnostic accuracy of the latter can be described as good.

To assess possibilities of predicting recurrence of laryngeal tumors on the basis of defining cyclin D1, p21WAF and caspase-3, ROC analysis was made as well (Fig. 3). In cyclin D1 and p21WAF statistical significance of AUR is not reliable ($P > .05$). Statistically significant results were obtained only for caspase-3: sensitivity - 84.6%, specificity - 49.1%; area under the ROC-curve - 0.668 ($P = .018$), by classification of diagnostic accuracy it can be interpreted as medium.

In all studied markers there was observed low specificity and predictive value of positive results. Predictive value of negative result and sensitivity is sufficiently high, this indicate that markers do not miss patients with possible relapses, as almost do not give false negative results.

Discussion. TNM classification remains one of the most convenient and reliable method for determining probability of laryngeal cancer metastasis. However, it does not include molecular properties of the tumor at the stages of carcinogenesis. Recently, studies on disorder of the cellular cycle, particularly of expression of marker Cyclin D1 are being conducted. For example in his study Pignataro L. (2005) proved that increased expression of Cyclin D1 was observed more in stage T3-4 than in stage T1-2, he also studied the relationship of this molecular marker with metastatic laryngeal cancer. Futhermore, the reverse correlation with p27 has been proven; this significantly worsened prognosis for the patients.¹⁸ At this time Morshed K. et al. (2007) considers cyclin D1 as an independent prognostic factor.¹⁹ This divergence of results demands explanation.

Our data on statistically significant increase in expression of cyclin D1 in the

presence of metastases in regional lymph nodes of the neck are confirmed by the studies of other authors. So, Pignataro L. (2005) who revealed correlation of cyclin D1 with regional metastases at the same time does not find association of expression of marker with the degree of tumor differentiation[16].

Inactivation of programmed cell death plays a very important role in the functioning of multicellular organisms. Cellular death is regulated by family of caspases, in particularly caspase 3 (cysteine protease) [20-22]. Role of caspase 3 was studied in the samples of LSCC by means of homonymous molecular marker and increase in the level of its expression with presence of metastases was proven.

p21 WAF1 is a protein, its expression was also analyzed in our work together with caspase-3 in the group of pro-apoptotic markers. This marker is responsible for arrest of the cellular cycle in the phase G1 in response to hypersecretion of protein p53 and DNA lesion. Increased activity of p53, in turn, may enhance signal of apoptosis through the impact on expression of genes Bax and Bcl-2, if they do not mutate and continue to perform their function. Impact of a wide range of factors for p21 WAF1 makes it interesting for investigation of LSCC.

Thus, the study of the cellular cycle, especially investigation of oncosuppressor p53 is the gold standard for LSCC prediction together with proliferative activity of the marker Ki-67, while others need further study.

The study of molecular markers of cyclin D1 showed its expression at the level of 58.3 (18.5)% (M (SD)), for the group of LSCC without metastases - 48.6 (17.7)%, for the group with metastases - 66.0 (15.3)% ($P < .001$), the investigated level of expression of p21 WAF1 ranged from 18% to 94% and on average made up 55.3 (22.1)%, for the group without metastases - 45.2 (26.0)%, for the group with metastases - 63.3 (14.3)% ($P < .001$). High expression of caspase 3 was revealed in 40 (57.1)% and was significantly lower ($P < .001$) in the group without metastases - 10 (32.3)% as compared with the group of laryngeal cancer patients with regional metastases - 30 (76.9)%. In the statistical analysis of data the largest value of AUC (0.780) was established for cyclin D1, somewhat lower (0.719) – for caspase-3 and the lowest value of AUC (0.694) - for p21 WAF1 ($P < .01$). Cyclin D1 has a good diagnostic value and can be used as an additional method for prediction of regional metastases of laryngeal cancer. However, the practical use of this marker as a prognostic one towards choosing the extent of surgical intervention on lymph structures of the neck requires further study. Separately, it should be noted that this study revealed no interrelationship between cyclin D1, caspase 3 and p21 WAF1 and the degree of differentiation of laryngeal cancer and its recurrences, but more in-depth study of molecular markers in the future may reveal other patterns of tumor growth and be used in practice for prediction and personalization of treatment.

Table 1

Characteristics	Total Sample (No. 70)	Main group with regional metastases (No. 39)	Group of comparison without regional metastases (No. 31)	P value
Demographics characteristics				
Males, No. (%)	69 (98.6)	38 (97.4)	31 (100.0)	.369
Mean age M (SD) Age, Mean (SD)	61.9 (9.5)	61.0 (8.5)	63.0 (10.7)	.385
Clinical Values				
Degree of tumor differentiation, No. (%)				
Hogh G1	14 (20.0)	6 (15.4)	8 (25.8)	.523
Moderate G2	45 (64.3)	26 (66.7)	19 (61.3)	
Low G3	11 (15.7)	7 (17.9)	4 (12.9)	
Tumor recurrences, No. (%)	13 (18.6)	11 (28.2)	2 (6.5)	.020
Cyclin D1 %, Mean (SD)				
Degree of tumor differentiation				
Hogh G1	46.5 (14.9)	54.2 (15.7)	40.8 (12.3)	.098
Moderate G2	59.4 (18.4)	68.3 (14.0)	47.3 (16.9)	<.001
Low G3	68.5 (16.3)	67.6 (17.5)	70.3 (16.5)	.808
Total Cyclin D1	58.3 (18.5)	66.0 (15.3)	48.6 (17.7)	<.001
p21 ^{WAF} %, Mean (SD)				
Degree of tumor differentiation				
Hogh G1	35.0 (22.8)	53.3 (14.2)	21.3 (17.9)	.004
Moderate G2	58.1 (19.8)	63.8 (13.7)	50.3 (24.3)	.022
Low G3	69.7 (11.4)	70.1 (13.9)	69.0 (6.8)	.174
Total p21 ^{WAF}	55.3 (22.1)	63.3 (14.3)	45.2 (26.0)	<.001
Caspase-3, rate of high expression, No. (%)				
Degree of tumor differentiation				
Hogh G1	7 (50.0)	5 (83.3)	2 (25.0)	.106
Moderate G2	23 (51.1)	18 (69.2)	5 (26.3)	.011
Low G3	10 (90.9)	7 (100.0)	3 (75.0)	.770
Total caspase-3	40 (57.1)	30 (76.90)	10 (32.3)	<.001

Demographic and clinical characteristics of laryngeal cancer patients included in the study

Table 2

Prognosis of metastases	% (95 % CI)					P value	Optimal cut-off point
	Se	Sp	PPV	NPV	AUC		
Cycline D1	87.2 (72.6 to 95.7)	61.3 (42.2 to 78.2)	73.9 (56.6 to 90.3)	79.2 (59.7 to 89.6)	0.780 (0.665 to 0.870)	<.001	48 %
Degree of tumor differentiation							
High G1	66.7 (22.3 to 95.7)	87.5 (47.3 to 99.7)	80.0 (33.9 to 97.8)	77.8 (33.4 to 99.4)	0.729 (0.434 to 0.924)	.165	48 %
Moderate G2	84.6 (65.1 to 95.6)	73.7 (48.8 to 90.9)	81.5 (60.0 to 94.6)	77.8 (54.3 to 92.5)	0.843 (0.704 to 0.934)	<.001	58 %
Low G3	25.0 (0.6 to 80.6)	100.0 (59.0 to 100.0)	66.7 (12.7 to 93.2)	40.0 (10.1 to 90.2)	0.571 (0.254 to 0.850)	.725	74 %
p21 ^{WAF}	97.4 (86.5 to 99.9)	45.2 (27.3 to 64.0)	69.1 (50.5 to 98.9)	93.3 (70.3 to 96.8)	0.694 (0.573 to 0.799)	.004	40 %
Degree of tumor differentiation							
Hogh G1	100.0 (54.1 to 100.0)	87.5 (47.3 to 99.7)	85.7 (43.5 to NaN)	100.0 (57.9 to 100.0)	0.927 (0.659 to 0.998)	<.001	34 %
Moderate G2	96.2 (80.4 to 99.9)	42.1 (20.3 to 66.5)	69.4 (44.2 to 98.9)	88.9 (56.7 to 95.6)	0.676 (0.520 to 0.808)	.048	46 %
Low G3	71.4 (29.0 to 96.3)	0 (0.0 to 60.2)	100.0 (24.8 to 100.0)	44.4 (7.1 to NaN)	0.518 (0.213 to 0.813)	.928	82 %
Caspase-3	76.9 (60.7 to 88.9)	67.7 (48.6 to 83.3)	75.0 (57.5 to 87.8)	70.0 (51.9 to 84.7)	0.719 (0.599 to 0.820)	<.001	2++
Degree of tumor differentiation							
Hogh G1	83.3 (35.9 to 99.6)	75.0 (34.9 to 96.8)	71.4 (30.9 to 99.2)	85.7 (40.2 to 98.4)	0.792 (0.498 to 0.956)	.013	2++
Moderate G2	69.2 (48.2 to 85.7)	73.7 (48.8 to 90.9)	78.3 (55.1 to 90.5)	63.6 (42.0 to 86.1)	0.727 (0.573 to 0.849)	.001	2++
Low G3	0 (0.0 to 41.0)	100.0 (39.8 to 100.0)	70.0 (4.3 to NaN)	100.0 (17.1 to 100.0)	0.518 (0.213 to 0.813)	.935	2++

Se – Sensitivity

Sp – Specificity

PPV – Positive predictive value.

NPV – Negative predictive value.

AUC – Area Under Curve.

Optimal cut-off point

Evaluation of possibilities of predicting regional metastases of laryngeal cancer based on defining cyclin D1, p21 WAF and caspase-3 (operating characteristics by the data of ROC analysis)

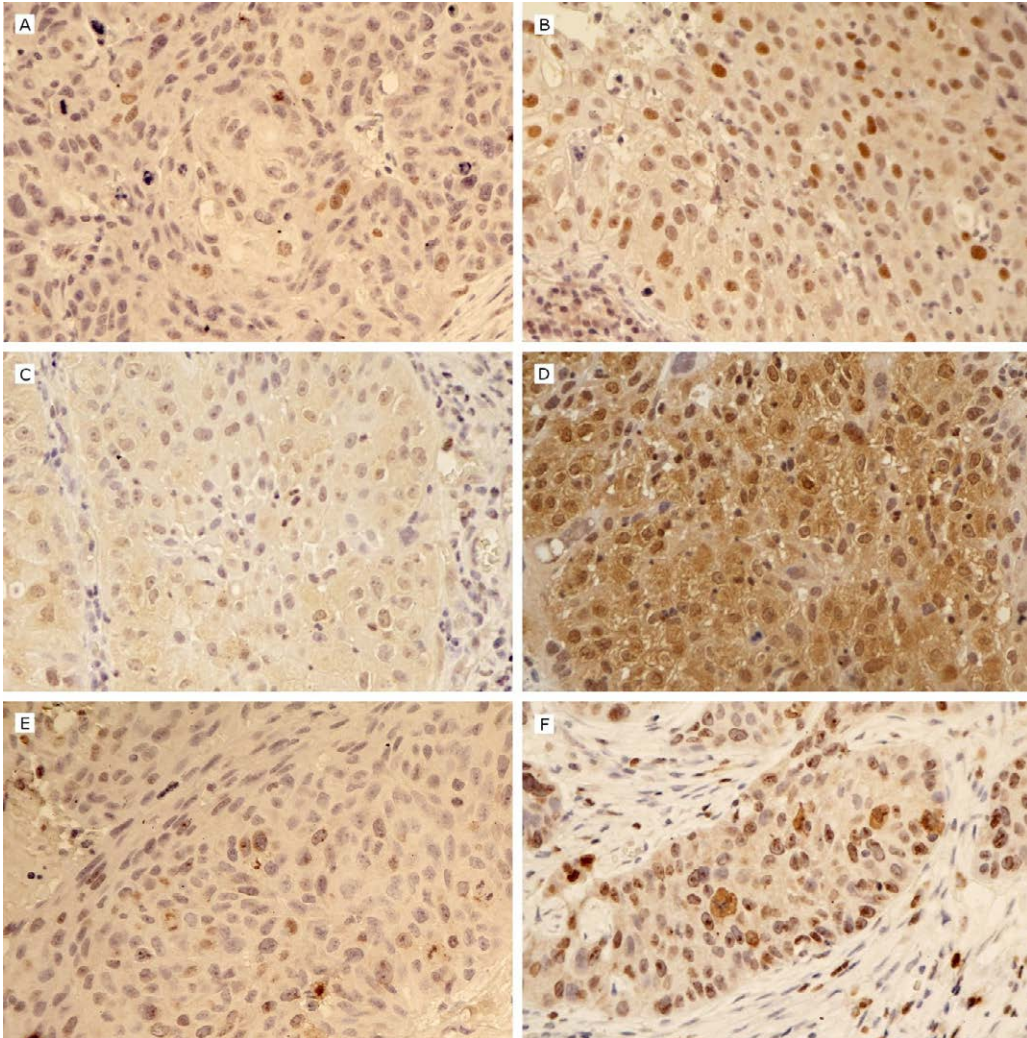


Figure 1.
Immunohistochemical investigation of laryngeal squamous cell carcinoma
Microscopic investigation of laryngeal squamous cell carcinoma showed high expression of immuno-
histochemical markers: cyclin D1 expression $\leq 50\%$ (A), expression $> 50\%$ (B); caspase-3 low level
of expression (C), high level of expression (D) and p21WAF1 level of expression $\leq 20\%$ (E), level of
expression $> 20\%$ (F) (image enlargement $\times 40$).

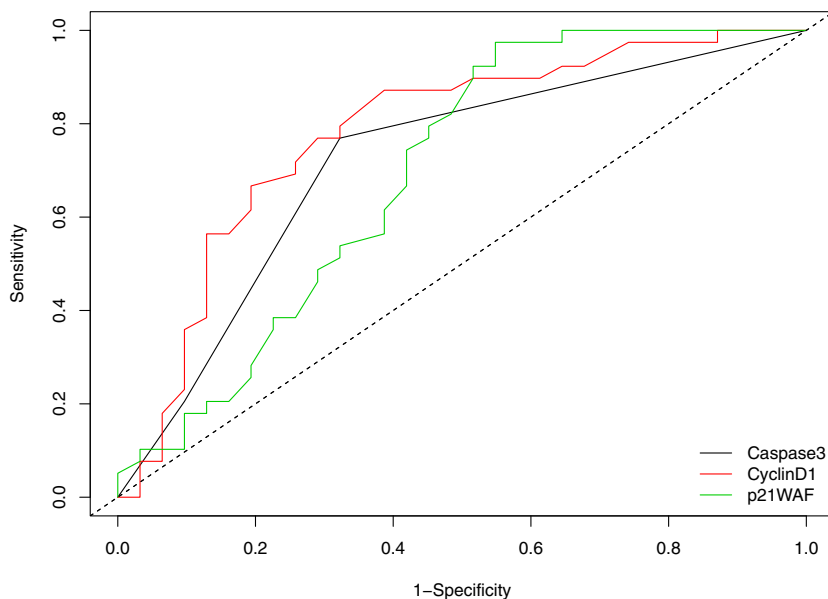


Figure 2
 ROC curves for predicting regional metastases of laryngeal cancer based on defining cyclin D1, p21WAF and caspase-3

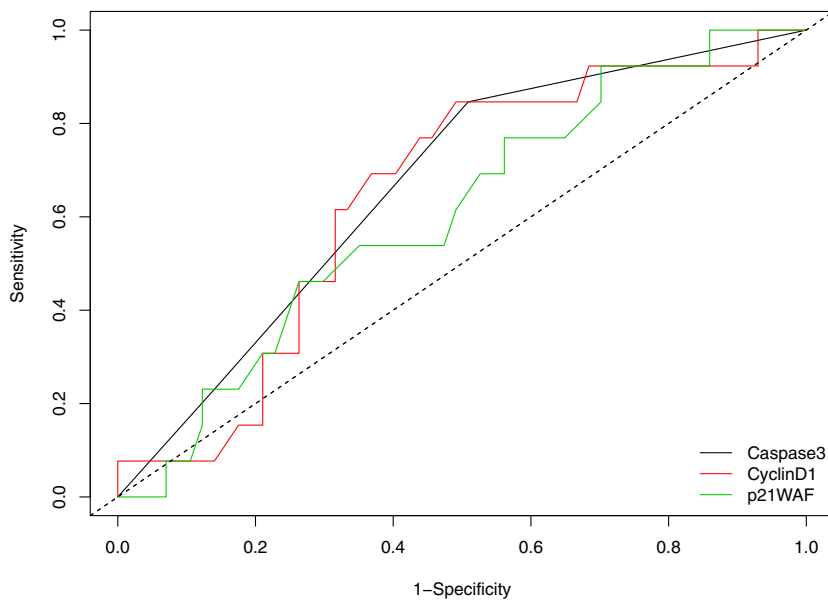


Figure 3
 ROC curves of predicting recurrence of laryngeal tumor based on defining cyclin D1, p21WAF and caspase-3

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