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Expression of cyclin D1, p27, p21, bcl-2, and p53 in laryngeal squamous cell carcinoma and an investigation of the correlation with conventional prognostic factors*

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Aim: Because it is a multistep process, in carcinogenesis it is necessary to determine key genes such as cyclin D1, p27, p21, bcl-2, and p53 that are responsible for uncontrolled proliferation and other mechanisms. The aim of this study was to examine the correlation between the anomalous accumulation of these proteins and the prognosis of laryngeal carcinomas.

Materials and methods: In our study, cyclin D1, p27, p21, bcl-2, and p53 expressions were analyzed immunohistochemically in laryngeal squamous cell carcinomas, and the findings were compared with conventional prognostic parameters such as patient age, sex, tumor localization and diameter, tumor differentiation, lymph node status, metastatic lymph node diameter, vascular and neural invasion, clinical stage, and survival in 50 patients.

Results: Our results showed a statistically significant correlation between the presence of cyclin D1 expression and shorter survival in patients with squamous cell carcinoma of the larynx. Patients with high p27 expression were found to have a higher risk of vascular invasion, and patients with high bcl-2 expression were found to have a higher risk of cervical lymph node metastasis.

Conclusion: A negative correlation was found between cyclin D1 and survival, and a positive correlation was found between both p27 and bcl-2 and laryngeal squamous cell carcinoma prognosis.

Key words: Larynx, squamous cell carcinoma, cyclin D1, p21, p27, p53, bcl-2

1. Introduction

Squamous cell carcinoma is the most common malignancy in the larynx (1). The prognosis of laryngeal cancer is typically evaluated on the basis of a number of conventional prognostic factors. Because it is a multistep process, in carcinogenesis it is necessary to determine key genes such as cyclin D1, p27, p21, bcl-2, and p53 that are responsible for uncontrolled proliferation and other mechanisms (2).

Cyclin D1, p27, and p21 are cell cycle regulators; they enable the cell to go through its normal cell cycle. bcl-2 is the protooncogene that blocks apoptosis. p53 is a tumor suppressor gene that has been claimed to play an essential role in the pathogenesis of human tumors by affecting not only the cell cycle but also apoptosis.

Many studies have examined the correlation between the anomalous accumulation of those proteins and the prognosis of laryngeal carcinomas, but conflicting results have been obtained.

In our study, we analyzed the expression of cyclin D1, p27, p21, bcl-2, and p53 immunohistochemically in laryngeal squamous cell carcinomas. The findings were compared with conventional prognostic parameters such as age and sex of the patients, tumor localization and diameter, tumor differentiation, lymph node status, metastatic lymph node diameter, presence or absence of vascular and neural invasion, clinical stage, and survival.

2. Materials and methods

2.1. Patient demographics and study protocols

A retrospective study was performed on 50 patients with laryngeal squamous cell carcinoma who underwent surgery with total laryngectomy and functional neck

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Patients' age, sex, and clinical stage were noted from the files; tumor localization and diameters, tumor differentiation, vascular and neural invasion, lymph node metastasis, and diameter of the metastatic lymph nodes were reevaluated from glass slides. Distributions according to tumor differentiation, tumor localization, and clinical stage are shown in the Table.

Patients were followed for periods from 3 months to 4 years. Patients' survivals were evaluated (median follow-up: 25.5 months).

2.2. Immunohistochemistry

All immunohistochemical analyses were performed with the peroxidase–antiperoxidase technique. Paraffin-embedded tissues were cut at 4–5 μm and dewaxed with xylene. Antigen retrieval was performed with EDTA solution for cyclin D1 antibody in a microwave oven and with citrate solution for p27, p21, bcl-2, and p53 in a pressure cooker.

Following the blocking of endogenous peroxidase activity with 3% hydrogen peroxide, cyclin D1 (clone DCS-6, Neomarker, 1/25), p27 (clone DCS-72.F6, Neomarker, 3/100), p21 (clone DCS-60.2, Neomarker, 3/100), bcl-2 (clone bcl-1/50/D5, Nova, 3/80), and p53 (clone DO-7+BP53-12, Neomarker, 1/50) were employed. Tissues then went through several steps of secondary antibody, AEC chromogen, and Mayer's hematoxylin.

Positive controls were selected as mantle cell lymphoma for cyclin D1; as colon carcinoma for p27, p21, and p53; and as normal tonsillar tissue for bcl-2. A negative control was not studied.

2.3. Scoring of preparations

Both histopathological reevaluation and observation of the immunohistochemical reactions were carried out by 2 pathologists. The positivity of the antibodies was determined by nuclear staining for cyclin D1, p27, p21, and p53 and by granular cytoplasmic staining for bcl-2 protein (Figures 1 and 2). Positivity with antibody was graded according to the proportion of positive tumor cells in areas where protein expression was more pronounced. A scale of 0 to 4 was used to score the proportion of positive cells with cyclin D1, p53, and bcl-2, with (0) indicating none or less than 2% of cells positive; (1+), 2% to 25% of tumor cells positive; (2+), 26% to 50% of tumor cells positive; (3+), 51% to 75% of tumor cells positive; and (4+), 76% to 100% of tumor cells positive. Patients were grouped for analysis according to either low-expression (0–2+) (<50%) or high-expression (3+–4+) (>50%) groups.

Immunohistochemical labeling of p27 and p21 antibodies was classified as positive when more than 5% of nuclei were stained.

2.4. Statistical analysis

The association of immunohistochemical expression of individual proteins with patients' age, sex, tumor localization and diameter, tumor differentiation, vascular and neural invasion, cervical lymph node metastasis, clinical stage, and survival was determined by chi-square analysis. The distribution of patient numbers according to immunohistochemical expressions and the comparison of them with clinicopathologic parameters are shown in the Table. The associations of the immunohistochemical expression of proteins with the diameters of the tumor and metastatic lymph nodes were also examined by variance analysis and t-test. Relative risk was measured for each immunohistochemical parameter by a 'risk estimate' test. Statistically significant cut-off values were not found for immune markers.

3. Results

Only one patient was female in the study group (n = 50). The distributions of age and other clinicopathological parameters are shown in the Table.

Advanced stage tumors were presented in patients of older age, and incidences of cervical lymph node metastasis were found to be increased in older patients. Tumor diameter was from 0.6 cm to 5 cm. Tumor localization and diameter did not show any statistical significant difference compared with other parameters.

Poorly differentiated tumors were presented in the advanced stage, and the relationship was found to be statistically significant (P = 0.00).

Lymph node metastasis was found to have a statistically significant inverse correlation with survival (P = 0.00), confirming the validity of the evaluation of lymph node metastasis as a prognostic factor. Metastatic cervical lymph nodes were found in 13 patients (26%). The biggest metastatic lymph node was measured as 2.5 cm, and the smallest was 1 cm in diameter. Metastatic lymph nodes were also found in patients with advanced clinical stage (P = 0.00).

Vascular invasion was found in 7 tumors (14%) and was associated with advanced tumor stage (P = 0.00) and cervical lymph node metastasis. Tumors with vascular invasion almost always had neural invasion, which was a statistically significant association (P = 0.00). A similar correlation was found between survival and vascular invasion.

Neural invasion was found in 3 patients (6%). There was a statistically significant association between neural invasion and metastatic lymph node diameter (P = 0.04). If the metastatic lymph node had a diameter of more than 2 cm, it was more likely to have a neural invasion. Patients who had tumors with neural invasion presented shorter survival (P = 0.00).

Table. Distribution of clinicopathological features of the study cases.

Clinicopathological features		Case numbers	Percentage (%)
Age	4th decade	5	(10%)
	5th decade	7	(14%)
	6th decade	20	(40%)
	7th decade	10	(20%)
	8th decade	8	(16%)
Tumor differentiation	Well	12	(24%)
	Moderate	36	(72%)
	Poor	2	(24%)
Tumor localization	Supraglottic	35	(70%)
	Glottic	10	(20%)
	Subglottic	4	(8%)
	Transglottic	1	(2%)
Clinical stage (TNM)	Stage 1	2	(4%)
	Stage 2	17	(34%)
	Stage 3	20	(40%)
	Stage 4	11	(22%)
Metastatic cervical lymph node (+ size of the metastatic lymph node)			
	Positive	13 (0.5–2.5 cm)	(26%)
	Negative	37	(74%)
Vascular invasion			
	Positive	7	(14%)
	Negative	43	(86%)
Neural invasion			
	Positive	3	(6%)
	Negative	47	(94%)
Survival			
	Alive	35	(70%)
	Deceased	15	(30%)
	Median follow-up period	25.5 months	

3.1. Cyclin D1

Cyclin D1 expression was observed in 70% of tumors (35/50 patients) (Figure 1 shows positive cyclin D1 expression of tumor). A statistically significant association was found between cyclin D1 positivity and survival (P = 0.043). There was a statistically borderline significance between cyclin D1 expression and tumor differentiation (P = 0.054).

3.2. p27

p27 was detected in 30% of the carcinomas (15/50 patients) (Figure 2 shows positive p27 expression of tumor). Among the cases with p27 positivity, the proportion of low expressors was 96%. Lack of any statistically significant association was observed between variables and detection of p27. An analysis of relative risk using risk estimate revealed higher vascular invasion in the higher p27

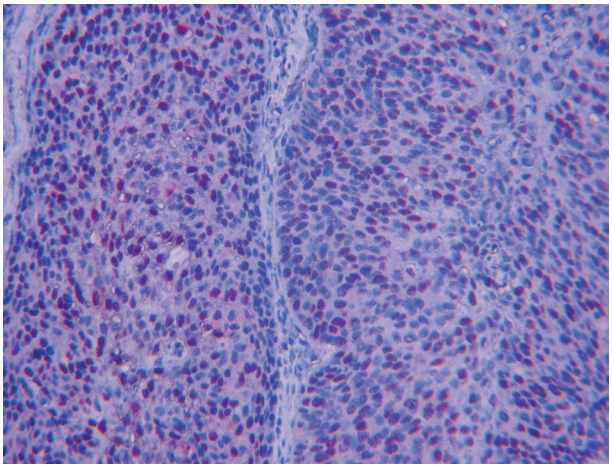

Figure 1. High nuclear cyclin D1 expression in the squamous carcinoma cells.

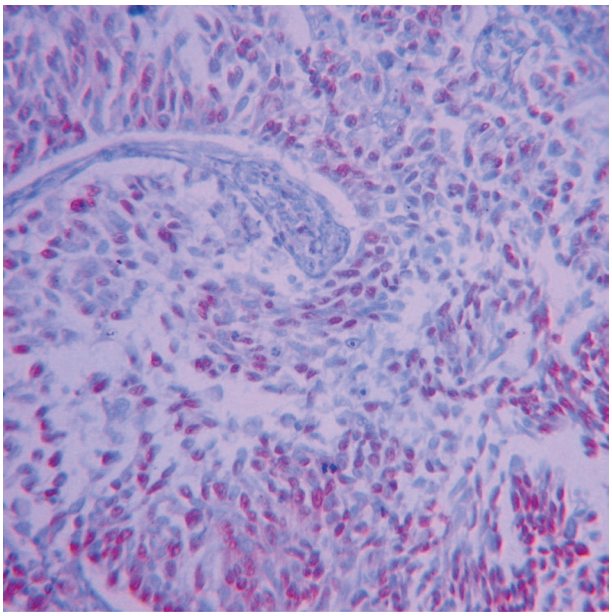

Figure 2. High nuclear p27 expression in the squamous carcinoma cells.

expression group (RR = 7.000), although this relationship was not statistically significant (P = 0.263).

3.3. p21

Patients with p21-positive tumors accounted for 20% of the cases. In 98% of all cases, p21 was expressed in less than 5% of neoplastic cells.

We found no statistically significant correlation between p21 expression and other clinicopathological parameters.

3.4. bcl-2

bcl-2 was expressed in 10% of the patients. Among them, a higher expression rate of bcl-2 was found (4%). Patients with high bcl-2 expression had a higher risk of cervical lymph node metastasis (RR = 3.000), but this correlation was not statistically significant (P = 0.456).

3.5. p53

Among 50 tumors, 26 were p53-positive. However, the high expression ratio was only 22%.

We found no statistically significant association between p53 expression and other protein expressions or parameters.

4. Discussion

We settled the question of the presence or absence of a correlation between the critical antiapoptotic cascade proteins that occur in carcinogenesis and the prognosis of squamous carcinoma of the larynx.

In the literature, most malignant lesions of the larynx are squamous cell carcinomas that are localized in the glottic region (approximately 60%) (3). Approximately 40% of them are localized in the supraglottic area, and only 1% are subglottic. In our study, 20% of the specimens were glottic and 70% were supraglottic. Supraglottic carcinomas have been reported to have the worst prognosis (3); however, in our study tumor localization showed no correlation with other parameters that we analyzed due to the small number of subglottic and transglottic cases (10%).

In laryngeal carcinomas, the clinical stage determined preoperatively is a prognostically important parameter (1). In our series, this parameter also shows prognostic relevance in tumor differentiation, neural and vascular invasion, and lymph node metastasis.

A particularly important variable within TNM classification is the presence of lymph node metastasis (1,4–8). In our study, we found the presence of lymph node metastasis to be significantly correlated with a poor prognosis, which is consistent with the findings of other studies (8,9). Additionally, there was a statistically significant inverse correlation between lymph node metastasis and survival (P = 0.00). We found a statistically significant association between neural invasion and metastatic lymph node diameter (P = 0.04).

Another commonly used prognostic criterion is the degree of histologic differentiation. In our study, differentiation was found to correlate with clinical stage. This parameter was also studied in other studies and found to be statistically significant in association with survival (5,7).

Vascular and neural invasion are important parameters that influence prognosis of cancer patients. Yılmaz et al. found that these 2 parameters were associated significantly with disease-free survival and recurrence of tumor (10). In another study, perineural invasion was found to be correlated with increased local recurrence and lymph node metastasis (11). The results in our study corroborate these findings.

Although some of the conventional prognostic factors that we have mentioned are used to predict the behavior of a laryngeal tumor, some attempts have been made to find some "biological" prognostic variables (12). Among these are proteins such as cyclin D1, p27, p21, and p53, which play a role in cell cycle control (13).

Cyclin D1 expressed in abnormally high concentrations has found to be associated with a bad prognosis among laryngeal tumors (14–17). In a study by Yoo et al. (14), a statistically significant association was found between cyclin D1 expression and local extension of the tumor. However, these researchers found no correlation between its expression and regional lymph node metastasis. Vielba et al. (3) found no statistically significant association between the overexpression of cyclin D1 and the survival or disease-free interval of the patients. We found that tumors with high expression of cyclin D1 presented shorter survival times and poorly differentiated tumors presented higher cyclin D1 expression; these relationships were statistically significant. According to this analysis, the variable most closely associated with survival was cyclin D1, corroborating the results of many other studies.

In addition, we also investigated the relevance of cell-cycle inhibitors such as p27 and p21 expression by immunohistochemistry. Low levels of p27 and p21 expressions have been found to correlate with a poor prognosis in lung, breast, prostate, gastric, bladder, uterine, cervix, and endometrial carcinomas (18,19), but their importance is still controversial in head and neck cancers. A higher vascular invasion was shown in the group with high expression of p27 (RR = 7.000), though this relationship was not statistically significant. We found no correlation of p21 expression with the parameters that we analyzed.

It has been reported that in most cases, at least one of the antiapoptosis genes is highly expressed in laryngeal cancer (20). In our study, patients with high bcl-2 expression had a higher risk of cervical lymph node metastasis, but this correlation was not statistically significant. In the literature, bcl-2 expression is usually associated with chemotherapy response: a higher expression of bcl-2 is said to correlate with a higher rate of complete response (20). However, there are a number of examples of bcl-2 expression being nonpredictive, or of low levels of bcl-2 being associated with increased survival (20–23). In our study, we found no statistically significant correlation of bcl-2 protein expression with any other parameter.

The alteration of p53 has been the subject of a variety of studies (24,25). There have been conflicting reports regarding the clinical value of p53 analysis. In one study, a statistically significant association was found between a high expression of p53 and tumor differentiation (26). In another study, no prognostic correlation was found regarding the role of p53 (27). We found no statistically significant association between p53 expression and other protein expressions or parameters.

In conclusion, our results show a statistically significant correlation between the presence of cyclin D1 expression assessed by immunohistochemical analysis and shorter survival in patients with squamous cell carcinoma of the larynx, although this study comprises a small series of patients without long-term follow-up and randomized controls. Patients with high p27 expression were found to have a higher risk of vascular invasion, and patients with high bcl-2 expression were found to have a higher risk of cervical lymph node metastasis, but these correlations were not statistically significant. In our study, there was a statistically significant inverse correlation between lymph node metastasis and survival and a statistically significant association (P = 0.04) between neural invasion and metastatic lymph node diameter. Future studies with larger series should be directed towards finding genetic defects in laryngeal tumors and their correlation with conventional prognostic factors, so that we can select what is likely to be the most conservative, least morbid treatment for each patient.

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