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Cyclin D1 Expression in Patients with Laryngeal Squamous Cell Carcinoma

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ABSTRACT

Background & Objective: Laryngeal squamous cell carcinoma (LSCC) is considered to be one of the most common cancers of the head and neck, accounting for roughly 90% of all malignant tumors of the larynx. To have a timely diagnosis for a better and practical therapy, molecular markers have to be investigated. The aim of this study was to determine the expression of Cyclin D1 (CD1) in patients with laryngeal squamous cell carcinoma.

Methods: In this study the demographic data of 82 patients with laryngeal squamous cell carcinoma, including age, gender and geographical region history of smoking and drug abuse, paraclinical findings, surgical description, and pathologic reports were extracted from their medical records. The stage and grade of the disease and tumor location were determined using their medical records. An appropriate tissue sample was selected. Then, the selected cancerous tissue samples stored as formalin-fixed paraffin-embedded tissue then were (Immunohistochemistry) IHC stained and analyzed in terms of the expression of CD1.

Results & Conclusion: According to the results, 75 out of 82 (91.5%) investigated samples were positive for CD1 expression. There was a significant relationship between stage of the disease (P=0.041) and CD1 expression in patients with laryngeal squamous cell carcinoma. There was no significant relationship between gender (P=0.055), age (P=0.256), history of smoking and drug abuse (P=0.192), location of the tumor (P=0.90), grade of the disease (P=0.515) and geographical region (P=0.466) and CD1 expression in patients with laryngeal squamous cell carcinoma. The results of the present study showed that CD1 expression was higher (91.5%) in patients with laryngeal squamous cell carcinoma in comparison to the other studies. According to the results we can conclude that stage of the disease can significantly affect CD1 expression in patients with squamous cell carcinoma.

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Introduction

Laryngeal squamous cell carcinoma (LSCC) accounts for roughly 90% of all malignant tumors of the larynx and is the second most prevalent malignancy of the respiratory system (1,2). In recent decades, the total number of patients that are diagnosed with LSCC has increased drastically leading to as low as 5 years or less in the survival rate of LSCC patients especially in advanced stages (3). Some of the early symptoms of LSCC are hoarseness, dysphagia and cervical lymph node metastasis (1). Given that hoarseness is the primary symptom of glottis involvement, 85% of the patients with glottis involvement are diagnosed at the initial stage of the disease, but since patients with supraglottic involvement present with nonspecific symptoms such as dysphagia and odynophagia, about 66% of them are diagnosed in the stages 3 and 4 of the disease (4).

The primary strategy for treating LSCC is surgical intervention or total laryngectomy followed by radiotherapy and chemotherapy; however, these treatment modalities are effective for the patients in the initial stages of the disease and the prognosis is poor in the patients with advanced-stage laryngeal cancer (LC) (1,2,5,6). Therefore, there is a strong need to identify markers that help the physician decide on the appropriate patient treatment. Studies on the molecular mechanisms involved in carcinogenesis can play an

important role in advancing the diagnosis and treatment of the patients with LC (7,8).

Cyclin, Cyclin-Dependent Kinase (CDK), and Cyclin-Dependent Kinase Inhibitor (CDKI) are considered as the regulators of the cell cycle. The types of CDK, Cyclin, and CDKI involved in the regulation of the cell cycle are different for each stage. Cyclin identifies specific kinases (CDKs), attaches to them, and forms complexes to yield the event of the cell cycle. Within the transition from G1 phase to S phase, first, the cyclin D1-3 complex then cyclin E are involved by activating CDK4-6 and CDK2, respectively (9). Current researches suggested that CD1 overexpression might help with the early identification of the risk of head and neck cancer (10-12). Moreover, studies implied that the overexpression of CD1 may be implicated in the biological behavior of LSCC and have a valuable prognostic significance (6). Hence, this study is to determine the expression of CD1 in the patients with laryngeal squamous cell carcinoma aiming toward a better insight into LSCC.

Materials and Methods

After approval by local Ethics Committee, this cross-sectional study was done on 82 patients with laryngeal squamous cell carcinoma who previously referred to ENT ward (Shahid Sadoughi Hospital, Yazd, Iran, during 2011-2016) for the diagnostic and therapeutic measures (by taking a census). Patients with incomplete demographic data and the specimens which were insufficient to perform IHC were excluded from the study. The Patients' demographic data including age, gender and geographical region as well as information about history of smoking and drug abuse, paraclinical findings, surgical descriptions and pathologic reports were extracted from their medical records. The stage and grade of the disease and tumor location were determined using their medical records. The appropriate tissue sample was selected from laryngeal squamous cell carcinoma. Cancerous tissue samples were selected from paraffin-embedded blocks and cut into 4-μm-thick sections. They were then de-paraffinized in xylene, dehydrated through graded alcohols, and placed in 0.1 % hydrogen peroxide to quench any endogenous peroxidase activity. Antigen retrieval was performed using a 750 W microwave oven for 15 min in 10 mmol sodium citrate buffer (10 mmol/L, pH 6.0). The sections were blocked with 10% normal goat serum for 30 min at room temperature to prevent the non-specific binding of antibodies. The slides were then incubated with anti-human CD1 monoclonal rabbit antibody (Dako Autostainer/ Autostainer Plus, USA) in a humidified chamber at 4°C overnight. The sections were then incubated with biotin-labeled goat anti-rabbit secondary antibody (Master polymer plus HRP, Granada 18016, Spain) for 30 min at 37°C, followed by the reaction with streptavidin–biotin horseradish peroxidase complex. The reaction products were observed by immersing the slides in a freshly prepared diaminobenzidine solution for 10 min and counterstaining them with hematoxylin before dehydration and mounting. All the slides were evaluated by light microscopy scanning the entire tissue specimen under low magnification (×40) and then confirmed under high magnification (×200 and ×400). They were examined by the experienced pathologists who were blinded to the study design. Positive immunostaining of CD1 protein was determined from the staining intensity as well as the percentage of immunoreactive cells in the most highly stained area of each slide based on a previously reported method with some modifications. The intensity score was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (severe staining). All data were recorded in a checklist. The data were analyzed using SPSS 18 (SPSS Inc., Chicago, Ill., USA). The statistical analysis included Chi-Squared test and Fischer's exact test. The P-value≤0.05 was considered statistically significant.

Compliance with Ethical Standards

Authors have no conflicts of interest. The study protocol was in accordance with the latest declaration of Helsinki for medical research involving human subjects and was approved by the local Ethics Committee. This article does not contain any studies with animals performed by any of the authors. Informed consent was obtained from all participants of the study.

Results and Discussion

This cross-sectional study was conducted on 82 patients with laryngeal squamous cell carcinoma. During this study, 75 out of 82 (91.5%) of the investigated patients were immunohistochemically positive for CD1 expression (Figure 1). Twenty out of 82 patients aged less than 50 years, 31 patients aged between 51 to 60 years, and 31 cases aged over 60 years old. Most of our patients were over the age of 50 years old. Consistent with the results, there was no significant relationship between age and CD1 expression (*P*=0.256). Considering gender, 69 were male and 13 were female. In accordance with our results, no significant relation was observed between CD1 expression and gender in the patients with laryngeal squamous cell carcinoma (*P*=0.055) (Data are shown in Table 1). Our results indicated no significant relationship between the history of smoking and CD1 expression (*P*=0.192). Moreover, our results showed no significant relationship between the location of the tumor and CD1 expression (*P*=0.90). Furthermore, no significant correlation was detected between the geographical region and CD1 expression (*P*=0.46) (Table 2). Also, the results revealed no significant relationship between the grade of the disease and CD1 expression (*P*=0.515). A significant correlation was found between CD1 expression and the stage of the disease in patients with laryngeal squamous cell carcinoma (*P*=0.041) (Table 3).

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Table 1. Frequency distribution of CD1 protein expression in patients with laryngeal squamous cell carcinoma according to the age and gender.

CD1 expression	Age			Gender	
	≤ 50	51 – 60	>60	Male	Female
Negative	1	2	4	5	2
	5.0%	6.5%	12.9%	7.2%	15.4%
Weak	6	9	9	18	6
	30.0%	29.0%	29.0%	26.1%	46.2%
Moderate	7	12	10	26	3
	35.0%	38.7%	32.3%	37.3%	23.1%
Severe	6	8	8	20	2
	30.0%	25.8%	25.8%	29.0%	15.4%
Total	20	31	31	69	13
P-value	0.256			0.055	

Table 2. Frequency distribution of CD1 protein expression in patients with laryngeal squamous cell carcinoma according to the smoking, location of the tumor and the geographical region.

CD1 expression	Smoking				Location			Geographical region		
	No smoking	Only Cigarette	Only Opium	Both	Supraglottic	Glottic	Subglottic	Central	Southern	Northern
Negative	2	1	1	3	4	3	-	5	2	-
	28.6%	6.7%	25.0%	5.4%	8.0%	10%	-	8.6%	8.7%	-
Weak	1	5	1	17	17	7	-	18	6	-
	14.3%	33.3%	25.0%	30.4%	34.0%	23.3%	-	31.0%	26.1%	-
Moderate	2	6	1	20	19	9	1	19	9	1
	28.6%	40.0%	25.0%	35.7%	38.0%	30.0%	50.0%	32.8%	39.1%	100%
Severe	2	3	1	16	10	11	1	16	6	-
	28.6%	20.0%	25.0%	28.6%	20.0%	36.7%	50.0%	27.6%	26.1%	-
Total	7	15	4	56	50	30	2	58	23	1
P-value	0.192				0.90			0.466		

Table 3. Frequency distribution of CD1 protein expression in patients with laryngeal squamous cell carcinoma according to the grade and stage of the tumor.

CD1 expression	Grade (differentiation)			Stage				Total
	1	2	3	1	2	3	4	
Negative	4	2	1	3	1	3	-	7
	13.3%	4.5%	12.5%	25.0%	2.6%	13.6%	-	8.5%
Weak	7	13	4	1	15	6	2	24
	23.3%	29.5%	50.0%	8.3%	39.5%	27.3%	20.0%	29.3%
Moderate	12	16	1	3	11	7	8	29
	40.0%	36.4%	12.5%	25.0%	28.9%	31.8%	80.0%	35.4%
Severe	7	13	2	5	11	6	-	22
	23.3%	29.5%	25.0%	41.7%	28.9%	27.3%	-	26.8%
Total	30	44	8	12	38	22	10	82
P-value	0.515			0.041				

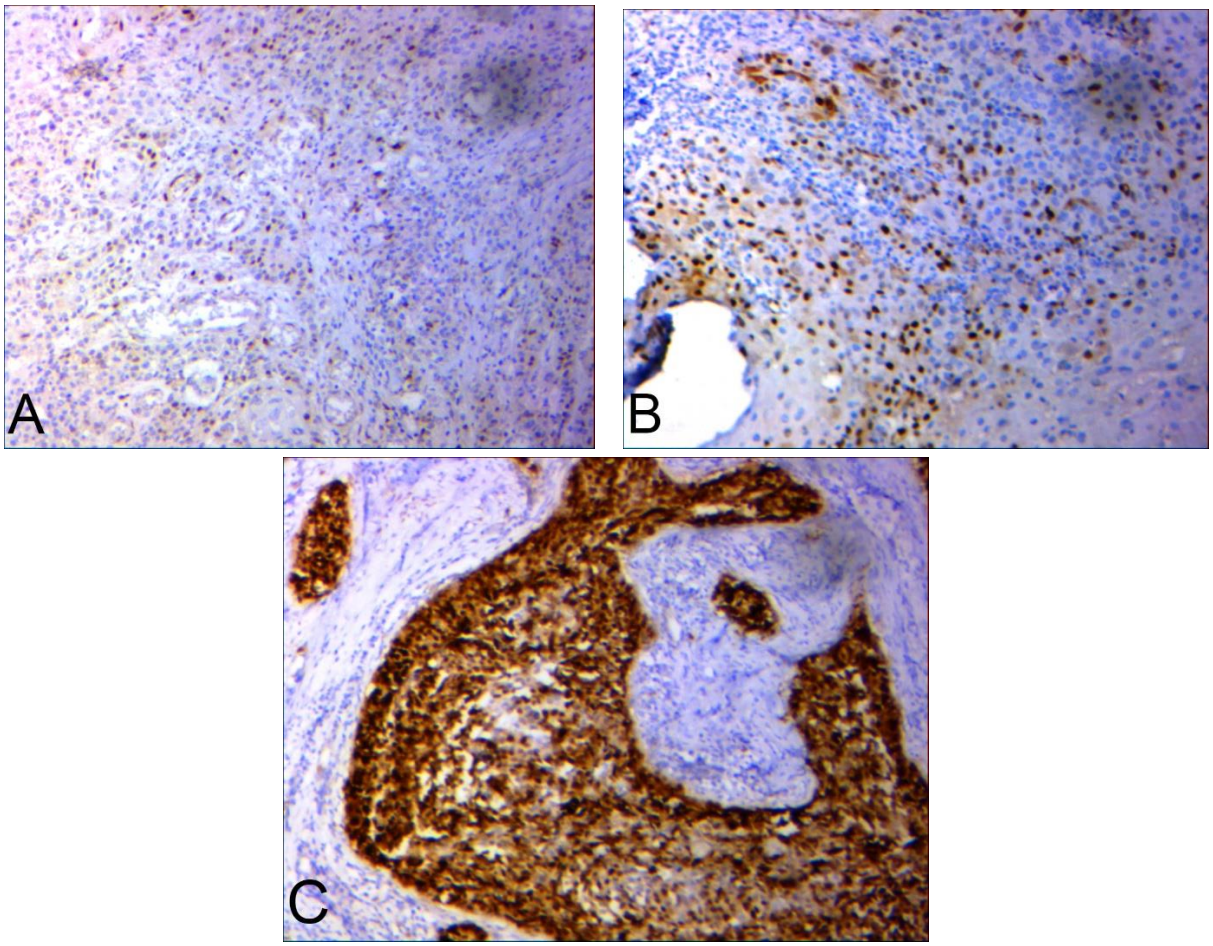

Fig. 1. Immunohistochemical staining of representative laryngeal squamous cell carcinoma (LSCC) with rabbit monoclonal antibodies against cyclin D1 with different intensity score grades in different stages of tumor. (A) Weak-intensity (Stage 1), (B) Moderate-intensity (Stage 2), and (C) Severe-intensity (Stage 3) staining and expression (Cyclin D1, ×200).

The aim of this study was to determine the expression of CD1 in the patients with laryngeal squamous cell carcinoma. In our study, 75 out of 82 91.5% of the investigated patients were positive for the CD1 expression that it was high compared to the other studies. A study done by Duan *et al.*, indicated that the circMYLK/miR-195/cyclin D1 regulatory axis could potentially affect the carcinogenesis of LSCC cells (13). In a clinical review done in 2018, nine studies showed a statistically significant association between CCND1/cyclin D1 amplification/overexpression in tumorigenesis of head and neck squamous cell carcinoma (11). A mini-review by Bakr *et al.*, demonstrated the amplification of cyclin D1 gene (CCND1) in 17%-55% of head and neck squamous cell carcinoma (HNSCC) in several studies. Also, overexpression of CD1 protein has been shown in 21%-64% of HNSCC (12). A recent study in 2020 collected 76 matched LSCC and normal tissues (101 cases in total) to assess the circ-CCND1 expression. The results showed that circ-CCND1 was markedly up-regulated in LSCC tissues compared to the precancerous tissues (3). In a study on the expression of immunohistochemical markers in non-oropharyngeal HNSCC in Ghana, the commonest primary site was the larynx 41.3% with 4.7% positivity for CD1 expression (14). Our study disclosed that 12.9% of the patients aged over 60 years were negative for CD1 expression; whereas, only 5% of the patients aged less than 50 years were negative for CD1 expression. Based on our findings, it seems that with increasing in age, the chance of being positive for CD1 expression decreases but not statistically significant. In the study in Ghana, the age of patients ranged from 15–98 years with mean 59.1 years but no significant correlation was noted between CD1 expression and age (14), which is in line with our study. Our results showed that CD1 expression was higher in males than females, but not suggesting a significant relationship between gender and CD1 expression. Along with our study, Zhang *et al.*, reported no relationship between gender and the expression of CD1 (15). In a study by Owusu-Afriyie *et al.*, 70.1% of the patients were males but no significant correlation was detected between CD1 expression and gender (14). Considering smoking and drug abuse, 7 out of 82 patients had no history of smoking and drug abuse. Most of the patients 68.3% had a history of smoking and drug abuse and 18.3% only smoked. The percentage of negative CD1 expression was higher in the patients with no history of smoking and drug abuse. Thus, this study showed that smoking and drug abuse resulted in positive CD1 expression; the extent of association between smoking

and CD1 expression was greater than the association between drug abuse and CD1 expression but not statistically significant. In line with our study, the results of a review study by Cornean *et al.*, revealed that cancer risk rises in the cases where environmental toxins, such as alcohol and smoke consumption are involved (16). Considering the location of the tumor, the supraglottic region had the highest frequency 60.97% but subglottic tumors had the lowest frequency 2.4%. We also found that supraglottic involvement was related to the positive CD1 expression (Out of 50 patients with supraglottic involvement, 4 patients were negative for CD1 expression and 46 patients were positive). Both patients with subglottic involvement were positive for CD1 expression. According to our results, 90% of the patients with glottis involvement were positive for CD1 expression. Overall, our results showed that there was no significant relationship between the location of the tumor and CD1 expression. Findings of a study done at Adnan Menderes University, Turkey, showed that 8 patients 32% had glottic, 6 24% had supraglottic and 11 44% had transglottic laryngeal cancer (5). The study of Cornean *et al.*, revealed that cancer attacks the glottic and supraglottic levels in LSCC (16). On the contrary to our study, a co-expression network analysis of Yang *et al.*, indicated that the expression levels of CCND1 protein are related to the site of the tumor and depth of tumor invasion. Also, associated with the existence of LSCC located in the supraglottic larynx are CCND1 immunopositivity (2). Forty-four out of 82 patients had a moderate degree (grade 2) of differentiation and eight patients had poor degrees (grade 3) of differentiation. One out of 8 patients in grade 3 (12.5%) and 2 out of 44 patients in grade 2 (4.5%) were negative for CD1 expression. Four patients in grade 1 (13.3%) were negative for CD1 expression. Accordingly, it seems that with an increase in the grade of the disease, the rate of being positive for CD1 expression also increases, but not significantly. In the study by Owusu-Afriyie, the histological grade of the tumors was mostly moderately differentiated or poorly differentiated grade (14). According to our results, 38 out of 82 patients were in stage 2 and 10 patients were in stage 4. Also, the results showed that as the stage of the disease progressed, the number of positive samples for CD1 expression significantly increased 25% of the patients at stage 1 of the disease were negative for CD1 expression while 0% of the patients at stage 4 had a negative expression of CD1 expression). Eryilmaz *et al.*, study implied that CD1 was correlated with tumor stage, metabolic tumor volume (MTV), and total lesion glycolysis (TLG) suggesting that it may have the potential to be used as a marker in the diagnosis and follow-up of laryngeal cancers (5). A recent study in 2020 has shown high circ-CCND1 was positively correlated with the advanced TNM stage (P=0.004) (3). Also, Yang *et al.*, explicated that expression levels of CCND1 are related to highly invasive (T3 and T4) malignant neoplasms of the larynx and tumors being at an advanced clinical stage (III and IV) (2). Considering the geographical region, 58 out of 82 patients were from Central provinces of the country, 23 patients were from Southern provinces, and just one patient was from the Northern region. Our study suggested no significant correlation between the geographical region and CD1 expression level. The discrepancy between our study and the other studies results may be due to these facts that the level of CD1 expression in tumors can vary according to the geographical location, race, and climate; moreover, the method of IHC staining and examining the tissue samples cannot be exactly the same across different countries and the differences between the applied kits may affect the IHC staining outcomes. On the other hand, the lack of a significant relationship between CD1 expression and variables can be for the small number of patients in some subgroups. At this time, there is no consensus among the various clinical studies and there might be other factors that could potentially play role which does require further evaluation.

Recommendation

Further studies are suggested to investigate the relationship between the level of CD1 expression and prognosis of patients with laryngeal squamous cell carcinoma.

Conclusion

The results of the present study showed that CD1 expression was high (91.5%) in the patients with laryngeal squamous cell carcinoma compared to the other studies. According to the results we can conclude that higher stages of the disease can significantly affect CD1 expression in the patients with laryngeal squamous cell carcinoma. Also, our results determined that cyclin D1 can be used in future treatment options as a diagnostic method as well as targeted therapy in the patients with LSCC.

Conflict of Interest

The authors declared that there is no conflict of interest regarding the publication of this article.

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