Expression Profile of Survivin and p16 in Laryngeal Squamous Cell Carcinoma: Contribution of Tunisian Patients

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Abstract
The objective of this study was to evaluate the expression of survivin and p16 in laryngeal squamous cell carcinoma (LSCC) in order to analyze their pathogenesis and prognostic significance in Tunisian patients. A total of 70 patients with LSCC collected at the Salah Azaiez Cancer Institute of Tunis were retrospectively evaluated. Expression of survivin and p16 was examined using immunohistochemistry, and the correlations with clinicopathological parameters, overall survival (OS), and disease-free survival (DFS) were statistically evaluated. The positive expression of survivin and p16 were found in 58.6% and 51.43% of LSCC cases, respectively. The p16 expression was not associated with either clinical parameters or patient survival, whereas there was a strong correlation of survivin expression and lymph node metastases ($P = .002$), alcohol consumption ($P = .024$), and therapeutic protocol (with or without chemotherapy; $P = .001$). Kaplan-Meier survival curves showed that patients with LSCC having positive survivin expression have shorter OS ($P = .026$) and shorter DFS ($P = .01$) than those with negative expression. Positive survivin expression was also correlated with high recurrence rate ($P = .014$). Therefore, survivin is a poor prognostic marker for LSCC but the therapeutic protocol remains, in multivariate study, the most decisive for the OS and DFS of our patients with $P < .01$. Our data indicated that, in Tunisian laryngeal squamous cell carcinoma, survivin expression is associated with unfavorable outcomes and represents a predictor marker of recurrence and chemoresistance. However, p16 expression has no prognosis value.

Keywords
survivin, p16, laryngeal squamous cell carcinoma, immunohistochemistry, prognostic impact, chemoresistance

Introduction
Laryngeal carcinoma is the second most common malignancy of the head and neck region, 90% to 95% of which is laryngeal squamous cell carcinoma (LSCC). This disease is much more common in male gender, and the most important risk factors are tobacco and alcohol. The incidence of laryngeal cancer is increasing annually worldwide. In Tunisia, it increased, between 2006 and 2009, from 5.6 to 6.1 for men and from 0.34 to 0.4 for women per 100 000 inhabitants. Moreover, the overall survival (OS) of LSCC did not significantly improve over recent decades. These results indicate that classical prognostic parameters are not sufficient for evaluating the malignancy of these tumors. However, many researchers have focused on identifying expression of molecular markers involved in cell proliferation and apoptosis as predictive factors for clinical management and prognosis, such as survivin and p16 proteins that have several activities in normal and malignant tissues.

Survivin protein inhibits apoptosis and plays an important role in regulating cell division. It is expressed in fetal tissues but it is restricted during development and seems to be negligible in the majority of terminally differentiated adult tissues. However, strong survivin expression is largely observed in the vast majority of cancers. Many studies have investigated the clinical usefulness of survivin expression as a predictive or a prognostic factor in the majority of malignant tumors. P16 protein, member of the inhibitor kinase family (INK4), has a tumor suppressor activity. This protein contributes to the regulation of the cell cycle progression and prevents tumor
development. Furthermore, several studies have shown that p16 is expressed in the majority of malignant tumors. This study aims to evaluate for the first time the expression of survivin and p16 in LSCC tumors and to assess their correlation with the pathogenesis and prognosis impact in Tunisian patients.

Patients and Methods

Patients

This study was accomplished in Salah Azaiez Institute of Cancer in Tunisia and concerned 70 patients with LSCC diagnosed and treated between 2002 and 2011. Clinical data were collected from their medical records, including sex, age at diagnosis and histological grading (G1: well differentiated, G2: moderately differentiated, and G3: poorly differentiated), tumor size, and tumor primitive node metastasis (pTNM) staging. Other parameters, such as history of smoking, alcohol consumption, and therapeutic attitude, were also collected.

Immunohistochemistry

Immunoperoxidase staining of paraffin sections from LSCC surgical specimens was routinely carried out. Tissue sections (4 μm) were cut from each paraffin blocks and were deparaffinized in xylene and dehydrated through a graded series of ethanol solutions. Antigens were retrieved by heating sections for 30 minutes at 98°C in an unmasking solution. Then slides were washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The slides were then incubated at room temperature with the specific primary antibody for 30 minutes with anti-survivin (clone D-8; Santa Cruz Biotechnology, Santa Cruz, California; 1:50 diluted). Specimens were then washed with PBS and incubated with the detection kit (Novolink Max Polymer Detection System, RE7280-KLeica Biosystems Newcastle, UK), according to the manufacturer’s protocols. Staining was visualized with 3,3-diaminobenzidine and slides were counterstained with Mayer’s hematoxylin. The sections were then dehydrated, cleared, and mounted.

For p16 analysis, the CINtec Histology kit with monoclonal mouse antibody (clone E6H4; Mtm laboratories AG, Heidelberg, Germany) was used, according to the manufacturer’s instructions.

The results of the immunohistochemical staining were evaluated by an anatomicopathologist. We considered 5% the cutoff for survivin expression and 1% for p16 expression as described in previous studies. The survivin immunostaining was scored as follows: score 0: <5%; score 1: 5% to 25%; score 2: 26% to 50%; score 3: >50%. The p16 immunostaining was scored as follows: score 0: absent, score 1: 1% to 4% tumor cells stained, score 2: 5% to 25% tumor cells stained, score 3: 26% to 50% tumor cells stained, and score 4: >50% tumor cells stained.

For statistical purposes, the specimens were classified into negative (−) and positive (+) categories. A score of 0 was considered to be negative and the other scores were considered to be positive.

Statistical Analyses

The correlation between clinicopathological parameters and survivin and p16 expressions was performed using the Fisher exact and Pearson χ² tests. Both OS and disease-free survival (DFS) probabilities were estimated by the Kaplan-Meier and log-rank test. The OS was defined from the time of initial diagnosis to the date of death. The DFS was measured from the date of the initial diagnosis to the date when recurrence was diagnosed. The Cox regression analysis was used to determine the independent prognostic predictors. A P value less than .05 was considered to be statistically significant in all statistical analyses. For statistical analysis, the samples were categorized into 2 groups: negative and positive reactions.

Results

Clinicopathologic Features

The study population consisted of 67 (95.7%) males and 3 (4.3%) females, with a mean age of 63 years (standard deviation = 10.06, 95% confidence interval [95% CI], 45-88). Fifty-four (77.14%) patients were treated with total laryngectomy and radiotherapy (TL + RT), while 15 (21.43%) were treated with chemotherapy in addition to TL and RT (TL + RT + CT). Just 1 (1.43%) was only treated with partial laryngectomy and was canceled from the statistical treatment study. A history of alcohol consumption was observed in 30 (42.9%) patients and tobacco smoking in 62 (88.6%) patients. They were, also, divided according to histological grade as follows: 66 (94.3%) cases having G1 type, 3 (4.3%) cases with G2 type, and 1 (1.4%) case with G3 type. According to the stage T of tumor, we found that the majority of patients (56, 80%) were in T4 stage, 12 (17.14%) patients were in T3 stage and 2 (2.86%) patients were in T2 stage. According to TNM stage, 61 (87.14%) patients were in the TNM stage IV, 7 (10%) patients were in the TNM stage III, and only 2 (2.86%) patients were in the TNM stage II. Thirty (42.86%) of the 70 tumors invaded the entire larynx, 15 (21.43%) tumors were glottic and supraglottic, 17 (24.28%) tumors were glottic and subglottic, and 8 (11.43%) tumors were only glottic. Cervical lymph node metastases were detected in 15 (21.43%) patients (Table 1).

Survivin Expression

Survivin expression was detected in 41 (58.6%) patients, while no expression was noted in 29 (41.4%) cases. The majority of survivin expressing tumors (37/41 cases) showed less than 50% positive cells. Furthermore, the survivin immunostaining was nuclear in 33 cases, and both nuclear and cytoplasmic in only 8
cases (Figure 1A). Moreover, the expression of survivin was significantly correlated with the lymph node metastasis \( P = .002 \), alcohol consumption \( P = .024 \), and therapeutic protocol \( P = .001 \). However, no significant correlation was found between the expression of survivin and the other clinicopathologic features (Table 2).

### The p16 Expression

Thirty-six (51.43%) cases of our 70 patients were p16 positive (Figure 1B). The majority of p16 expressing tumors (22/36 cases) showed more than 50% positive cells. In all these positive cases, p16 expression was observed in nuclei and cytoplasm of tumor cells. However, no significant correlation was found between the expression of p16 and clinicopathological features \( P > .05 \); Table 3).

### Survival Analysis

Survival data were available for 64 patients with LSCC. Forty-three (67.19%) patients are still alive and 21 (32.81%) died within an OS mean time of 88.22 months (95% CI: 72.55-103.90 months). Twenty (31.25%) patients had tumor recurrence within a DFS mean time of 89.53 months (95% CI: 73.76-105.31 months).

In univariate survival analysis, we showed that the OS and the DFS, in survivin-positive expression cases, were significantly shorter than that in the negative ones \( P = .026 \) and \( P = .01 \), respectively; Figure 2A and B). Our data demonstrated, also, that the mean times of OS in survivin-positive versus survivin-negative cases were 66.06 months (95% CI: 49.17-82.96 months) and 110.61 months (95% CI: 91.05-130.16 months), respectively. Furthermore, the mean times of DFS in survivin-positive versus survivin-negative cases were 64.88 months (95% CI: 47.87-81.88 months) and 115.86 months (95% CI: 97.91-133.80 months), respectively. On the other hand, the recurrence rate was significantly higher among patients with LSCC with survivin-positive expression than among those with negative expression \( P = .014 \). Moreover, the odds of recurrence were estimated to be 4 times higher for patients with a positive survivin expression than for those with negative expression (odds ratio = 4.38; 95% CI: 1.26-15.21). Our results demonstrated, also, that only the lymph node metastasis and the treatment type significantly correlated with poor patients' OS and DFS with \( P < .05 \) (Figures 3A, B and 4A, B). In multivariate Cox regression analysis, only the treatment type (chemotherapy) was an independent prognosis factor for patients with LSCC \( P = .021 \) for DFS and \( P = .040 \) for OS) and the survivin and lymph node metastasis were not independent factors of prognosis for our patients \( P < .05 \); Tables 4 and 5).

Furthermore, our results did not demonstrate, in univariate statistical analysis, any significant difference concerning OS \( P = .79 \) and DFS \( P = .57 \); Figure 5A and B) of patients with p16-positive expression compared to those with negative expression. The mean times of OS and DFS among patients with positive expression of p16 compared to those not expressing p16 were 84.74 months (95% CI: 63.73-105.76 months) versus 84.73 months (95% CI: 64.75-104.72 months) and 83.76 months (95% CI: 62.77-104.76 months) versus 89.3 months (95% CI: 69.78-108.83 months), respectively.

### Discussion

In this study, we examined the pathogenesis and prognostic roles of survivin and p16 in Tunisian patients with LSCC. In fact, these markers have enormous interest in cancer research because they were often upregulated in malignant lesions and

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**Table 1.** Clinicopathological Characteristics of 70 Patients With Laryngeal Squamous Cell Carcinoma.

|                        | Number | Percent |
|------------------------|--------|---------|
| Sex                    |        |         |
| Male                   | 67     | 95.7    |
| Female                 | 3      | 4.3     |
| Age                    |        |         |
| <60 years              | 29     | 41.4    |
| ≥60 years              | 41     | 58.6    |
| Smoking habit          |        |         |
| Yes                    | 62     | 88.6    |
| No                     | 3      | 4.3     |
| Unspecified            | 5      | 7.1     |
| Alcohol consumption    |        |         |
| Yes                    | 30     | 42.9    |
| No                     | 19     | 27.1    |
| Unspecified            | 21     | 30      |
| Tumor site             |        |         |
| Glottic + supra + subglottic | 30   | 42.86   |
| Glottic + supraglottic | 15     | 21.43   |
| Glottic + subglottic   | 17     | 24.28   |
| Glottic                | 8      | 11.43   |
| pT stage               |        |         |
| T1                     | 0      | 0       |
| T2                     | 2      | 2.86    |
| T3                     | 12     | 17.14   |
| T4                     | 56     | 80      |
| pTNM stage             |        |         |
| I                      | 0      | 0       |
| II                     | 2      | 2.86    |
| III                    | 7      | 10      |
| IV                     | 61     | 87.14   |
| Lymph nodes            |        |         |
| N−                     | 54     | 77.14   |
| N+                     | 15     | 21.43   |
| Unspecified            | 1      | 1.43    |
| Histological grade     |        |         |
| G1                     | 66     | 94.3    |
| G2                     | 3      | 4.3     |
| G3                     | 1      | 1.4     |
| Treatment              |        |         |
| TL + RT                | 54     | 77.14   |
| TL + RT + CT           | 15     | 21.43   |
| PL                     | 1      | 1.43    |

**Abbreviations:** PL, partial laryngectomy; TL + RT, total laryngectomy and radiotherapy; TL + RT + CT, chemotherapy in addition to TL and RT.
Survivin was included in the inhibitor of apoptosis protein family with a potential dual role in apoptosis inhibition and regulation of mitosis. Expression and prognostic role of this marker were investigated in LSCC only in some earlier studies. In our study, we have shown that survivin expression was detected in more than half of the tumors. Similar results on laryngeal cancer have been reported by other authors. Some of them detected survivin in the cytoplasm and others in the nuclei. In our series, the intracellular localization of survivin was predominantly nuclear, in contrary, cytoplasm and nuclear staining were noted at the same time in only a few cases. It has been reported that the nuclear pool of survivin is involved in promoting cell proliferation, whereas the cytoplasmic pool may participate in controlling cell survival, but not cell proliferation. Based on these observations, the important nuclear presence of survivin detected in our series may suggest increased proliferative activity in Tunisian LSCC.

On the other hand, the survivin expression in our study significantly correlated with alcohol consumption. A few studies have been performed on alcohol effect on survivin expression in otorhinolaryngology (ORL) cancers. In fact, alcohol consumption causes a series of mutations at gene level that induce cell proliferation and promote carcinogenesis. Recently, many studies revealed that the increased p16 expression was highly correlated with the presence of human papillomavirus (HPV)-related neoplasms or with the alterations of the p16-Rb pathway. The expression of p16 was detected by immunohistochemistry that was proven to be the best and cost-effective method with a good sensitivity and specificity of 100% and 79%, respectively. Our results showed that half of tumors expressed the p16 protein suggesting that p16 upregulation is a frequent event in Tunisian laryngeal cancer. Previous works reported that p16 positivity was detected in 3% to 97% of LSCC. This controversial result might be due to biological behavior differences of tumors and variability in technical protocols. The immunostaining of p16 in our study was found in the nucleus and cytoplasm for all cases. This finding is explained by the fact that the only function associated with increased tumor aggressiveness. They have also became attractive molecules for evaluation of prognostic and therapeutic in cancer.

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attributed to p16, the cell cycle regulation, takes place in the nucleus.40 Moreover, cytoplasmic p16 has been associated with tumor progression and prognosis in some kinds of neoplasms.40 Our data demonstrated no significant correlation between p16 expression and different clinicopathological parameters, OS, and DFS. These results are in agreement with previous studies,41,42 but at variance with others.43,44 Indeed, there has been conflicting results as to whether p16 is a prognostic marker in LSCC. Some authors proved that P16 overexpression is associated with better prognosis44; therefore, multiple other retrospective studies did not find p16 to function as a significant outcome predictor.42

In summary, we have evaluated for the first time the immunophenotyping of survivin and p16 in Tunisian patients with LSCC. Unlike the p16 protein, survivin could be a poor prognostic factor and seems to be considered as a predictor marker

| Survivin Positive | Survivin Negative | P Value |
|-------------------|-------------------|---------|
| No. of patients   | 41                | 29      | 58.6 | 41.4 | .67 |
| Sex               |                   |         |      |      |     |
| Male              | 41                | 26      | 61.19| 38.81|     |
| Female            | 0                 | 3       | 0    | 100  |     |
| Age               |                   |         |      |      | .11 |
| <60 years         | 14                | 15      | 48.28| 51.72|     |
| ≥60 years         | 27                | 14      | 65.85| 34.15|     |
| Smoking habit     |                   |         |      |      |     |
| Yes               | 36                | 26      | 58.06| 41.94|     |
| No                | 2                 | 1       | 66.66| 33.33|     |
| Unspecified       | 3                 | 2       |       |      |     |
| Alcohol consumption|                 |         |      |      | .024|
| Yes               | 21                | 9       | 70   | 30   |     |
| No                | 6                 | 13      | 31.58| 68.42|     |
| Unspecified       | 14                | 7       |       |      |     |
| Tumor site        |                   |         |      |      | .18 |
| Glottic + supra + subglottic | 14 | 16 | 46.66 | 53.33 |     |
| Glottic + supraglottic | 8  | 7 | 53.33 | 46.66 |     |
| Glottic + subglottic | 13 | 4 | 76.47 | 23.53 |     |
| Glottic           | 6                 | 2       | 75   | 25   |     |
| pT stage          |                   |         |      |      | .67 |
| T1                | 0                 | 0       | 0    | 0    |     |
| T2                | 2                 | 0       | 100  | 0    |     |
| T3                | 7                 | 5       | 58.33| 41.66|     |
| T4                | 32                | 24      | 57.14| 42.86|     |
| pTNM stage        |                   |         |      |      | .43 |
| I                 | 0                 | 0       | 0    | 0    |     |
| II                | 2                 | 0       | 100  | 0    |     |
| III               | 3                 | 4       | 42.86| 57.14|     |
| IV                | 36                | 25      | 59.02| 40.98|     |
| Lymph nodes       |                   |         |      |      | .002|
| N−                | 26                | 28      | 48.15| 51.85|     |
| N+                | 14                | 1       | 93.33| 6.66 |     |
| Unspecified       | 1                 |         |      |      |     |
| Histological grade|                   |         |      |      |     |
| G1                | 38                | 28      | 57.57| 41.41|     |
| G2                | 2                 | 1       | 66.66| 33.33|     |
| G3                | 1                 | 0       | 100  | 0    |     |
| Treatment         |                   |         |      |      | .001|
| TL + RT           | 26                | 28      | 48.15| 51.85|     |
| TL + RT + CT      | 14                | 1       | 93.33| 6.66 |     |

Abbreviations: TL + RT, total laryngectomy and radiotherapy; TL + RT + CT, chemotherapy in addition to TL and RT.

| P16 Positive | P16 Negative | P Value |
|--------------|--------------|---------|
| No. of patients | 36           | 34      | 51.43 | 48.57 | .60 |
| Sex           |              |         |       |       |     |
| Male          | 35            | 32      | 52.24| 47.76 |     |
| Female        | 1             | 2       | 33.33| 66.66|     |
| Age           |              |         |       |       | .46 |
| <60 years     | 13            | 16      | 44.83| 55.17|     |
| ≥60 years     | 23            | 18      | 56.10| 43.9 |     |
| Smoking habit |              |         |       |       | .87 |
| Yes           | 32            | 30      | 51.61| 48.39|     |
| No            | 1             | 2       | 33.33| 66.66|     |
| Unspecified   | 3             | 2       |       |      |     |
| Alcohol consumption |        |         |       |       | .27 |
| Yes           | 12            | 18      | 40   | 60   |     |
| No            | 11            | 8       | 57.89| 42.11|     |
| Unspecified   | 13            | 8       |       |      |     |
| Tumor site    |              |         |       |       | .48 |
| Glottic + supra + subglottic | 16 | 14 | 53.33 | 46.66 |     |
| Glottic + supraglottic | 7  | 8 | 46.66 | 53.33 |     |
| Glottic + subglottic | 7  | 10 | 41.12 | 58.82 |     |
| Glottic       | 6             | 2       | 75   | 25   |     |
| pT stage      |              |         |       |       | .66 |
| T1            | 0             | 0       | 0    | 0    |     |
| T2            | 1             | 1       | 50   | 50   |     |
| T3            | 8             | 4       | 66.66| 33.33|     |
| T4            | 27            | 29      | 48.21| 51.78|     |
| pTNM stage    |              |         |       |       | .71 |
| I             | 0             | 0       | 0    | 0    |     |
| II            | 1             | 0       | 50   | 50   |     |
| III           | 5             | 2       | 71.42| 28.57|     |
| IV            | 30            | 31      | 49.18| 50.81|     |
| Lymph nodes   |              |         |       |       | .88 |
| N−            | 28            | 26      | 51.85| 48.14|     |
| N+            | 7             | 8       | 46.66| 53.33|     |
| Unspecified   | 1             |         |      |      |     |
| Histological grade |         |         |       |       | .80 |
| G1            | 34            | 32      | 51.51| 48.48|     |
| G2            | 2             | 1       | 66.66| 33.33|     |
| G3            | 0             | 1       | 0    | 100  |     |
| Treatment     |              |         |       |       | .52 |
| TL + RT       | 27            | 27      | 50   | 50   |     |
| TL + RT + CT  | 8             | 7       | 53.33| 46.66|     |

Abbreviations: TL + RT, total laryngectomy and radiotherapy; TL + RT + CT, chemotherapy in addition to TL and RT.

Table 2. Correlation of Survivin Positivity With Clinicopathological Parameters.

Table 3. Correlation of p16 Expression With Clinicopathological Parameters.
Figure 2. Kaplan-Meier curves of (A) overall survival ($P = 0.026$) and (B) disease-free survival ($P = 0.01$) of survivin expression in laryngeal squamous cell carcinoma.

Figure 3. Kaplan-Meier curves of (A) overall survival ($P = 0.001$) and (B) disease-free survival ($P = 0.003$) of lymph node metastasis in laryngeal squamous cell carcinoma.

Figure 4. Kaplan-Meier curves of (A) overall survival ($P = 0.000$) and (B) disease-free survival ($P = 0.000$) of type of treatment in laryngeal squamous cell carcinoma.
of recurrence and chemoresistance. However, p16 accumulation is not a useful tool for prognosis in Tunisian LSCC. Therefore, immunohistochemical expression of p16 will be investigated, in our future studies, as a surrogate marker for HPV infection in Tunisian LSCC.

Authors’ Note
We have the agreement of the Ethics Committee of Salah Azaiz Institute, for a retrospective research, on using laboratory, clinical data, and tissue samples of laryngeal carcinoma patients, recruited from Salah Azaiz Cancer Institute. It is a retrospective study; for this type of study formal consent is not required.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table 4. Multivariate Cox Regression Analysis for Disease-Free Survival of Laryngeal Squamous Cell Carcinoma.

|          | B   | SE  | Wald  | df | Sig. | Exp(B) | 95% CI     |
|----------|-----|-----|-------|----|------|--------|------------|
| Survivin | 0.775 | 0.629 | 1.518 | 1 | .218 | 2.171 | 0.632 7.452 |
| Lymph node metastasis | 0.240 | 0.238 | 1.018 | 1 | .313 | 1.271 | 0.797 2.027 |
| Treatment | 1.335 | 0.576 | 5.360 | 1 | .021 | 3.798 | 1.227 11.757 |

Table 5. Multivariate Cox Regression Analysis for Overall Survival of Laryngeal Squamous Cell Carcinoma.

|          | B   | SE  | Wald  | df | Sig. | Exp(B) | 95% CI     |
|----------|-----|-----|-------|----|------|--------|------------|
| Survivin | 0.567 | 0.588 | 0.931 | 1 | .335 | 1.763 | 0.557 5.582 |
| Lymph node metastasis | 0.168 | 0.219 | 0.589 | 1 | .443 | 1.183 | 0.770 1.819 |
| Treatment | 1.193 | 0.582 | 4.202 | 1 | .040 | 3.296 | 1.054 10.313 |

Abbreviations: B, β coefficient; CI, confidence interval; df, difference; SE, standard error; Sig., significance; Wald, Wald test statistic.

Figure 5. Kaplan-Meier curves of (A) overall survival (P = .79) and (B) disease-free survival (P = .57) of p16 expression in laryngeal squamous cell carcinoma.
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