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Original Article

Combination of IMP3 and PCNA Expression in Diagnosis of Laryngeal Squamous Cell Carcinoma

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Abstract

Background: Laryngeal carcinoma is one of the most common head and neck cancer worldwide. It has a high mortality rate and poor prognosis. Insulin-like growth factor II m-RNA-binding protein 3 (IMP3) is one of IMP family members which plays an important role in cell growth and migration during embryogenesis and has a role in carcinogenesis. Proliferating Cell Nuclear Antigen (PCNA) is a nuclear protein that is considered as a cell proliferation marker and has an important role in DNA duplication. Aim of the work: This study aimed to evaluate IMP3 and PCNA expression in laryngeal squamous cell carcinoma (LSCC), carcinoma in situ (CIS) and hyperplastic lesions. Patients and methods: Fifty cases of LSCC plus 8 cases of CIS and 9 cases of hyperplastic lesions were stained with IMP3 and PCNA and its immunoexpression results were statistically evaluated. Results: There is a significant positive correlation between IMP3 and PCNA expression in LSCC ($p < 0.001$ and $r= 0.860$) which indicates that IMP3 synchronously acts with PCNA to promote uncontrolled cell growth and proliferation. Conclusions: Combined use of IMP3 and PCNA increases the sensitivity and specificity of diagnosis of LSCC.

Key words: Immunohistochemical expression IMP3, PCNA, laryngeal squamous cell carcinoma.
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Introduction

After lung cancer, laryngeal carcinoma—typically a squamous cell carcinoma (SCC)—is thought to be the second most prevalent tumor in the respiratory tract. Alcohol and tobacco use are common risk factors. Despite advancements in treatment, the overall five-year survival rate for this malignancy has stayed at roughly 50%, and many patients had metastases at the time of initial diagnosis. [1]

Insulin-like growth factor mRNA binding protein 3; IMP3 is an oncofetal protein which has a role in embryogenesis and carcinogenesis of some tumors. [2] It is believed that IMP3 is a powerful posttranscriptional oncogene that is highly expressed in malignant tumors, increasing cell proliferation and aggressiveness of tumor cells. [3]

Cell proliferation rate is one of the most important indicators of tumor's aggressiveness. Cell proliferation is regarded as an important mechanism in carcinogenesis. [4] This has led to the study of other proliferation markers, such as PCNA, which is thought to be a predictive factor in various cancers. PCNA, or proliferating cell nuclear antigen, is a nuclear protein that plays a key part in both DNA replication and cell division. It first emerges in the nucleus in the late Gl phase, peaks in the S phase, and then fades in the G2 and M phases. Thus, PCNA is associated with DNA synthesis and cellular proliferation. [5]

In this study, we aim to evaluate expression of IMP3 and PCNA in benign, CIS and LSCC and to differentiate between LSCC and its mimickers.

Patients And Methods

This partially prospective, partially retrospective study was done using 50 laryngeal carcinoma specimens from patients who were diagnosed clinically as laryngeal carcinoma. In addition, eight cases previously diagnosed as in situ carcinoma (five cases obtained by laryngectomy and were associated with invasive component of LSCC, and the other 3 cases were biopsy specimens) and nine cases of hyperplasia in association with laryngeal polyp (two biopsy specimens associated with laryngeal polyps with apparently normal epithelium and 7 specimens of epithelium nearby invasive tumors removed by laryngectomy with mild to moderate dysplasia). The Clinical Trials.gov identifier (NCT number) for this trial is: NCT05293327.

Immunohistochemistry

Four micron tissue sections were cut from formalin-fixed paraffin-embedded tissue blocks for immunohistochemistry (IHC). Tissue sections were stained using rabbit monoclonal antibodies, ready to use for IMP3 (Catalog number; Cat # 2-IN090-13, Quartett, Berlin, Germany) and PCNA (Cat # RM- 2-PR049-13, Quartett, Berlin, Germany).

Using the Cell Marque trilogy and a pressure cooker, the heat-induced epitope retrieval approach was used to retrieve antigen for both antibodies. The primary antibody was incubated on tissue sections for one hour at room temperature. Each tissue section received one to two drops of biotinylated goat polyvalent, which were then incubated for ten minutes at room temperature before being twice rinsed in PBS. After applying one to two drops of streptavidin peroxidase, tissue sections were allowed to sit at room temperature for ten minutes before being rinsed twice in PBS.

Positive control was included with each run. Positive controls were placental and tonsilar tissue for IMP3 and PCNA respectively; the negative control was performed on the same tissue without applying the primary antibodies.

Immunohistochemical Evaluation

At low power magnification (×40), IMP3 protein expression was demonstrated in tumor cells as brownish cytoplasmic staining. Staining intensity and extent (percentage) were used for evaluation. Depending on the stain degree, IMP3 staining intensity was rated as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Depending on the proportion of cells that were stained positively, the staining percentage was graded as follows: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The total of the staining percentage and staining intensity was the final score. Based on their final scores, the cases were categorized into three groups: 0-1, negative; 2-4, weak positive; and 5-7, strong positive. [6]

The expression of PCNA protein was demonstrated as brownish nuclear staining. At high power magnification (x 400), the proliferation index (PI)

was calculated based on PCNA brown nuclear-stained cells in the hot spots. It was then calculated as a percentage of positively stained tumor nuclei per 1000 tumor cells. 0 indicates no reactivity, 1+ indicates 1-9% cell positivity, 2+ indicates 10-40% cell positivity, and 3+ indicates >40% cell positivity (5).

Statistical Analysis

Version 25.0 of IBM SPSS software was used to analyze the data (IBM Corp., 2017; IBM SPSS Statistics for Windows, Version 25.0). New York, NY: IBM Corp. Using the chi-square test, categorical variables were examined. Tests for diagnostic validity were conducted, including tests for diagnostic accuracy, sensitivity, specificity, PPV, and NPV.

To test the best way to use IMP3 and PCNA in the diagnosis of LSCC, we combined the results of their use once in test in series (which means that if both IMP3 and PCNA were positive this was considered positive and if any of them was negative this was considered negative) and once in test in parallel (which means that if either IMP3 or PCNA was positive this was considered positive and the negativity of both was required to consider it negative). Scatter plot and ROC curves graphs were produced. Significant differences were considered at $p < 0.05$.

Results

Clinicopathological data

Tables 1 and 2 provide a summary of the clinicopathological information for the patients under study. The patients' ages ranged from 45 to 100 years, with the majority being over 60. Males made up the majority of the cases (64/67).

Table (1): The demographic data of laryngeal carcinoma studied patients

Description		Studied patients (N= 50)	
		N	%
Gender	Male	48	96.0%
	Female	2	4.0%
Age group	≤ 60 years	19	38.0%
	> 60 years	31	62.0%
Age (years)	Mean± SD	64.86± 9.49	
	Median	63.0	
	Range	45.0 – 100.0	

Table (2): Data of hyperplastic and CIS studied patients.

Description		Studied cases (N= 17)	
Gender	**Male**	**16**	**94.12%**
	ıale		**%**
Age group	**≤ 60 years**	**7**	**41.17%**
	> 60 years		**3%**
Age (years)	**Mean± SD**	**62.29± 9.1907**	
	lian		
	Range	48.0 – 75.0	
Type of lesion	**CIS**	**8**	47.0%
	perplastic cases		53.0%

Immunohistochemical expression of IMP3

There was positive expression of IMP3 in 58/67 (86.57%) of all the studied cases **(Table 3),** most of them (49) were LSCC.

Table (3): Difference in IMP3 expression in the three studied groups

IMP3 expression	Histopathological type			Total	p-value
	Malignant	*In situ* lesions	Hyperplastic lesions		
Positive	49	7	2	58	<0.0001*
Negative	1	1	7	9	
Total number of cases	50	8	9	67	

*P-value was highly significant Chi square test was used

There was significant difference in IMP3 expression in hyperplastic lesions and in *in situ* carcinomas compared with its expression in LSCC (p<0.0001, =0.015 respectively). However, there was no significant difference in IMP3 expression in and in *in situ* carcinomas compared with LSCC (p =0.26), as shown in **tables (3&4).**

Table (4): Difference in IMP3 expression between each two studied groups

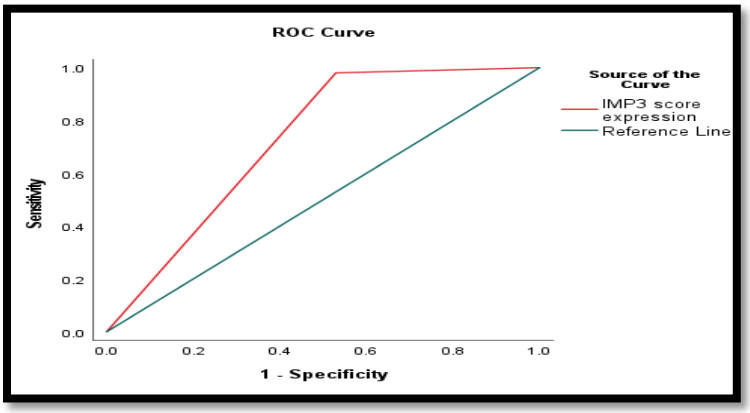
Histopathological type		P-value
Malignant	Hyperplastic lesions	<0.0001** (Fisher)
In situ lesions	Malignant	=0.26 (Fisher)
Hyperplastic lesions	In situ lesions	=0.015* (Fisher)

Assessment of the sensitivity, specificity and diagnostic accuracy of IMP3 in the studied cases:

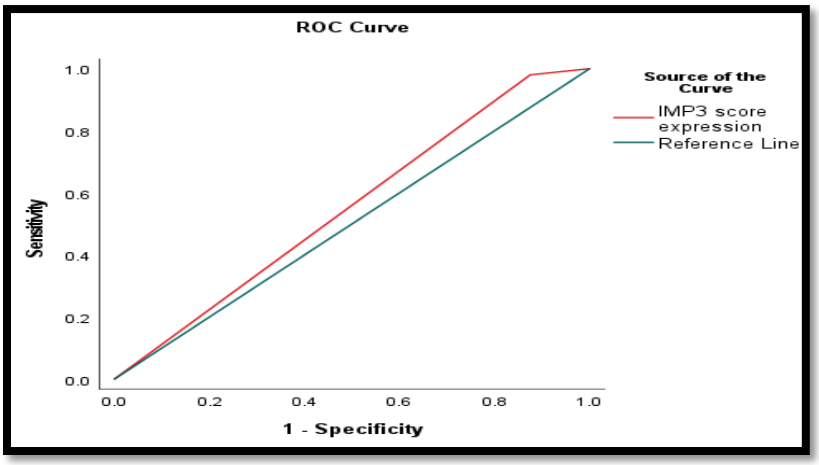
We found that IMP3 score had an overall sensitivity, specificity, and diagnostic accuracy of 98%, 47.1% and 85.1% respectively in detecting LSCC. The PPV was 84.5%, while the NPV was 88.9%. Assessment of the sensitivity, specificity, PPV, NPV and diagnostic accuracy of IMP3 between each two studied groups was shown in **table (5) & graphs (1& 2).**

Table (5): Accuracy measures of IMP3 score in detection of LSCC

Diagnosis	Sensitivity	Specificity	PPV	NPV	Accuracy
LSCC vs others (CIS and hyperplastic)	98%	47.1%	84.5%	88.9%	85.1%
LSCC vs hyperplastic lesions	98%	77.8%	96.1%	87.5%	94.9%
LSCC vs CIS	98%	12.5%	87.5%	50%	86.2%
CIS vs hyperplastic lesions	87.5%	77.8%	77.8%	87.5%	82.4%



Graph (1): ROC curve showed diagnostic accuracy of IMP3 in LSCC vs other laryngeal lesions with area under curve (AUC) = 0.73.



Graph (2): ROC curve show diagnostic accuracy of IMP3 in LSCC vs *in situ* lesions with AUC= 0.55.

Immunohistochemical expression of PCNA

There was positive expression of PCNA in 58/67 (86.57%) of all the studied cases **(Table 6);** all the 50 LSCC cases were PCNA positive.

Table (6): The difference in PCNA expression between the three studied groups

PCNA expression	Histopathological type			Total	p-value
	LSCC	*In situ*	Hyperplastic		
Positive expression	50	7	1	58	**<0.0001***
Normal basal expression	0	1	8	9	
Total number of cases	50	8	9	67	

Chi square test was used *p-value was highly significant

There was significant difference in PCNA expression in hyperplastic lesions and in situ lesions compared with its expression in LSCC (p<0.0001, 0.003 respectively). However, there was no significant difference in PCNA expression in in situ lesions compared with its expression in LSCC (p =0.14), as shown in **table (7).**

Table (7): The difference in PCNA expression between each two studied groups

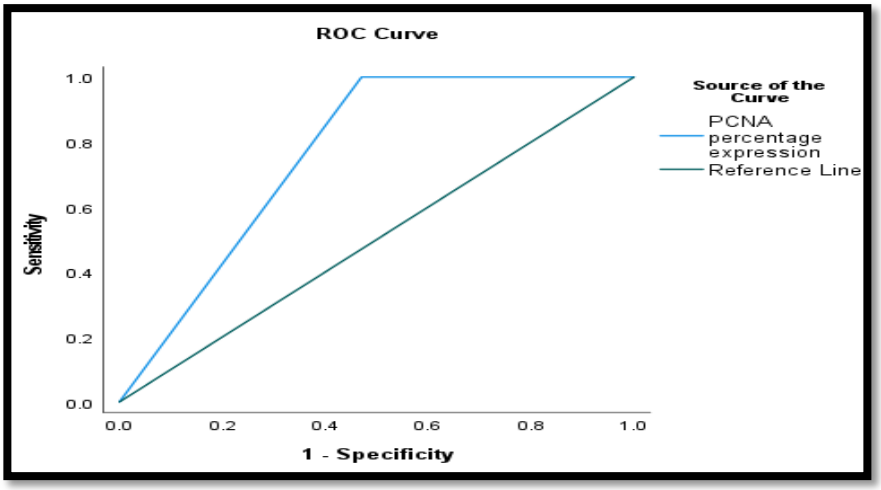
Histopathological type		**P-value**
Malignant	**Hyperplasia**	<0.0001*(Fisher)
***In situ* carcinomas**	**Malignant**	0.14 (Fisher)
Hyperplasia	***In situ* carcinomas**	0.003* (Fisher)

Assessment of the sensitivity, specificity and diagnostic accuracy of PCNA in the studied cases:

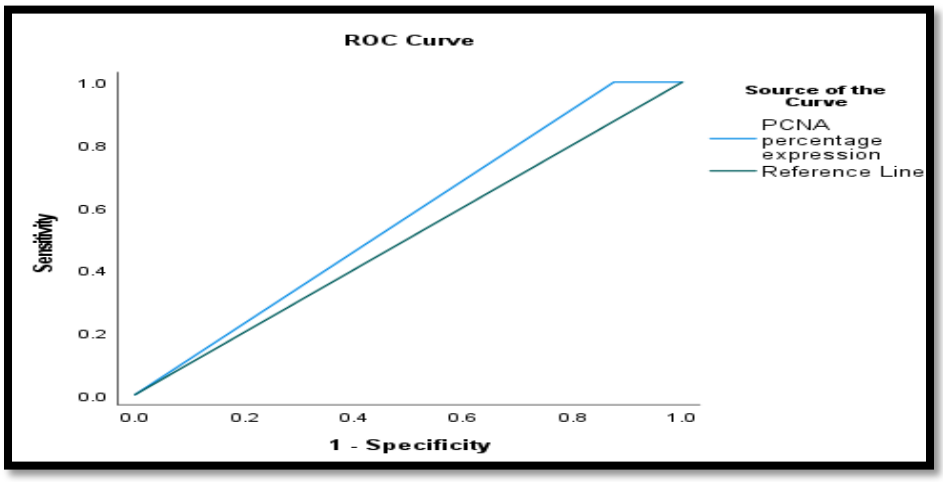
PCNA percentage had an overall sensitivity, specificity, and diagnostic accuracy of 100%, 52.9% and 88.1% respectively in detecting LSCC. The PPV was 86.2.9%, while the NPV was 100%. Assessment of the sensitivity, specificity, PPV, NPV and diagnostic accuracy of PCNA between each two studied groups was shown in **table (8) and graphs (3& 4).**

Table (8): Accuracy measures of PCNA percentage in detection of LSCC

Diagnosis	Sensitivity	Specificity	PPV	NPV	Accuracy
LSCC vs others (CIS and hyperplastic)	100%	52.9%	86.2%	100%	88.1%
LSCC vs hyperplastic lesions	100%	88.9%	98%	100%	98.3%
LSCC vs CIS	100%	12.5%	87.7%	100%	87.9%
CIS vs hyperplastic lesions	87.5%	88.9%	87.5%	88.9%	88.2%



Graph (3): ROC curve showed diagnostic accuracy of PCNA in LSCC vs other laryngeal lesions with AUC= 0.77.



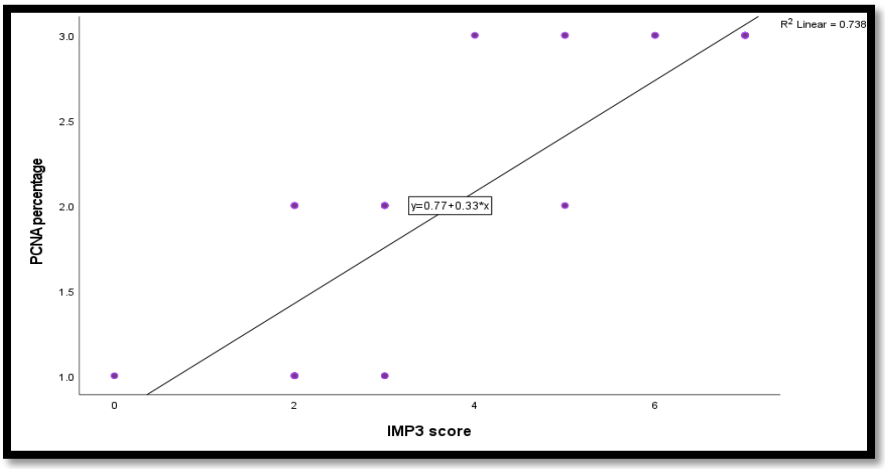
Graph (4): ROC curve showed diagnostic accuracy of PCNA in LSCC vs *in situ* lesions with AUC= 0.56.

Correlation between IMP3 and PCNA expression in LSCC:

It was found that there was high significant positive correlation between IMP3 score and PCNA expression in LSCC, as shown in **table (9) and graph (5).**

Table (9): Correlation between IMP3 score and PCNA expression in LSCC studied cases.

	IMP3 score	
	R	**p- value**
PCNA expression	0.860	**<0.001 (HS)**



Graph (5): Scatterplot showing strong positive correlation between IMP3 score and PCNA percentage in LSCC studied cases.

Assessment of the sensitivity, specificity and diagnostic accuracy tests of both IMP3 and PCNA in combination in the diagnosis of LSCC

To determine the most effective use of IMP3 and PCNA in diagnosis of LSCC, we combined their results by using test in series and test in parallel, number of positive and negative cases according to these tests were illustrated in **tables (10-12) and graph (6).**

Table (10): Number of positive and negative cases according to test in series and test in parallel in the studied cases

Description		Histopathological type			Total
		Malignant	*In situ*	Hyperplastic	
Test in series	Positive	49	6	1	56
	Negative	1	2	8	11
Test in parallel	Positive	50	8	2	60
	Negative	0	0	7	7
Total		50	8	9	67

By using the test in series, sensitivity, specificity, PPV, NPV and diagnostic accuracy of combination of both markers in differentiating LSCC from other laryngeal lesions were shown in **table (11).**

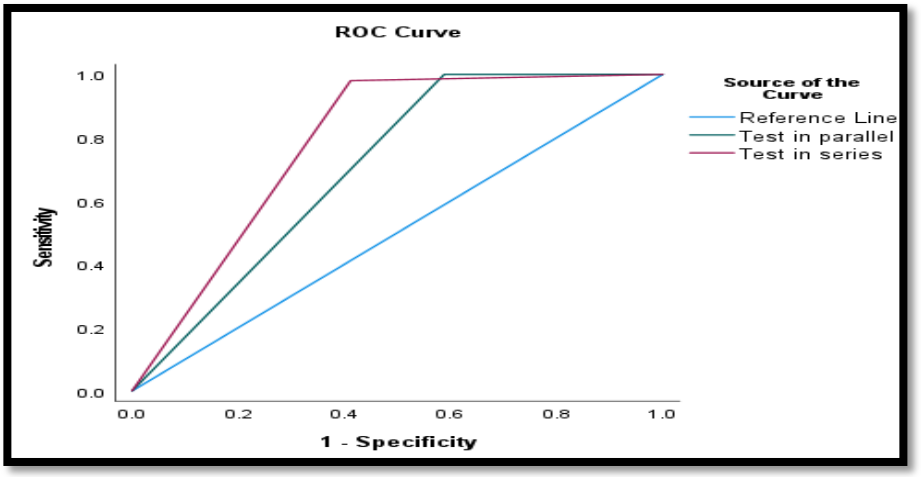
By using the test in parallel, sensitivity, specificity, PPV, NPV and diagnostic accuracy of combination of both markers in differentiating LSCC from other laryngeal lesions were shown in **table (12).**

Table (11): Test in series of both markers in combination in the diagnosis of LSCC vs other laryngeal lesions

Diagnosis	Sensitivity	Specificity	PPV	NPV	Accuracy
LSCC vs other laryngeal lesions	98%	58.8%	87.5%	90.9%	88.1%

Table (12): Test in parallel of both markers in combination in the diagnosis of LSCC vs other laryngeal lesions

Diagnosis	Sensitivity	Specificity	PPV	NPV	Accuracy
LSCC vs other laryngeal lesions	100%	41.2%	83.3%	100%	85.1%



Graph (6): ROC curve showed test in series and test in parallel of both IMP3 and PCNA in LSCC vs other laryngeal lesions with AUC= (0.78, 0.71 respectively).

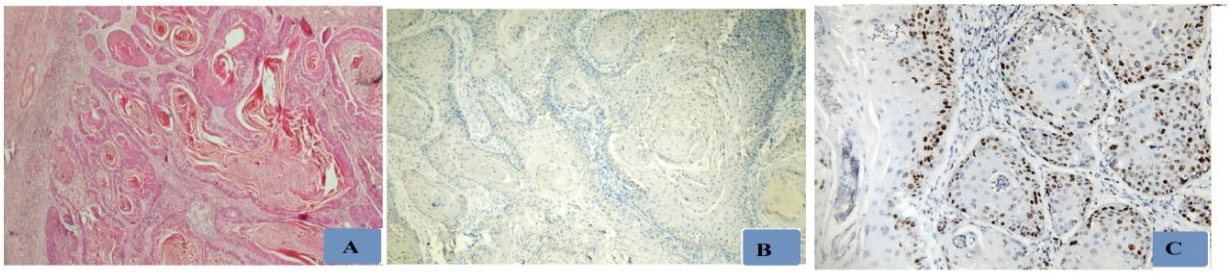


Figure (1): Showing LSCC grade I, (A) H&E x40, (B) IMP3 showing negative expression "score 0" x100, (C) PCNA showing percentage "1", 200

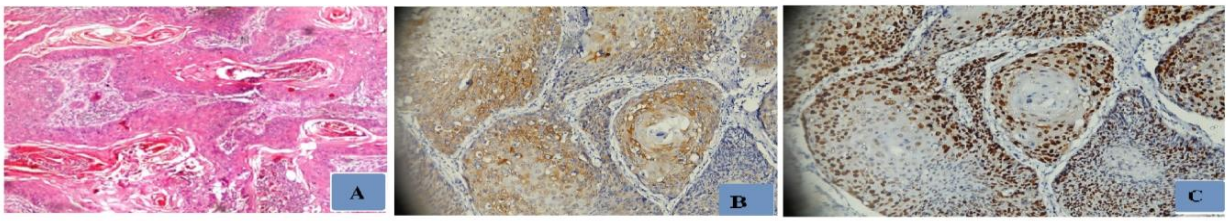


Figure (2): Showing LSCC grade I, (A) H&E, x100, (B) IMP3 showing weak expression "score 3", (C) PCNA Showing percentage "2".

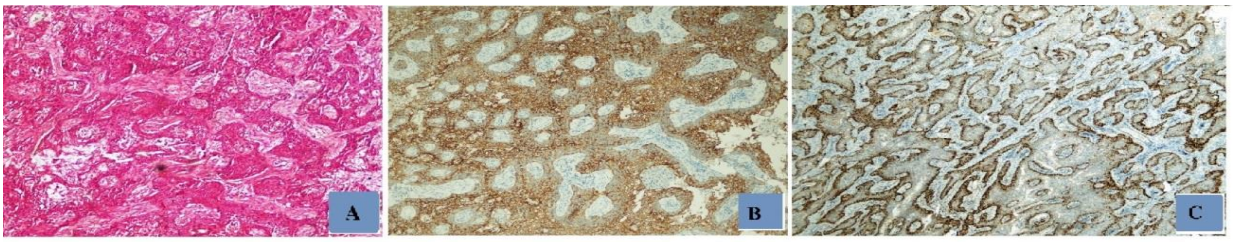
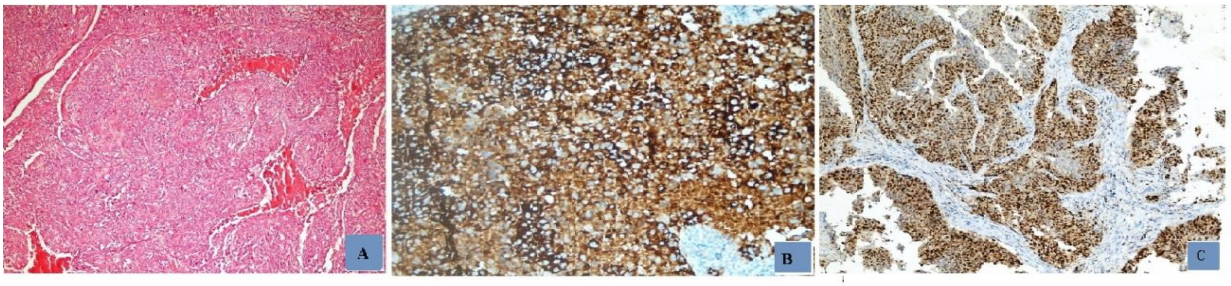


Figure (3): Showing LSCC grade II, (A) H&E x100, (B) IMP3 showing srong expression "score 5", (C) PCNA shoeing percentage "3'.



Fiure (4): Showing LSCC grade III, (A) H&E, x100, (B) IMP3 showing strong expression "score 6", (C) PCNA showing percentage "3".

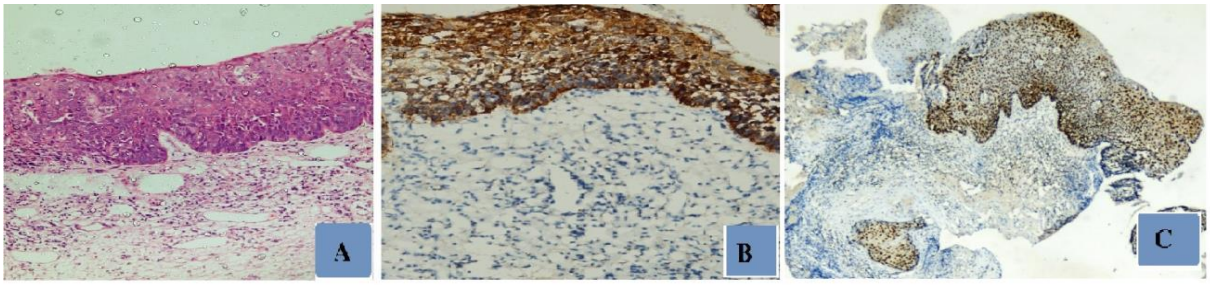


Figure (5): Showing severely dysplatic laryngeal epithelium (CIS), (A) H&E, 200, IMP3 showing positive expression, x200, (C) PCNA showing positive expression up to the surface, x40

Figure (6): (A) H&E showing benign- looking surface epithelium with underlying USCC grade III" arrow", x40, (B) IMP3 showing negative surface epithelium and strong IMP3 expression of invasive LSCC " score 7" ,x100, (C) PCNA showing weak basal epithelium and underlying LSCC showing pereentage "3".

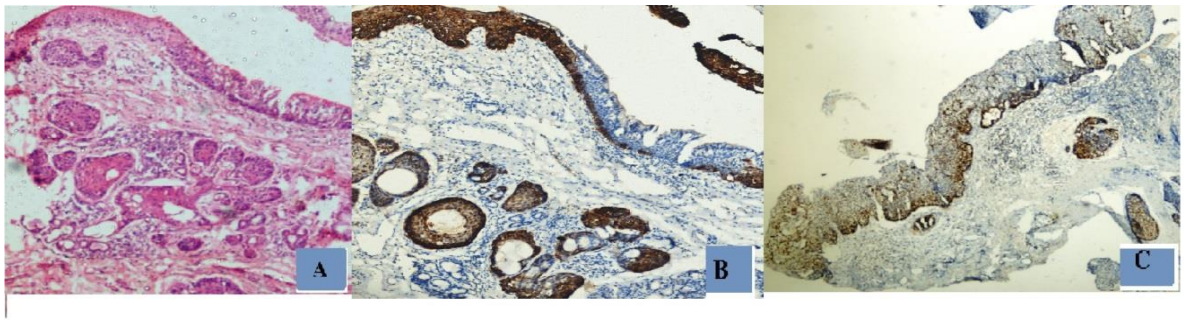


Figure (7): Laryngeal surface epithelium showing severe dysplasia on the left side and adjacen benign epithelium on the right side, (A) H&E, x100, (B) IMP3 showing positive expression in severe dysplasia and negative in benign epithelium, x100, (C) PCNA showing positive expression up to the surface in severe dyspllasia and in benign epithelium positivity is basal and in adjacent layers, x40.

Discussion

The most prevalent head and neck cancer in the world, laryngeal carcinoma has a poor prognosis and a high death rate. [7] Nearly 95% of all malignant tumors in the larynx are squamous cell carcinomas. [8] Despite major advancements in treatment, the long-term survival rate for individuals with laryngeal cancer has not changed significantly. [9]

A member of the IMP family, insulin-like growth factor II mRNA binding protein-3 (IMP3) has been found to be a significant indicator for a number of cancerous tumors. According to **Tarsitano et al.** [10], it plays a part in the invasion, adhesion, and proliferation of cells in malignant neoplasms.

In this study, IMP3 was negative in most hyperplastic cases (7/9; 77.7%) and positive in 2/9 of hyperplasia with mild to moderate dysplasia where they showed weak cytoplasmic IMP3 staining. Whereas 7/8 (87.5%) of CIS cases showed positive membranous IMP3 staining, near to the findings of **Chen et al.** [2]. They found that all cases of hyperplasia were negative for IMP3, and 84% of CIS cases were IMP3 positive.

This finding is important to distinguish between malignant tumors and their mimics of non-malignant laryngeal surface epithelium. It also highlights the progression in expression of IMP3 from non-neoplastic up to dysplastic epithelium and later to LSCC. This finding put a suggestion of a possible role to IMP3 in early carcinogenesis in the larynx.

There was significant difference in IMP3 expression in the three studied groups (LSCC, CIS and hyperplastic lesions). This was in harmony with **Maržić et al.** [11], who reported statistically significant differences between the three groups.

Concerning the sensitivity and specificity of IMP3 in detection of LSCC from other lesions as CIS and hyperplasia; they were 98% and 47.1% respectively. PPV was 84.5% and NPV was 88.9%. Our results were near to Chen et al., (2013) report regarding IMP3 sensitivity. They reported that the IMP3 sensitivity in LSCC was 92%.

On the other hand, **Abd-Elaziz et al.** [3] discovered that IMP3 had a sensitivity of 60.66% and a specificity of 60.32%. Additionally, **Maržić et al.** [11] reported IMP3 sensitivity and specificity of 80.0% and 89.3%, respectively, which differed somewhat from the results of the current study.

DNA replication, chromatin remodeling, DNA repair, and cell-cycle regulation are all significantly impacted by PCNA, a proliferating cell nuclear antigen . [12]Survival and prognosis are strongly correlated with PCNA.[13]

Our study showed that PCNA was basally expressed in 8/9 (89%) of hyperplastic cases, the remaining case with moderate dysplasia showed that in addition to basally located PCNA expression, the mid zonal layers of laryngeal epithelium showed PCNA expression too. CIS cases showed positivity up to the epithelial surface in 6/8 (75%) of cases, one case showed positivity till the mid layers and the last one showed only basal PCNA expression.

This finding is in harmony with what was published by **Laitakari et al.** [14] , who found that in hyperplastic epithelium, PCNA staining was positive in multiple layers in a basal position, but absent in

the superficial epithelial layers. They also found that this expression was gradually increased in the mid-zonal layers in preneoplastic conditions; dysplasia grades I and II. In dysplasia grade III, PCNA-positive nuclei were seen diffusely up to the surface epithelium.

There was significant difference in PCNA expression in our three groups (LSCC, CIS and hyperplastic lesions). This was near to the results previously published by **Saraç et al.** [15] who showed statistically significant differences between LSCC and control group (benign laryngeal epithelium).

Regarding the sensitivity and specificity of PCNA in differentiating LSCC from CIS and hyperplastic lesions, our results showed gradual increase in levels of PCNA in those lesions progressing to invasive cancer. The sensitivity was 100% while the specificity was 52.9% for diagnosing LSCC. **Ye et al.**[16] results' were away from ours, as they reported that the sensitivity and the specificity of PCNA were 84% and 76% respectively for diagnosing LSCC. This may be explained by different tumor type they studied (they analyzed the marker in Non-small cell lung carcinoma). To the best of our knowledge, no previous studies analyzed the PCNA sensitivity and specificity for detection of LSCC.

There was highly significant positive correlation between PCNA expression and IMP3 score ($p<0.001$ & $r= 0.860$) in LSCC. To the best of our knowledge, no previous studies discussing these correlations. This suggests that IMP3 could affect cellular proliferation and PCNA expression in the carcinogenesis and progression of LSCC.

Using a combination of both markers, we assessed the sensitivity and specificity for diagnosis of LSCC compared to CIS and hyperplasia by using tests in series and in parallel. The sensitivity, specificity and diagnostic accuracy by test in series were 98, 58.5 and 88.1% respectively. The sensitivity, specificity and diagnostic accuracy using test in parallel were 100%, 41.2% and 85.1% respectively. This indicates that using both markers together in the diagnosis of LSCC improves sensitivity and specificity and overall diagnostic accuracy. To the best of our knowledge, this is the first study in which combination of those markers were used in LSCC.

However, **Maržić et al.** [17] found a positive statistically significant correlation ($r=0.497$, $p<0.001$ and $r=0.190$, $p<0.026$, respectively) between IMP3 and markers of cell proliferation and cell cycle, specifically Ki-67 and cyclin D1. This suggests that IMP3 acts in tandem with these markers to promote unchecked cell growth and proliferation. Therefore, we might propose that IMP3 acts in conjunction with PCNA to increase cell proliferation in LSCC, as PCNA is a proliferating marker.

According to **Oka et al.** [18], proliferating cells expressed both PCNA and Ki-67, but there were significant distinctions between the two. Ki-67 expression begins in the middle of the G1 phase, progresses through the S and G2 phases, peaks in the M phase, and then degrades extremely quickly at the conclusion of the M phase. PCNA, on the other hand, has an extremely extended half-life. That would suggest improved specimen detection.

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Abbreviations: HNSCC: head and neck squamous cell carcinoma, carcinoma CIS; carcinoma in situ, SCC squamous cell carcinoma, IMP3: insulin-like growth factor II m-RNA-binding protein 3, PCNA: proliferating cell nuclear antigen, IHC: immunohistochemistry, AJCC: American Joint Committee on Cancer, PPV; positive predictive value, NPV; negative predictive value