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THE RELATION BETWEEN THE PROGNOSTIC MARKERS (P53 AND KI67) IN BENIGN AN PREMALIGNANT LARYNGEAL LESION

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Abstract

Background: The histological classification of precancerous lesions depends on the degree of epithelial dysplasia.Grade1 lesions; it is designated as simple hyperplasia or keratosis with or without mild dysplasia. Grade2 lesions, it is designated as moderate dysplasia. Grade3 lesion, it is designated as severe dysplasia. Simple hyperplasia with low risk of malignancy, atypical hyperplasia with a high risk of malignancy. Benign laryngeal lesion (non neoplastic); Vocal cord nodule and Amyloidosis. Tumors and Tumor like Conditions; Papilloma and Papillomatosis. The main risk factors are smoking which enhanced by heavy alcohol consumption, nutritional factors and the viral infections Human papillomavirus (HPV). All have paly an implicated role as predisposing factors in the pathogenesis. The incidence of P53 and KI67 tumors has been diagnosed in patients with laryngeal premalignant lesion has to be increase. **Tumor suppressor protein (P53)**, also known as ***TP53***, the TP53 gene is the most frequently mutated gene in human cancer (>50%), indicating that it plays a critical role in cancer prevention. The TP53 gene codes proteins that link to DNA and control gene expression in order to avoid genome mutations. **Marker of Proliferation (Ki-67 or MKI67)**, is a nuclear protein encoded by the MKI67 gene in humans, it is among the most useful. **Aim of study:** This study was designed to assess the relation between the prognostic markers (P53, KI67) and then finding correlation between these markers. **Materials and Methods:** This research was conducted at the Faculty of Medicine, Department of Pathology and forensic medicine. A total of (35) formaldehyde fixed and paraffin-embedded tissues (FFPET) were included in this study**.** The cases were classified as; 10 cases premalignant laryngeal lesions (SIL) and 25 cases benign laryngeal lesion (papilloma and singer's nodules). Cases were revised by sectioning and staining with hemotoxyllin and eosin (H&E) for confirming diagnosis.

Then Immunohistochemical technique was applied in order to assess the expression of P53 and ki67 proteins in all premalignant and benign laryngeal lesion. The Combined Positive Score (CPS) is used to assess the expression . P53 protein expression was determined by score index; which is positive if SI ≥ 1%. Ki67 protein expression was determined also by score index with cut off 4%, as low and high expression. Statistical analysis was done to assess the significance of expression, association and correlation between these markers. **Results:** The current study revealed that P53 protein was shown 40% of premalignant laryngeal lesions and 12% of benign laryngeal lesions. **While** looking at the ki67 protein, discovered that 40% of

premalignant lesions had high ki67 expression and 20% of benign laryngeal lesions had high ki67 expression. **Conclusion:** p53 and ki67 expression have an essential role in pathogenesis of laryngeal carcinoma and noticed in both premalignant and benign laryngeal lesions without statistical significant difference and it is associated and correlated to P53 and Ki-67. This finding assists the target therapy and the screening surveillance of any laryngeal lesion particularly premalignant lesions.

Introduction

The histological classification of precancerous lesions depends on the degree of epithelial dysplasia.Grade1 lesions, it is designated as simple hyperplasia or keratosis with or without mild dysplasia. Grade2 lesions, it is designated as moderate dysplasia. Grade3 lesion, it is designated as severe dysplasia. Simple hyperplasia with low risk of malignancy, atypical hyperplasia with a high risk of malignancy (1). Benign laryngeal lesion (non neoplastic); Vocal cord nodule and Amyloidosis. Tumors and Tumor like Conditions; Papilloma and Papillomatosis (2).

The incidence of P53 and KI67 tumors has been diagnosed in patients with laryngeal premalignant lesion to increase (3), and there is closely relation (4, 5). The expression of P53 positive and high expression of KI67 is a strong confirmation of biologically relevant and correlated to laryngeal premalignant and benign lesion (as prognostic markers) (6, 7).

Aims of study

1. Stand out the relationship between the occurrence of laryngeal lesions (benign, premalignant) and p53,ki67 expression, as a causal factor together with development of laryngeal SCC.
2. Finding association and correlation between P53 and KI67 expression as surrogate indicators for patients with premalignant and benign laryngeal lesions.

Normal Anatomy of larynx:

The larynx is consider as a complex organ that compound of various stromal and epithelial tissues. The supraglottic region is composed of the third and fourth branchial pouches, while the glottis and subglottis are composed of the sixth branchial pouch (8).

The major cartilages of the larynx (cricoid, thyroid, and arytenoids) are of hyaline type, whereas the epiglottis is of elastic type, with numerous fenestrations(8).

Histology:

Depending on the location, the natural laryngeal epithelium ranges from stratified squamous to ciliated respiratory epithelium. The union between two epithelial types separated by a transition zone (8).

Patches of squamous epithelium can be found in the respiratory-like epithelial areas on a frequent basis. These are more prominent in smokers . Dendritic melanocytes can be found in the basal layer, especially the black ones. Around the larynx, seromucinous glands can be observed (8).

The platelet like structure for true vocal cords termed as Reinke space, that is surrounded by the voiced ligature from single aspect and the base of ranked lining from another aspect. It contains some capillaries but lacks lymph vessels (9).

Laryngeal lesions:

I. Benign:

- Non neoplastic Lesions:

***Vocal Cord Nodule.** Represents a peculiar noninflammatory reaction to injury causing hoarseness. People who abuse their voices are more likely to have this problem. It's also known as singer's nodule since it mostly affects the anterior third of the vocal cords (10).

***Contact granuloma** (contact ulcer). Is a lesion that is frequently mistakenly confused with laryngeal nodule but having a different presentation, microscopic appearance, and behavior. The most prevalent causes are severe phonotrauma, gastric reflux illness, and intubation. It is almost found at the level of the posterior commissure, near the vocal process of the arytenoid cartilage, at a site subglottic region of the trachea and the larynx (11).

***Amyloidosis**. The tracheobronchial tree may be involved as a rare occurrence. It is normally asymptomatic, although it can cause hoarseness and bleeding. The most common location is the false vocal cords., but any portion of the larynx can be involved, often in a multifocal fashion (12).

***Eosinophilic angiocentric fibrosis**. Is a strange upper respiratory tract inflammatory lesion that appears more commonly in the nasal cavity and is thought to represent a mucosal variation of granuloma faciale (20).

- Tumors and Tumor like Conditions (Papilloma and Papillomatosis):

Papilloma and Papillomatosis. Juvenile laryngeal papillomas are papillary tumors seen on the true cords of children and adolescents that can migrate to the false cords, epiglottis, subglottic region, and in rare cases the migrate to trachea and bronchi. When extensive, Papillomatosis can lead to severe respiratory problems and even death. Ultrastructural examination, immunohistochemical demonstration of HPV antigens, and in situ hybridization have all been used to prove the viral etiology of juvenile laryngeal papillomatosis. Viral DNA has also been found in non-involved sites in individuals with active illness and as well as in patients in remission. HPV-11 and HPV-6 are two forms of HPV that have been related to laryngeal papillomas (13).

Premalignant:

Squamous Intraepithelial Lesions (SIL) Keratosis of the larynx most commonly affects the true cords and interarytenoid area. It is also called simple or epithelial hyperplasia and leukoplakia. It frequently occurs in smokers, singers, and other people who frequently use their voices. Hoarseness is a common complaint, and a laryngoscopic examination of the affected areas can reveal white thickening. The presence of reddening should alert the clinician that a more serious lesion (dysplasia/CIS) may be present. Cases of keratosis that have an irregular

warty configuration are referred to as verrucous keratosis or papillary keratosis. Benign keratotic lesions are characterized by hyperkeratotic epithelium (often with a granular layer) and acanthosis, without dysplastic under the microscope. Cases of keratosis are characterized by varying degrees of loss of normal growth and development, cellular atypia, and decrease of stratification. It is grouped under generic terms such as (SIL), laryngeal intraepithelial neoplasia (LIN) and squamous intraepithelial neoplasia (SIN), as they are in other sites, these embracing the traditional categories of dysplasia and CIS. The degree of nuclear anomalies (including polarity alterations) and the extent of stratification loss in the epithelium are used to grade these changes (14).

The World Health Organization (WHO) Classification of Tumors described and illustrated the criteria for their grading system (referred to as the "dysplasia system") as follows (15):

1. Mild dysplasia. The nuclear abnormalities are minor, and they are more visible in the basal third of the epithelial thickness. In the upper layers, where the cells have matured and stratified, they are minimal. The parabasal layers may contain a few mitoses. In most cases, keratosis and chronic inflammation are present.

2. Moderate dysplasia. Nuclear abnormalities are more noticeable than in mild dysplasia, and nucleoli are more visible. These changes are most noticeable in the epithelial thickness's lower two-thirds. Moderate nuclear abnormalities may persist up to the surface, but in the upper layers, cell maturation and stratification are visible. Mitoses can be found in the intermediate and parabasal layers. Keratosis may be present in the lesion.

3. Severe dysplasia. More than two-thirds of the epithelial thickness is affected by nuclear abnormalities and loss of maturation, with some stratification of the most superficial layers. Pleomorphism of the nucleus is common, and some cells may have bizarre nuclei. The nucleoli are prominent in some areas, but all of the nuclei are hyperchromatic in others. Mitoses can be found high up in the epithelium, including atypical mitoses. The lesion is distinguished from CIS by the presence of some maturation and stratification of the cells in the most superficial layers. Keratosis is frequently associated with the lesion.

4. CIS. A lesion in which the entire thickness of the squamous epithelium shows the cellular characteristics of carcinoma without stromal invasion. The majority of laryngeal CIS is made up of keratinized cells (spinous or well-differentiated), while a small percentage is made up of basal-like cells similar to those found in uterine cervix CIS (basal type). Papillary CIS is a type of CIS characterized by papillary fronds with a fibrovascular stroma covered by squamous epithelium and cytologic characteristics similar to the conventional variety. It's also worth remembering that a dysplasia/CIS lesion could simply be the periphery of an invasive cancer.

The Ljubljana classification, an alternative grading system, divides the classes into the following categories: 1.Hyperplasia of the squamous epithelium. 2.Hyperplasia of the basal and parabasal areas. 3.Hyperplasia atypical. 4.CIS. The first two are considered benign, the third is considered potentially malignant, and the fourth is considered genuinely malignant. The authors mention the lack of significance of the presence of a surface keratin layer in the differential diagnosis between CIS and smaller lesions, an important difference with the WHO

approach. The Ljubljana categories are difficult to translate into those used by the WHO system, and no convincing evidence has been brought forward that this system will improve the admittedly unsatisfactory rate of interobserver agreement with the WHO system or that it can provide more accurate prognostic information (16). A third proposal is that of a binary system, by which lesions are divided as follows: 1. Hyperplasia/keratosis, SIN I (low grade), 2. SIN II (high grade**)** (17).

Prognostic markers:

2.4.1 P53:

The p53 protein is a nuclear phosphoprotein that helps keep the DNA intact. The p53 gene mutation is one of the most common genetic abnormalities seen in malignant tumors in humans. Due to its extended half-life, the inactive p53 protein from the mutant gene affects the mechanism of genetic defect repair and the opportunity for apoptotic elimination of cells with damaged DNA. The inactive p53 protein may be discovered using immunohistochemistry methods. Its high expression in head and neck cancers has been associated to tumor aggressiveness and a poor prognosis (18).

2.4.2 KI67:

KI67 is a nuclear non-histone protein that may be found in both normal and cancerous cells that are rapidly growing. The protein is employed in the estimation of the tumor's so-called growth fraction of the tumor. In numerous forms of malignant tumors, the prognostic relevance of KI67 antigen has been emphasized. Ki-67 expression has also been found to connect with clinical and pathological factors in head and neck malignancies (12, 19).

KI67 is one of the most useful markers to evaluate cell proliferative activity and has been widely used in tumor treatment and research. However, its role in human laryngeal carcinoma remains poorly defined. Ki67 expression levels are linked to cervical lymph node metastases and clinical outcomes (12, 19).

The pretreatment apoptotic index and the Ki-67 proliferation assessment are beneficial in predicting the clinical outcome of patients with laryngeal cancer who are referred for radiation (20).

Study design and setting:

The cross sectional study was accomplished by the field of Pathology and Forensic Medicine, faculty of Medicine in University of Kufa (the laboratory's materials and equipment were standardized to our study as well qualified) in 2022 within 6 months of study. All cases incorporated in current study are collected in random way from Al-Sadder Teaching Hospital and also from private laboratories at Al-Najaf governorate. So, we have 35 cases (25 case benign lesions and 10 cases premalignant lesions) can be divided according to the type of surgical procedure (punch biopsy and excisional biopsy).

*When Statistical equation (21);

$$\text{Sample size} = \frac{Z_{1-\alpha/2}{}^2 p(1-p)}{d^2}$$

Here

$Z_{1-\alpha/2}$ = Is standard normal variate (at 5% type 1 error ($P<0.05$) it is 1.96 and at 1% type 1 error ($P<0.01$) it is 2.58). As in majority of studies P values are considered significant below 0.05 hence 1.96 is used in formula.
p = Expected proportion in population based on previous studies or pilot studies.
d = Absolute error or precision – Has to be decided by researcher.

Selection of Samples:

A) Sample collection: 35 cases of benign, premalignant and malignant laryngeal lesions are incorporated within this study, they were random collected from many histo-pathological labs in AL- Najaf city. Tissue was saved with formaldehyde and embedded in paraffin blocks**.**

B) inclusion and exclusion criteria:

***inclusion criteria**:

- ✓ Cases already diagnosed as benign, premalignant lesion by H and E stain.
- ✓ Cases with available clinical data including: age, gender, subtype and grade.
- ✓ ***exclusion criteria:**
- ✓ Biopsies with inadequate pathological material.
- ✓ Biopsies with abnormal and poor quality of pathological material.

Materials and Equipment:

A) H and E stain procedure:

For review and differentiation between benign, and premalignant . After sectioning of blocks by microtome dewaxing in 60 C° 50 for minutes followed by rehydration and staining.

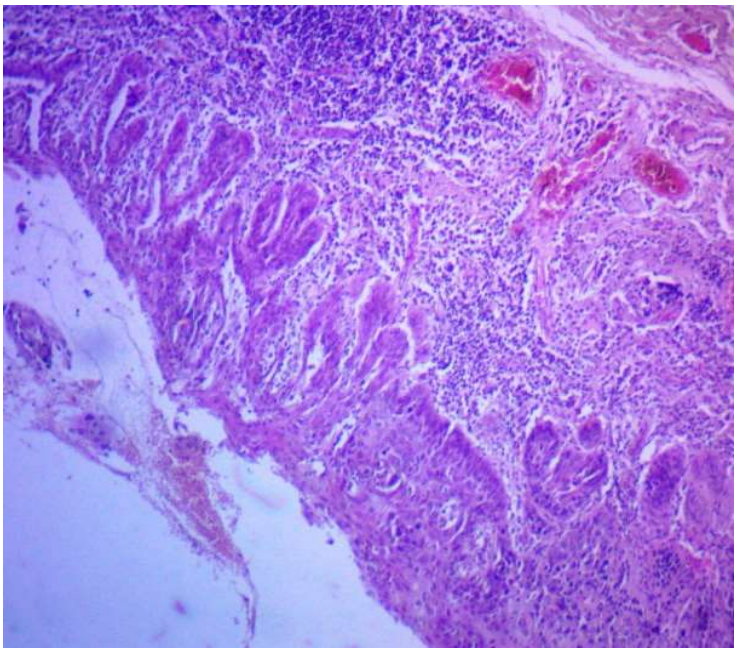

Fig. 1: SIL (premalignant lesion) of larynx H and E stain. 10 x 10.

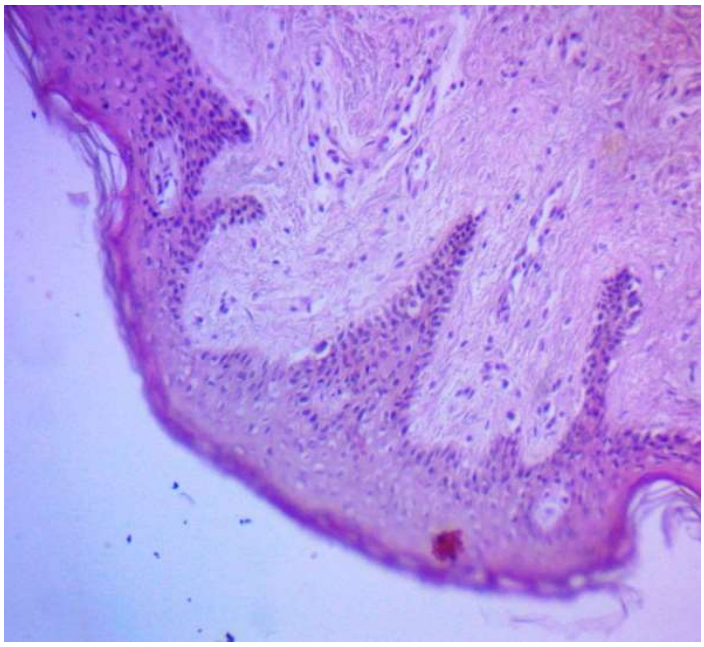

Fig. 2: Vocal Cord Nodule (benign lesion) of larynx H and E stain. 10 x 10.

Table 1: Materials.

Materials	Company
Xylene	Leica
hematoxylin stain	Dako
Distilled water	Iraq

Retrieval solution	Dako
Peroxidase	Dako
HRP (Hourse raddish peroxidas)	Dako
Absolute ethanol, analytical grade	Leica
Mounting medium: Distyrene- Plasticizer-Xylene (DPX)	Leica
Primary antibodies (PDL1,P53 and KI67)	BIO SB, DAKO
DAB (Diaminobenzidine) chromogen and buffer solution.	Dako

Table 2: Equipment.

Equipment	Company/origin
Hot air oven	Memert/Germany
Water bath	Memert/Germany
Regular micro pipettes.	Eppendorf/Germany
Gloves, cotton swabs, as well as tissue paper	China
Tubes	China
Positive charge slides	Dako
Light microscopy.	Leica
Glass and plastic staining jars	China
Microtome	Leica
Cover slips	China
Digital timer	China
moisture and boiling serving dish	Iraq

Tissue section: The formaldehyde conserved and paraffin immersed specimen were exposed to thin section microtome to (four micron) slices in thickness, and assorted on positive charge slides.

Deparaffinization and Rehydration:

This step has been carried out previously by interest in the following:

1. Wax removal in the oven at 58°C (for 50 mint).
2. The slides were carried to xylene jar and perform two times alteration of xylene for 5 min.

3. Rehydrated for 3 minutes in a two-times modification of 100% ethanol.
4. 4 Slides should be immersed in 90% ethanol for 3 minutes.
5. Slides should be immersed in 80% ethanol for 3 minutes.
6. Sides wash with water for 30 seconds.

Antigen Retrieval:

1. Heat antigen retrieval method by putting the slides in antigen retrieval solution in 95° water bath for 20 minutes.
2. Allow 20 minutes for the slide to cool at room temperature before proceeding to the next step.

Immunohistochemical Schedule:

1. Polydetector Peroxidase endogenous of slides for five minutes.
2. Immunohistochemical cleaning buffer washed two times.
3. Applied the Primary Antibody to the samples for 20 hours according to the industrialization-recommended timetable and our lab optimization.
4. Immunohistochemical cleaning buffer washed two times.
5. Covered the specimen with a Polydetector Hourse raddish peroxidase tag and waited 30 minutes for it to hatch.
6. Immunohistochemical cleaning buffer was used two times.
7. Blend one droplet of Polydetector Diaminobenzidine Chromogen per ml of Polydetector Diaminobenzidine Buffer to make Diaminobenzidine.
8. Incubated for 10 minutes after covering specimen with Diaminobenzidine substrate-chromogen liquid.
9. Rinsed with distilled water five times.
10. Hematoxylin dripping (6 mints).
11. Distal water cleaning (2 mints).
12. a hundred percent alcohol (2 mints).
13. The drying process.
14. xylene.
15. Mounted with cover slip (20).

Tumor Marker Used:

- **Anti-P53,** monoclonal Mouse Anti-Human P53 Protein, 11 ml, Ready-To-Use, DAKO, Clone DO-7, Code N1581, LOT 00005848, Dako North America, Inc. 6392 Via Real Carpinteria, CA 93013 USA (22).
- **Anti-KI67,** monoclonal mouse anti-human KI67 antibody (Dako, clone MIB-1, Ref NI633, Lot 00002106, Carpinteria, CA) (23) .

Quantitative Scoring Methods:

For P53:

I. Intensity score (number of stained cells):

0	**No stain**
1	**Weak stain**
2	**Moderate stain**
3	**Strong stain**

II. Proportion score (percentage of stained cells):

0	**0- < 10 %**
1	**10– 25 %**
2	**26– 50 %**
3	**51– 75 %**
4	**> 75 %**

*Score index : intensity score x proportion score = score index, range (0 -12).

Positive: if sore index ≥1.

*Quick H.score (Q):

Intensity score multiplying by the proportion percentage.

Formula: Q = P x I, average (0- 300) (24, 25).

Staining site: (Nuclear stain) these proteins were mostly produced in the nucleus, and their levels were greater in tumor tissue than in non-tumor tissue, however the difference in P53 staining between tumor and non-tumor tissues was not significant. Although nuclear localization of P53 might indicate a mutant version of P53, our work implies that nuclear localization of P53 may play a role in the progression of human laryngeal squamous cell carcinoma (26).

For KI67 :

I. Intensity score (number of stained cells):

0	**No stain**
1	**Weak stain**
2	**Moderate AND strong stain**

II. proportion score:

0	**≤ 10%**
1	**11-30%**
2	**31-60%**
3	**> 60%**

High or low expression:

The sum of the scores provide the final score for KI-67 expression in each sample, in which a final score of < 4 was defined as low/negative expression and a final score of ≥ 4 was defined as high expression.

Staining site: Ki-67 is an antibody that binds to a cell nucleus antigen expressed in all phases of the cell cycle except G0 (27, 28).

Statistical Analysis:

Data were summarized as tables and expressed in frequency and percentage; chi square test, Fisher's exact probability test, sensitivity and specificity test, predictive values, correlation and regression test. Where used to find diagnostic efficiency of P53 and KI67. $P<0.05$ was regarded as a statistically significant value. And measured to assess the association between different categorical variables.

Project layout:

Cases were distributed as follow; the mean age of the study population was 53 years ranging from 31 to 74 years. Total cases 35 case (20 males and 15 females). 25 case of benign lesions (singers nodules and papilloma), 10 cases of premalignant lesions (SIL). Immunohistochemical assessment of P53 revealed that it was positive in 4 (40%) cases with premalignant laryngeal lesions, while it was encountered in 3 (12%) cases with benign laryngeal lesions. There was a statistically significant difference ($P<0.05$) among these three categories (Table 1). The proliferative index of these tumors were also achieved by using ki67 tumor marker test, high expression of nuclear brown stain was encountered in high expression of Ki-67 was encountered 4 cases (40%), low expression was seen in 6 cases (60%) with premalignant laryngeal lesions, while high expression was seen in 5 cases (20%) and low expression was seen in 20 cases (80%) with benign laryngeal lesions. There was no statistically significant difference ($P<0.05$) (Table 2). The correlation between P53 expression and KI67 in benign and pre malignant lesions is clarified well correlated, $R = 0.44$ (Fig. 1). The linear regression test between P53 and KI67 expression in benign and pre malignant lesions, can find the missing parameter value from other parameter Fig. (2),

Table 4: P53 expression in laryngeal lesions that are benign, premalignant (N=35).

Types of lesion	P53 expression		Total n(%)	P-Value
	Positive n(%)	Negative n(%)		
Benign	3 (12%)	22 (88%)	25 (71.43%)	0.03
Premalignant	4 (40%)	6 (60%)	10 (28.57%)	
Total	7 (20%)	80 (68%)	35 (100)	

Table 5: KI67 expression in laryngeal lesions that are benign and premalignant (N=35).

Types of lesion	KI67 expression		Total n(%)	P-Value
	High n(%)	Low n(%)		
Benign	5 (20%)	20 (80%)	25 (71.43%)	0.41
Premalignant	4 (40%)	6 (60%)	10 (28.57%)	
Total	9 (25.71%)	26 (74.29%)	35 (100)	

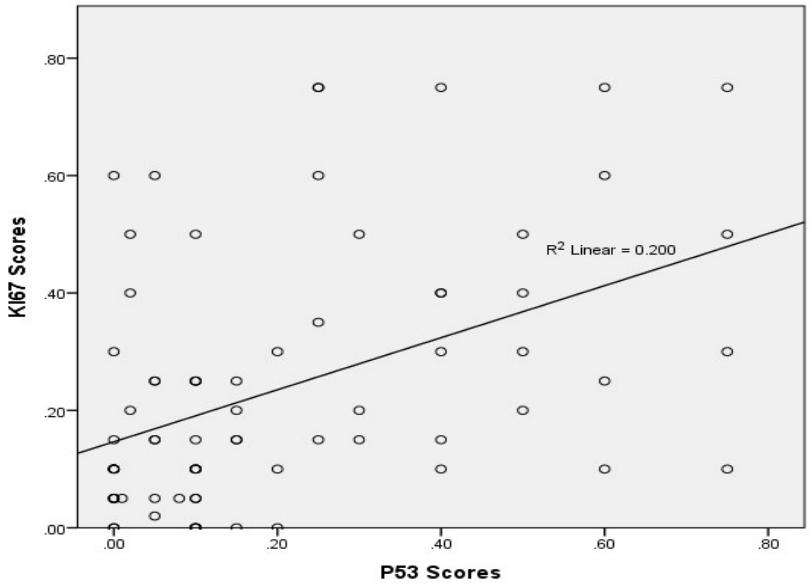

Fig. 4: Linear correlation test between KI67 expression and P53 expression in laryngeal lesion.

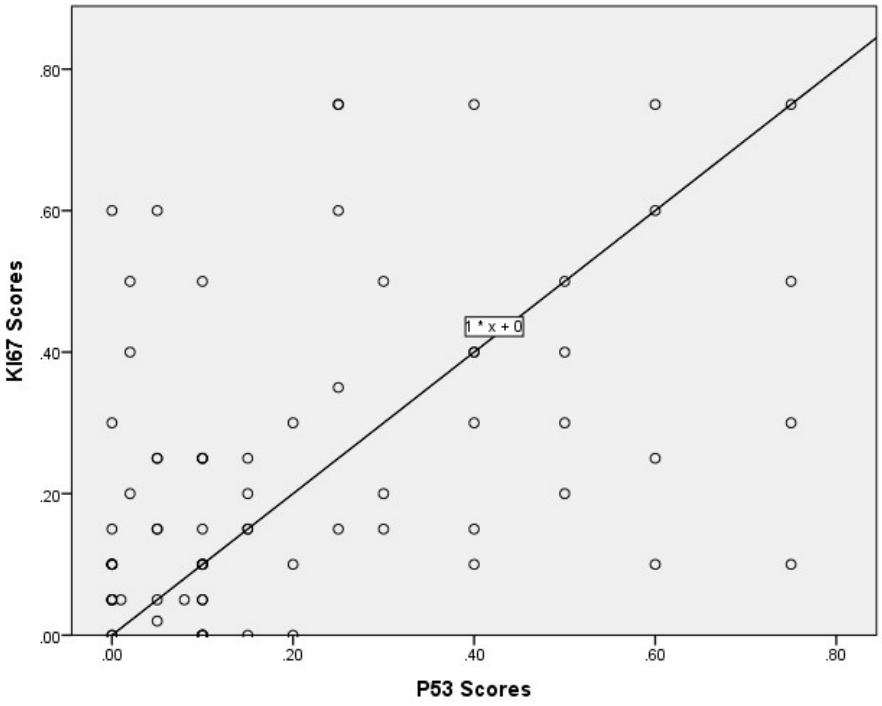

Fig. 5: Linear regression test between KI67 expression and P53 expression in laryngeal lesion.

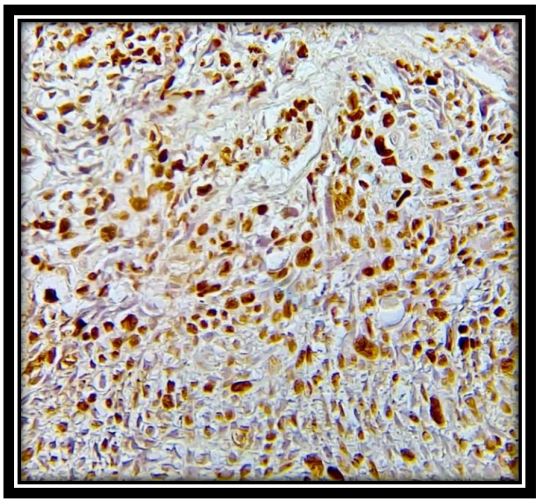

Fig. 6: P53 positive, diffuse strong nuclear stain IHCA: 10 x 4.

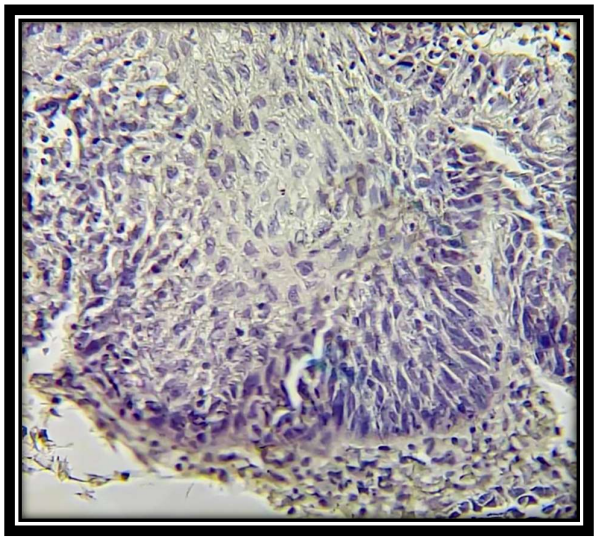

Fig. 7: P53 negative, nuclear stain IHC of SIL. 10 x 20

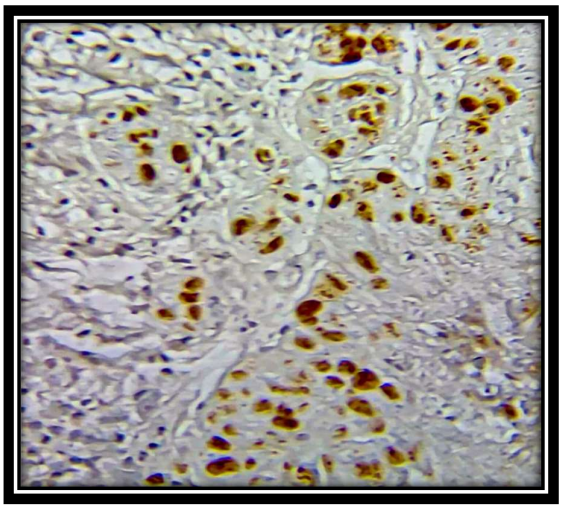

Fig. 8: KI67 high expression, nuclear stain IHC. 10 x 20.

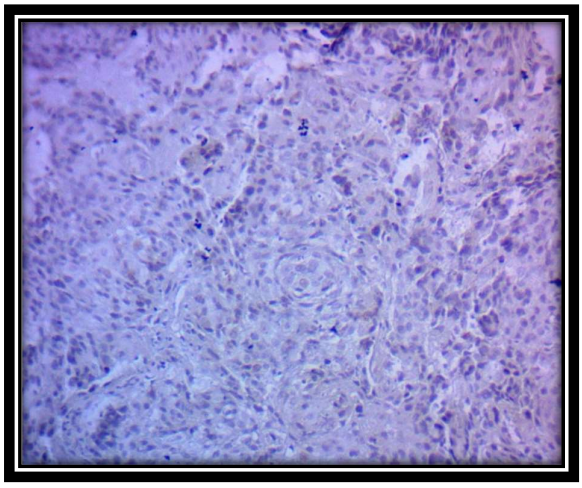

Fig. 9: KI67 (negative) low expression, nuclear stain IHC. 10 x10

Discussion

The correlation between P53 expression and KI67 in malignant lesions is clarified as well correlated (R= 0.44). This agrees with a study done by Wojciech Pastuszewski et al. 2007, as a result; Ki-67 antigen expression was strongly connected with p53 protein expression (r=0.477; p less than 0.05), and expression of either marker was favorably correlated with grading of malignancy (r=0.47, p less than 0.05; r=0.31, p less than 0.05; for Ki-67 and p53, respectively) (77).

Limitation of the Present Study:

1. Lack of information about smoking and alcohol.
2. Small sample size.

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