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RESEARCH ARTICLE

Evaluation of the prognostic significance of P53 and Proliferating cell nuclear antigen (PCNA) in laryngeal squamous cell carcinoma

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ABSTRACT

Background: Laryngeal tumors are considered to be the most frequent neoplasm of head and neck cancers, they account for almost 25% of the malignant neoplasms involving this region. Even though it shows better clinical outcome, its prognosis still worse due to its local invasiveness, lymph node and distant metastasis. Thus, a better realization of the molecular pathways developing laryngeal carcinogenesis may help in the identification of new predictive and prognostic biomarkers, and developing novel management strategies for this cancer. Material and methods: This study included 40 cases of laryngeal squamous cell carcinoma (LSCC), 38 cases were received as punch biopsies and only 2 cases were received as excisional biopsies. Immunohistochemical staining of all cases using P53 and PCNA was done. **Results**: The immunohistochemical expression of p53 was positive in the nuclei of 31 out of 40 (77.5%) LSCC cases. PCNA expression was positive in 28 out of 40 (70%) LSCC cases. P53 expression was significantly correlated with the increase in tumor size, higher tumor stage and lymph node invasion. PCNA expression, as well, was significantly correlated with the increase in tumor size, higher tumor stage, tumor grade, tumor site and lymph node invasion. **Conclusions**: p53 and PCNA are properly independent predictors of the outcomes in patients with LSCC, and their immunohistochemical expression may aid in predicting the clinical course in patients with LSCC, and selecting patients who should be treated more cautiously.

Keywords: laryngeal carcinoma LSCC, p53, PCNA

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INTRODUCTION

Laryngeal tumors are viewed as the most common neoplasm of head and neck tumors, they represent about 25% of the threatening neoplasms in this area. ; 1-2.5% of all malignancies in the body and 2% of all cancer-related deaths.

Males are commonly affected than females at a rate of 7:1 (Bai *et al.*, 2016). Even though it shows better clinical outcome, its prognosis still worse, with a 5-year overall survival rate of ~60% due to its local invasiveness, lymph node and distant metastasis (Iovănescu *et al.*,2013). The complaints of patients with laryngeal carcinoma are usually non-specific and so, the diagnosis and proper treatment are usually delayed. In one study, only 27.8% of cases were observed to undergo surgery due to their late diagnosis (Traoré *et al.*, 2008). Consequently, a better realization of the molecular pathways developing laryngeal carcinogenesis may help in the identification of new predictive and prognostic biomarkers, and developing novel management strategies for this cancer.

The particular molecular pathways of malignant transformation in the larynx are not obvious. Carcinogenesis is suggested to be based on genetic damage that impairs the mechanisms of cell cycle regulation specifically the two main pathways regulating the cell cycle, tumor-suppressor gene p53 and retinoblastoma gene (pRb) pathways (Ashraf *et al.*, 2010).

The p53 gene, a proto-oncogene that regulates cell growth, has a fundamental role in the G1 phase of the cell cycle, as it can block the cell cycle in G1 if facing any genotoxic harm,

consequently, interruption of its pathways results in enhanced cell proliferation and further genetic injury. Mutations in p53, leads to the expression of a non-functional protein that seems to be the furthermost frequent aberration in human neoplasms and is present as an initial event in laryngeal carcinoma (Wayne and Robinson, 2006). Even though a relationship was revealed between p53 expression and decreased patients survival, incompatible results have been documented concerning its clinicopathological implications in laryngeal cancers (Nader *et al*., 2005).

One of the most significant measures that display the aggressive behavior of certain tumor is cell proliferation rate. Cell proliferation is considered as an essential biologic mechanism in tumorigenesis (Rodrigues *et al*., 2008). Accordingly, several proliferation markers have been investigated such as PCNA, which used as a prognostic factor in various tumors. Proliferating cell nuclear antigen (PCNA) is a protein presents in the nuclei. It has a serious role in DNA duplication and cell proliferation; it appears in the nucleus in late Gl phase. It peaks in S phase and subsides in G2 and M phases; therefore PCNA expression correlates with cellular proliferative activity and DNA synthesis (Su and Zheng, 2009).

PCNA is identified in malignancies, such as breast, gastric, pancreatic, renal, and ovarian carcinomas. An increased level of PCNA expression has been described to signify an aggressive tumor (Deniz *et al*., 2008). Head and neck malignancies with high mitotic activity are revealed to have a worse prognosis, especially in neoplasms invading structures adjacent to the tumor's site of origin (Pich *et al*., 2004)

This work aims to study immunohistochemical expression of p53 and PCNA in laryngeal carcinoma and to correlate our findings with different clinic-pathological parameters (age, tumor site, tumor size, histological grade, lymph node status, and disease staging) to evaluate their usefulness as prognostic indicators for laryngeal carcinoma.

MATERIAL AND METHODS

This study was done at Pathology Department, Faculty of Medicine, Tanta University. A total of 40 cases that had been diagnosed with laryngeal squamous cell carcinoma (LSCC) were included in our work. Thirty-eight cases were received as punch biopsies and only 2 cases were received as excisional biopsies specimens from the ENT (Ear, Nose, Throat) department, faculty of medicine, Kafrelsheikh University and the ENT department, faculty of medicine, Tanta University, during the period from 2016 to 2019. All clinical data, laryngoscopy mapping of the tumor, radiological data and biopsies details were provided by ENT senior surgeon. Our study included 40 male patients. Their age ranged from 29 to 73 (median 55 years).

Tissue sections were fixed in 10% formalin and embedded in paraffin and Sections 4 -5 µm thick were prepared and stained routinely with hematoxylin and eosin (H&E) for microscopic study. The clinicopathological characteristics were assessed for each patient including age, tumor size, site, tumor grade according to the WHO grade and cervical lymph node status. The pT staging was classified according to the American Joint Committee on Cancer (AJCC) - TNM classification (eighth edition) (Amin *et al.*, 2017).

Research involving human participants

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Before enrollment of the study, informed written consent was obtained from all patients. This protocol was approved by the ethical committee of the faculty of medicine, Tanta University.

Immunohistochemistry

The immunoperoxidase technique for immunostaining was used on 4-µm-thick sections from formalin-fixed, paraffin-embedded blocks. Pretreated sections were then incubated with a mouse monoclonal antibodies of PCNA (Clone PC10) (DAKO, Egypt, dilution 1: 200) and also with a mouse monoclonal antibodies for p53 (Clone DO7) (Thermo Scientific, Egypt, dilution 1:50).

The primary antibody was replaced with phosphate-buffered saline for the negative control group.

For both antibodies, antigen retrieval (PBS buffer; pH 7.4) was done for all sections and were incubated with the primary antibody for 2 h at room temperature. The sections were incubated with the secondary antibody (HRP-rabbit/mouse) for 15 min at room temperature.

Assessment of Immunohistochemical Staining

All slides were scanned by three different pathologists under magnification x100 and x400. P53 staining reactions were analyzed semi-quantitatively by counting 1000 tumor cells in each sample (original magnification, x 400) and assessing the percentage of positive nuclear-stained cells. P53 was considered negative if the staining was below 10% and positive if it was above 10% (Ashraf *et al*., 2010).

PCNA proliferation index was assessed according to the brown nuclear-stained cells in the region of maximum expression under high power magnification and recorded as a percentage of positively stained tumor nuclei per 1000 tumor cells and was considered as a proliferating index (PCNA PI) as follows (0, no reactivity; 1+, 1-9% cells positive; 2+, 10-40% cells positive, 3+, >40% cells positive). Cases were divided into two groups: low expression (<40%) and high expression (>40%) (Deniz *et al*., 2008).

Statistical analysis

Data were presented as mean ± standard deviation. Analysis was done using the chi-square test, person correlation in SPSS (version 15.0; SPSS Inc., Chicago, Illinois, USA) software. Significant differences were considered at $p < 0.05$.

RESULTS

Clinicopathological data

This study included 40 specimens of laryngeal squamous cell carcinoma. They were 40 males and no females. Their age ranged from 29 to 73 (median 55 years). Out of the studied 40 cases, 29 cases (72.5%) were in the glottic area and 11 (27.5%) were in supraglottic. Cases were graded according to the degree of keratinization, nuclear pleomorphism and presence or absence of intercellular bridges as follow: 17 (42.5%) cases were Grade I (well-differentiated), 14 (35%) cases were Grade II (moderately differentiated) (Fig. 1), and 9 (22.5%) cases were Grade III (undifferentiated). Thirty-one cases (77.5%) were staged T1 and 9 cases (22.5%) were T2 and T3. Ten cases (25%) had cervical lymph node involvement out of 40 cases.

Immunohistochemical staining results of p53

P53 positive expression was demonstrated as nuclear staining in 31 (77.5%) out of 40 cases. The relation between the immunohistochemical staining results of p53 expression and different clinicopathological parameters is summarized in (Table 1, Figures 2,3). We found statistically significant differences in p53 expression due to tumor staging ($p = 0.012$), tumor size ($p = 0.001$), and lymph node invasion ($p = 0.017$). No significant difference was detected in p53 expression due to patients' age, grade and tumor site.

Immunohistochemical staining results of PCNA

High PCNA expression appeared as nuclear staining in 28 (70%) out of 40 cases. The relation between the immunohistochemical staining results of PCNA expression and different clinicopathological parameters is summarized in (Table 2, Figures 4-8). There was a statistically significant differences in PCNA expression due to tumor staging ($p = 0.02$), tumor grade ($p = 0.002$), tumor size ($p = 0.01$), tumor site ($p = 0.01$) and lymph node invasion ($p = 0.04$). No significant difference was detected in PCNA expression due to patients' age.

DISCUSSION

Various clinico-pathological parameters affect the prognosis of laryngeal SCC. However, parameters are not dependable, as tumorigenesis and malignant behavior of cancer are not well identified. Accordingly, an increasing concern was paid regarding cancer progression and prediction by various molecular markers (Rivlin *et al*., 2011). Several genetic alterations are enrolled in the pathogenesis of laryngeal carcinoma. P53 as a tumor suppressor gene has an essential role in apoptosis and regulation of cell growth. Gene mutations deactivate this pathway resulting in reduced

Table 1. Relation of p53 expression with different clinicopathological parameters:

Parameters	Cases, no	Positive expression N = 31	Negative expression N = 9	χ2	P
Age					
> 50	25	19	6	0.028	0.86
< 50	15	12	3		
Tumor size					
<4 cm	11	8	3	5.68	0.001*
>4 cm	29	23	6		
Tumor site					
Glottis	29	20	9	4.87	0.29
Supraglottic	11	11	0		
Grading					
Grade (I)	17	13	4	1.87	0.34
Grade (II)	14	9	5		
Grade (III)	9	9			
pT Staging					
T1	31	23	8	5.47	0.012*
T2-T3	9	8	1		
Lymph node status					
Involved	10	9	1	0.369	0.017*
Not involved	30	22	8		

*****Statistically significant at (P < 0.05).

Table 2. Relation of PCNA expression with different clinicopathological parameters:

Parameters	Cases, no	High expression N = 28	Negative or low expression N = 12	χ2	P
Age					
> 50	25	16	9	0.002	0.241
< 50	15	12	3		
Tumor size					
< 4 cm	11	9	2	6.11	0.01*
> 4 cm	29	19	10		
Tumor site					
Glottis	29	23	6	4.87	0.01*
Supraglottic	11	5	6		
Grading					
Grade (I)	17	10	7		
Grade (II)	14	12	3	3.53	0.002*
Grade (III)	9	6	2		
pT Staging					
T1	31	20	11	5.47	0.02*
T2-T3	9	8	1		
Lymph node status					
Involved	10	6	4	5.23	0.04*
Not involved	30	22	8		

*****Statistically significant at (P < 0.05).

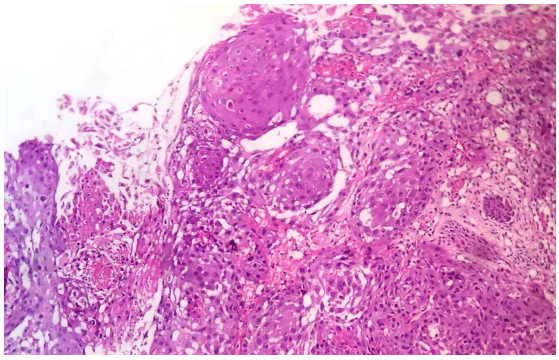

Figure 1. Squamous cell carcinoma of the larynx moderately differentiated (H & E x100).

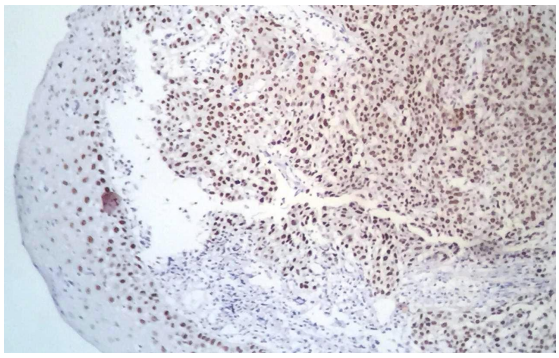

Figure 2. Squamous cell carcinoma of the larynx showing high p53 nuclear expression (Immunohistochemistry x100).

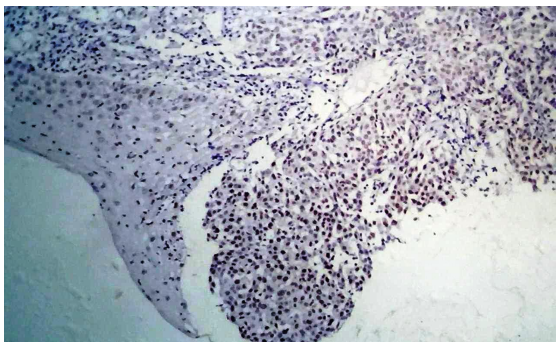

Figure 3. Squamous cell carcinoma of the larynx showing negative p53 nuclear expression (Immunohistochemistry x100).

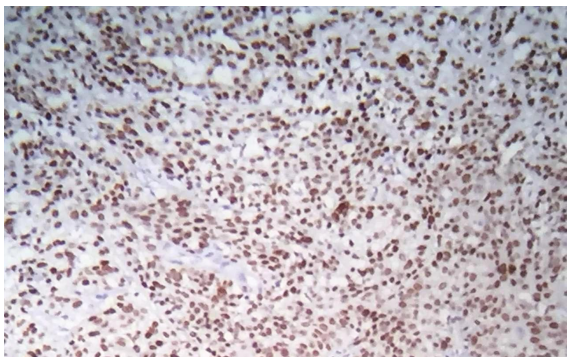

Figure 4. Squamous cell carcinoma of the larynx (poorly differentiated) showing positive (+3) PCNA expression (Immunohistochemistry x100).

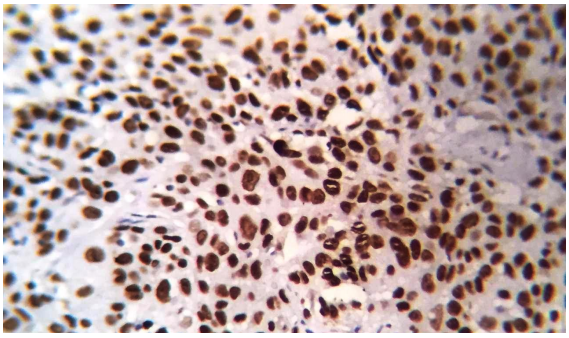

Figure 5. Squamous cell carcinoma of the larynx showing positive (+3) PCNA expression (Immunohistochemistry x400).

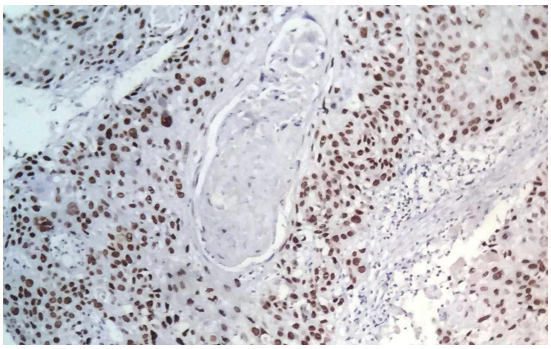

Figure 6. Squamous cell carcinoma of the larynx (moderately differentiated) showing positive (+2) PCNA expression (Immunohistochemistry x100).

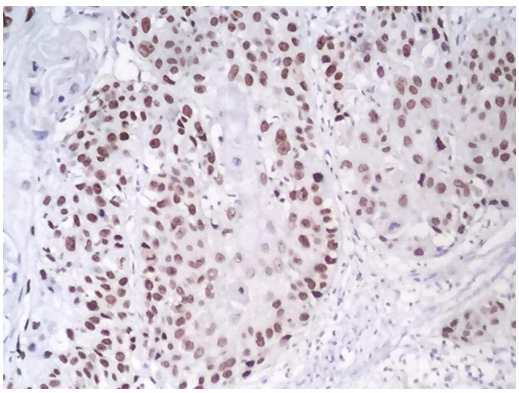

Figure 7. Squamous cell carcinoma of the larynx (moderately differentiated) showing positive (+1) PCNA expression (Immunohistochemistry x100).

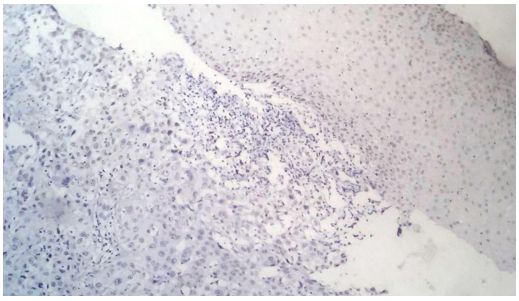

Figure 8. Squamous cell carcinoma of the larynx (poorly differentiated) showing negative PCNA nuclear expression (Immunohistochemistry x100).

apoptosis and increased cell proliferation thus allowing the accumulation of other genetic defects and contributing to the process of carcinogenesis. Such mutations present in about 70% of the head and neck malignancies and are associated with prognosis (Klatka, 2001). In the current study, immunehisto-chemical investigation of p53 was done in 40 cases of laryngeal SCC. Our results showed that p53 was positively expressed in 31 (77.5%) of cases which is consistent with other studies which reported p53 expression at frequencies ranging from 40% to 90% (Cabanillas *et al*., 2007; Mithani *et al*., 2007 and Simsek *et al*., 2013). Our results revealed a statistically significant relation between p53 expression and tumor staging (p=0.012). As high p53 immunoexpression was detected in high stage (in 88.8% of pT2-pT3, more invasive and metastatic tumors). Similarly, Ashraf *et al*. (2010) revealed a statistically significant relation between p53 expression and tumor staging. On the contrary, other studies reported no significant relation between p53 expression and pT stage (Kazkayasi *et al*., 2001; Klatka, 2001 and Rodrigues *et al*., 2008).

In the present study, 78% of p53 positive cases were high-grade tumors (II, III) and 90% had nodal metastasis, certainly signifying that p53 immunohistochemical expression in LSCC patients is related to poor prognosis (high histological grade and regional metastasis). But, we couldn't find a statistically significant relation between p53 expression and tumor grades. Likewise, Klatka. (2001) and Rodrigues *et al*. (2008) found no significant correlation between p53 expression and histological grades.

Regarding the relation between p53 expression and lymph node invasion, we revealed a statistically significant relationship between them (p = 0.017). This was in agreement with Kazkayasi *et al*. (2001), who reported that p53 expression was significantly associated with the presence of lymph node metastases. In another study, Perisanidis *et al.* (2012) found that the lymph node status and p53 overexpression were the only factors significantly associated with survival.

Simsek *et al*. (2013) found no statistically significant correlation between the level of p53 expression and regional lymph node metastases. Similarly, Rodrigues *et al*. (2008) found that 60% of the tumors were positive for p53, for both metastatic and non-metastatic disease regarding lymph node involvement, but it failed to achieve statistical significance between groups. Also, a statistically significant association was detected between p53 expression and tumor size (p=0.001), as p53 positive expression was high in cases with tumor size > 4 cm in comparison with cases with tumor size < 4 cm.

Our results revealed an insignificant relation between p53 expression and patients' age, grade and tumor site. Similarly, Kazkayasi *et al.* (2001) found no statistically significant relation between the expression of p53 and age of patients, tumor site and histopathological grade of the tumor. On the other hand, Cercelaru *et al*. (2017) documented a statistically significant association between p53 expression and the tumor grade but they failed to detect significant relations with gender, age, and disease staging (pT) so, they supposed that presence of p53 expression in early carcinomas may have important prognostic value. In another study by Anghelina *et al*. (2006), they stated that p53 expression was detected in 0%, 31%, and 52% of cases of epithelial hyperplasia without atypia, epithelial hyperplasia with atypia, and laryngeal SCC respectively. So they concluded that p53 expression had a major role in cancer pathogenesis and progression.

Rivlin *et al*. (2011) stated that accumulation of p53 is believed to be an early step in neoplastic progression as many laryngeal dysplasias express p53, so, it has an essential role in evoking of laryngeal tumors. However, conflicting results had been described on the prognostic significance of p53 in LSCC as, according to previous research, p53 expression was not a significant prognostic predictor in LSCC (Calli *et al*., 2005 and Micozkadioπlu *et al*.,2008). Such different findings regarding p53 expression might be attributed to the biological features of the LSCC, procedure issues, interpretation of the results, or a combination of these factors.

The degree of the multiplication of cells within tumors provides an accurate evaluation of their degree of biological aggressiveness. Rapidly growing tumors are mostly more serious and fatal than gradually growing ones (Pich *et al*., 2004). Having an essential role in regulating DNA synthesis and cell multiplication, PCNA is among the furthermost commonly studied proliferation markers together with Ki-67 and DNA flow cytometry. It is well established that the existence of an increased amount of PCNA positive cancer cells is certainly related to more aggressive malignant behavior (Sarafoleanu *et al*., 2009).

In the current study, immunohistochemical investigation of PCNA was done in 40 cases of LSCC. Our results showed that PCNA was positively highly expressed in 28 (70%) of cases. Our results revealed a statistically significant association between PCNA expression and tumor staging (p = 0.02) with high PCNA expression in the high tumor stages (88.8% of pT2-pT3, more invasive and metastatic tumors). Similarly, Liu *et al*., (2003), in a study of 59 patients, found that PCNA immunopositivity is correlated with pathologic T and N stages. Micozkadıoğlu *et al*. (2008) documented a statistically significant positive correlation between the PCNA staining and T stage, whereas stronger staining was seen in T3 and T4 stages than in T1 and T2 stages.

In the current work, 78% of high PCNA positive cases were high-grade tumors (II, III) versus 59% of low-grade tumors (I), definitely indicating that PCNA immunoexpression in LSCC patients is related to poor prognosis (high histological grade). Furthermore, our work revealed a statistically significant association of PCNA expression with tumor grading (p=0.002). Additionally, a statistically significant association of PCNA expression with lymph node invasion (p=0.04) was detected in our work. In agreement with our observations, Boran *et al.* (2003) in their study proposed that high PCNA positivity may increase the probability of lymph node metastases. Likewise, Zheng *et al*. (2002) displayed that PCNA index was correlated with the tumor grade, lymph node invasion. Besides, the association of PCNA index with the depth of tumor margins, neck metastases, and loco-regional recurrence that indicated more aggressive malignancy. Also, a statistically significant relation was detected between PCNA expression and tumor size (p=0.01) in the current study, as PCNA showed high expression in cases with tumor size >4 cm in comparison with cases with tumor size <4 cm. PCNA showed high expression in 79.3% of tumors in the glottic area compared to 45.5% of tumors in the supraglottic area which was statistically significant. Conversely, Micozkadıoğlu *et al*. (2008) reported that PCNA expression was significantly stronger in supraglottic tumors than in glottic tumors. Buyukbayram *et al*. (2004) showed significant associations between PCNA expression and tumor grade, stage, invasion pattern, nodal status, and recurrence. They stated that PCNA index had an essential role in predicting the disease-free survival, but of no value in predicting the overall survival.

CONCLUSIONS

P53 and PCNA could be used as independent predictors of the outcomes in patients with LSCC, and their immunohistochemical expression may aid in predicting the clinical course in patients with LSCC, and selecting patients who should be treated more cautiously.

Conflicts of interest

All authors have approved this article and declare no conflicts of interest.

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Egyptian Association for Cancer Research (EACR)
http://eacr.tanta.edu.eg/

EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcbr.journals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

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