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Prof. Mohamed Labib Salem, PhD

**Role of tumor-associated macrophages in
relation to angiogenesis in urothelial bladder
carcinoma**

Heba F. Harras and Hend S. Abo Safia



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Welcome letter from Editor-in-Chief



Welcome to the Int J Cancer and Biomedical Research (IJCBR)!

It is with great pleasure that I write this editorial to welcome you to the IJCBR. This journal provides a platform for publication of original and reviews research articles, short communications, letter to editor, thesis abstract, conference report, and case studies. These types of publication are directed at the interface of the fields of cancer and biomedical research.

The IJCBR relies on a distinguished expert of the Advisory and Editorial Board Members from the top international league covering in depth the related topics. They timely review all manuscripts and maintain highest standards of quality and scientific methodology and ethical concepts. Meanwhile, we take all possible means to keep the time of the publication process as short as possible.

I take this chance to welcome your contributions to the IJCBR and have every expectation that it will soon become one of the most respected journals in both the fields of cancer and biomedical research.

A handwritten signature in blue ink that reads "Mohamed L. Salem". The signature is written in a cursive, flowing style.

Mohamed L. Salem,

Editor in Chief

Role of tumor-associated macrophages in relation to angiogenesis in urothelial bladder carcinoma

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ABSTRACT

Background: Urothelial Bladder carcinoma is an aggressive tumor with male predominance. The tumor microenvironment is found to affect tumor progression and biology. One of its main constituents is tumor associated- macrophages (TAMs). Angiogenesis is a powerful factor in the development of many tumors and is supposed to be induced by tumor cells and some stromal components that regulate tumor behavior. **Aim:** To assess the correlation of TAMs and angiogenesis in the stroma of urothelial carcinoma and its correlation with the tumor grade and stage and nodal metastasis. **Material and Methods:** This retrospective study was carried out on 30 paraffin blocks of bladder urothelial carcinoma. Hematoxylin, and eosin staining was done for confirmation of the histopathological diagnosis. Immunohistochemical staining by CD68 antibody was used for counting of TAMs and CD34 antibodies was used for tumor associated angiogenesis (microvessel count; MVCs). **Results:** Significant higher TAMs count was detected in high grade and invasive bladder cancers as compared to low- grade and non- invasive types and in patients with nodal metastasis. Moreover, MVCs was significantly higher in higher grade, invasive bladder cancers and in patient with nodal metastasis as compared to controls. There was a positive correlation between the predominance of CD68⁺ TAMs and CD34⁺ MVCs in urothelial carcinoma. **Conclusion:** There is a positive correlation between TAMs and angiogenesis in the stroma of urothelial carcinoma and both are positively correlated with tumor grade, stage, and nodal metastasis. Our data indicated that both TAMs and MVCs could serve as prognostic markers for urothelial bladder cancer.

Keywords: angiogenesis; TAMs; Urothelial carcinoma

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INTRODUCTION

Bladder carcinoma (BC) is the tenth most common malignancy in the world, with urothelial carcinoma (UC) being significantly the most frequent histological variant of BC (>90%) (Bray et al., 2018). It is usually more common in males than females (Antoni et al., 2017). Unfortunately, The risk of recurrence, muscle invasion and distant metastasis was found to be high, despite effective early transurethral resection (Babjuk et al., 2011). Given that the response and prognostic value of the various treatment lines of bladder cancer cases differ greatly, there is a high need for biomarkers that can exactly prewise prognosis and treatment efficacy (van Wilpe et al., 2020).

The tumoral stroma that contains inflammatory cells, macrophages, blood vessels, and fibroblasts is called tumor microenvironment and is found to affect tumor progression and biology (Sica et al., 2008).

There are two types phenotypes of macrophages; the classic (M1) or alternative (M2) phenotype. The classic M1 phenotype is pro-inflammatory and tumoricidal, whereas the alternative M2 phenotype regulates tissue remodeling, releases some anti-inflammatory molecules that decrease the inflammatory process (Allavena et al., 2008). TAMs are mainly of M2type and are supposed to have anti-inflammatory effect, facilitate tumor growth by matrix remodeling, angiogenesis, and immunosuppression (Mantovani and Locati,

2013, Yang et al., 2019). TAMs have many aspects in the progression of different cancers, however their prognostic role in urothelial carcinoma is still indecisive (Wu et al., 2018).

Neovascularization that regulate tumor behavior are supposed to be induced by tumor cells and some stromal components. It was stated that TAMs may have a role in tumor angiogenesis and invasion (Leek et al., 1994). To address this debate, we carried out this study based on the notion that TAMs are characterized by expression of CD68 marker and MVCs identified by CD34 in urothelial bladder carcinoma. The aim of the present study was to assess the correlation between TAMs and angiogenesis in the stroma of urothelial carcinoma and their correlation with the grade and stage of the tumor and nodal status as well.

MATERIALS AND METHODS

This retrospective study was carried out on 30 paraffin blocks from bladder urothelial carcinoma patients collected from our database at pathology department, Tanta university during the period from February 2019 to January 2021. Samples were coded to protect the patient's privacy. All the available clinicopathological data of the patients were collected from the archival files including age, sex, and lymph node status. Approval from the research ethics committee (REC), Faculty of Medicine, was taken antecedent to have the permission to get an access for the patients' private clinical data.

Histopathological study: Serial sections were taken from each block. Histological sections of 4-mm thickness were stained by hematoxylin, and eosin (H&E) for confirmation of histopathological diagnosis, grade, stage and to select representative areas of the tumor for immunohistochemical study. Tumor staging was done according to The American Joint Committee on Cancer (AJCC) Staging System, 8th edition, 2017 (Magers et al., 2019) while tumor grading was conducted according to the criteria of 2016 World Health Organization classification. Current recommendations for grading urothelial carcinoma are to assign either low- or high-grade as part of a two-tier grading scheme (Grignon DJ, 2016).

Immunostaining: Tissue sections of 4µm thickness of the selected paraffin blocks were deparaffinized in xylene for 20 minutes, rehydrated in graded ethanol and incubated in 0.5% hydrogen peroxide/methanol for 10 minutes. To perform epitope retrieval, sections were subjected to hot water (60°C) in 10 mM citrate buffer, pH 6.0 for 10 minutes followed by cooling down for 20 minutes. After washing in phosphate buffered saline (PBS), sections from each case were incubated with CD68 Mouse Monoclonal Antibody for counting of tumor associated macrophages (Anti-CD68 antibody [KP1] (ab955), USA) and CD34 monoclonal mouse antibodies (Anti-CD34 antibody [9B10D4/4H5E7] (ab54208) , USA) for Tumor associated angiogenesis. In the following day, the slides were washed in PBS then incubated with the biotinylated secondary antibody for 10 minutes, then washed in PBS, incubated with streptavidin for 10 minutes, washed and stained with 3,3'-diaminobenzidine (DAB) to eliminate any insoluble brown deposit. Finally, counterstained with hematoxylin, washed in running water, dehydrated in graded alcohol ethanol and cover slipped.

Immunohistochemical evaluation: The results of IHC were performed by three pathologists blinded to the clinical information. The counting of immunohistochemical marker expression for CD34 and CD68 was performed at the central lab of microbiology Department, Tanta University using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England,). It is composed of Leica DM-LB microscope with JVC color video camera attached to a computer system Leica Q 500IW. We place the slide to be examined on the stage of the microscope and focus it at low power magnification (40X). For TAMs count, the highest density of macrophage areas (hotspots) that showed cytoplasmic staining was selected at three low power. The positively stained CD68 macrophages were counted at high power magnification (x400) then its mean count in the three hotspots was estimated for each case (Mantovani and Sica, 2010, Jamiyan et al., 2020) (Figure 1).

For estimation of Tumor-associated angiogenesis we estimated MVCs at three low power fields with maximal neovascularization positively stained with CD34, then counted at high power magnification (x400), with an estimation of its mean value (Weidner et al., 1991). Any single brown immunostained endothelial cell, or endothelial cell clusters discrete from other microvessels, was counted as a single microvessel regardless of the presence of a lumen. Vessels with thick muscle wall or large lumen (~50µm) were ruled out from counting (Zhang et al., 2017). The area of tissue in the field was measured, and the vessel count was expressed per square millimeter (Figure 2).

Statistical analysis: All statistical analyses were performed using StatView (ver. 5.0; SAS Institute Inc., Cary, NC, USA). Student's t-tests and Mann-Whitney U tests were used to evaluate the correlations between TAM, MVCs and the pathological parameters. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Clinicopathological results: This study included 25 males and 5 females; their age ranged between 45 – 70 years. Out of 30 cases, 11 case representing 36.7% were of low- grade tumor and 19 cases (63.3%) were of high grade tumor, besides there were 6 cases (20 %) showed no muscle invasion (pTa), 9 cases (30%) showed lamina propria invasion (pT1) and 15 cases (50%) showed muscle invasion (pT2). Lymph node metastasis was detected in 18 cases (60%) and absent in 12 cases (40%).

CD68 immunohistochemical results: TAMs were detected using CD68 antibodies in the stroma of bladder cancer (included the papillary axis, lymphoid aggregates, and the underlying tumor stroma) (Table 1). CD68 immunostaining was positively correlated with tumor stage, grade, and nodal metastasis. CD68 immunoexpression in superficial non-invasive cases was much lower than that in muscle-invasive cases and was higher in high grade tumors than that in low- grade ones (Figure 3).

CD34 immunohistochemical results (Table 2):

The MVCs were detected using CD 34 antibodies on tumor stroma of bladder cancer (included the papillary axis, lymphoid aggregates, and the underlying tumor stroma). CD34 immunostaining showed significant positive correlation with the tumor grade, stage and nodal metastasis as MVCs were significantly higher in invasive bladder carcinoma than in superficial non-invasive bladder carcinoma and higher in high grade tumors than low- grade ones (Figure 4).

Correlation between CD68 and CD34 immunoexpression: As regards the relationship between the CD68 expressed TAMs count and CD34 expressed MVCs, there was a significant positive correlation ($r=0.872$; $P=0.001^*$) between TAMs count and MVCs in the studied cases of urothelial bladder carcinoma (Table 3).

DISCUSSION

Bladder cancer is considered a heterogeneous tumor, it has a variable natural history, clinical behavior, and outcome. It was found that low-grade tumors and those which are not invading muscle had an indolent course, while tumors with high- grade and muscle invasion were mainly life-threatening (Kaufman et al., 2009). Although a multidisciplinary approach to deal with bladder cancer is usually done, its treatment and management remain challenging and controversial (Eccles and Welch, 2007; Wu et al., 2018). However, great corroborations have indicated an obvious role of immune cells in bladder carcinoma microenvironment which can help a better selection of its proper management (Kim, 2016).

Previous studies on bladder cancer were focused on microenvironment immune cells, such as tumor-infiltrating dendritic cells, tumor-infiltrating lymphocytes, and tumor-associated macrophages (Ayari et al., 2009, Sjudahl et al., 2014), Other studies had searched the bladder cancer-associated macrophages, especially after treatment of bladder cancer with Bacillus Calmette-Guerin (BCG), neoadjuvant chemotherapy and immunological checkpoint inhibitors (Takayama et al., 2009, Tervahartiala et al., 2017).

Table 1. Correlation between CD68 expression and tumor stage, grade and nodal status in the studied urothelial carcinoma cases (n=30).

Pathological parameter N=30		CD68		Test	P value
		Mean \pm SD	Range		
Stage N (%)	Ta 6 (20)	57.17 \pm 9.17	45.90-68.80	U: 40.886	0.001*
	T1 9 (30)	59.44 \pm 9.82	47.80-70.45		
	T2 15 (50)	103.33 \pm 16.44	70.04- 130.70		
Grade N (%)	High 19 (63.3)	99.67 \pm 21.83	75.80-130.70	t: 5.565	0.001*
	Low 11 (36.7)	62.20 \pm 14.26	48.9-86.20		
LN status N (%)	Positive 18 (60%)	97.50 \pm 26.21	70.28-130.70	t: 6.421	0.001*
	Negative 12 (40%)	72.01 \pm 22.60	46.9-100		

*Significant (P value<0.05), N = Number, LN = Lymph node

Table 2. Correlation between CD34 expression and tumor stage, grade and nodal status in the studied urothelial carcinoma cases (n=30).

Pathological parameter N=30		CD34		Test	P value
		Mean \pm SD	Range		
Stage N (%)	Ta 6 (20)	35.50 \pm 9.95	24.15-53.46	U: 23.881	0.001*
	T1 9 (30)	42.00 \pm 7.55	30.60 -59.21		
	T2 15 (50)	63.73 \pm 10.84	43.08-85.08		
Grade N (%)	High 19 (63.3)	64.13 \pm 10.49	40.16-99.9	t: 7.356	0.001*
	Low 11 (36.7)	39.0 \pm 8.06	24.15-52.92		
LN status N (%)	Positive 18 (60%)	70.04 \pm 24.14	48.02-99.90	t: 9.560	0.001*
	Negative 12(40%)	62.09 \pm 22.12	24.15-89.12		

*Significant (P value<0.05), N = Number, LN = Lymph node

Table 3. Correlation between CD68 expressed TAMs and CD34 expressed MVC in the studied urothelial carcinoma cases (n=30).

CD 68	CD34	
	r	P value
	0.872	0.001*

*Significant (p value<0.05).

It has been reported that Macrophages are able to adapt to many different activities. They are two types; proinflammatory (M1) and anti-inflammatory (M2) macrophages (van Wilpe et al., 2020). Previous studies have shown that TAMs are M2 phenotype and accelerate tumor metastasis and progression through stimulation of angiogenesis and matrix breakdown (Pollard, 2004).

In the present work, we studied the correlation between TAMs and tumor angiogenesis (MVCs) in the stroma of urothelial carcinoma as regards tumor grade, stage, and lymph node metastasis. As concern CD68 expression in TAMs, we found that the CD68+ TAMs count was significantly higher in muscle-invasive bladder cancers than in non-invasive cancers. Moreover, high-grade urothelial carcinoma showed significantly higher CD68 expression compared to low-grade ones. In addition, significant higher MVC (represented by CD34) was noted in high grade and invasive urothelial carcinoma compared to low-grade and non-invasive types. Both of CD68 and CD34 positive immunostaining showed positive correlation with lymph node metastasis.

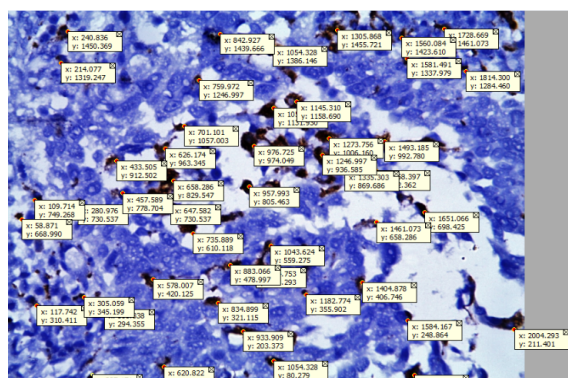


Figure 1. Window of image analysis showing counting of TAM expressing CD68 in a case of high-grade muscle – invasive urothelial carcinoma(T2) [x400].

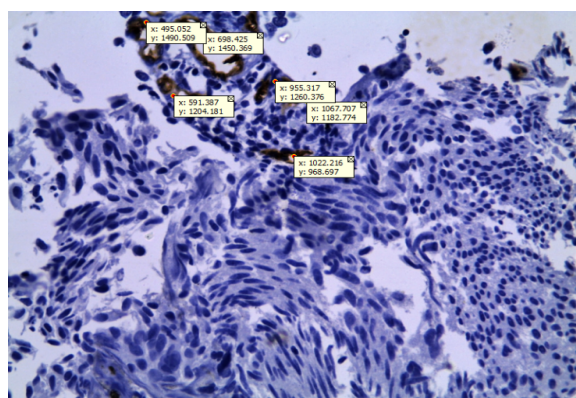


Figure 2. Window of image analysis showing MVD expressed by CD34 in a case of low-grade superficial urothelial carcinoma (Ta) [x400].

Our findings are in agreement with Bochner et al. (1995) , who examined tumor angiogenesis (determined by MVCs using CD34 immunomarker) in bladder cancer and reported that it is considered as an independent prognostic factor in invasive bladder cancer. Meanwhile, Ahmed et al. (2018) showed positive correlation of CD34+ MVCs with only tumor grade in invasive bladder cancer. These results are also supported by Xue et al. (2019) study who concluded that macrophages are the predominant immune cells in the stroma of bladder cancer, and their predominance is associated with high tumoral grade and stage. Moreover, Suriano et al. (2013) indicated that infiltration of the tumor microenvironment by macrophages was significantly correlated with bad survival rate of bladder cancer cases. On the other hand, Wu et al. (2018) demonstrated that high infiltration of the bladder cancer tissue by CD68⁺ tumor- associated macrophages showed no significant correlation with either tumor grade nor its stage.

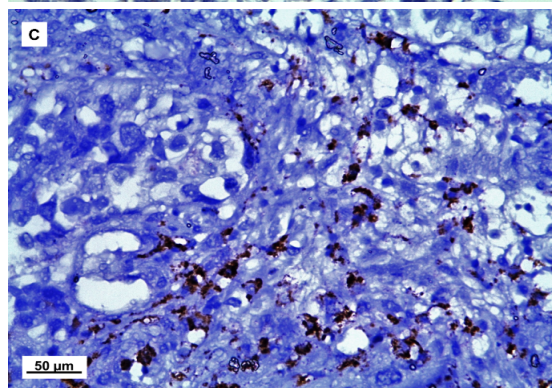
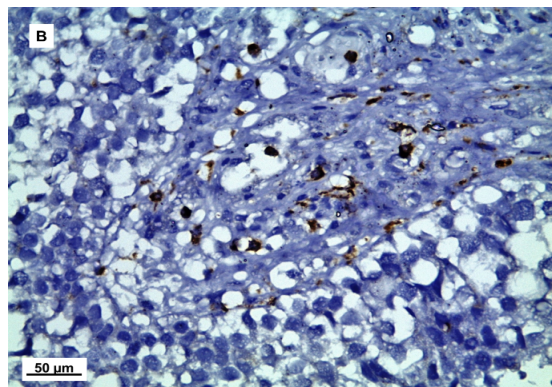
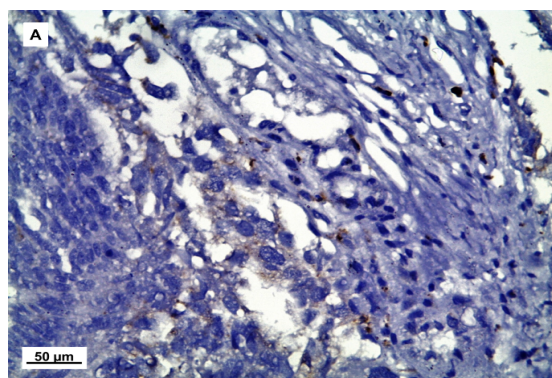


Figure 3. Expression of CD68 in Urothelial carcinoma studied cases: **(A)** Superficial (non- invasive) urothelial carcinoma (Ta) [x400]. **(B)** urothelial carcinoma invading core of papillae (T1) [x400]. **(C)** high grade urothelial carcinoma invading lamina propria (T1) [x400].

In this study there was a positive correlation between the predominance of TAMs and MVCs in urothelial bladder carcinoma ,this was in agreement with Hanada et al. (2000) study who demonstrated that there were higher count of TAMs in invasive bladder cancers than in non-invasive type in addition to positive correlation between TAMs and microvessels density.

Our results could be explained by Mantovani and Locati (2013) study which reported that TAMs have an angiogenic phenotype and are expressed in different manners in invasive and non-muscle invasive malignant bladder tumors.

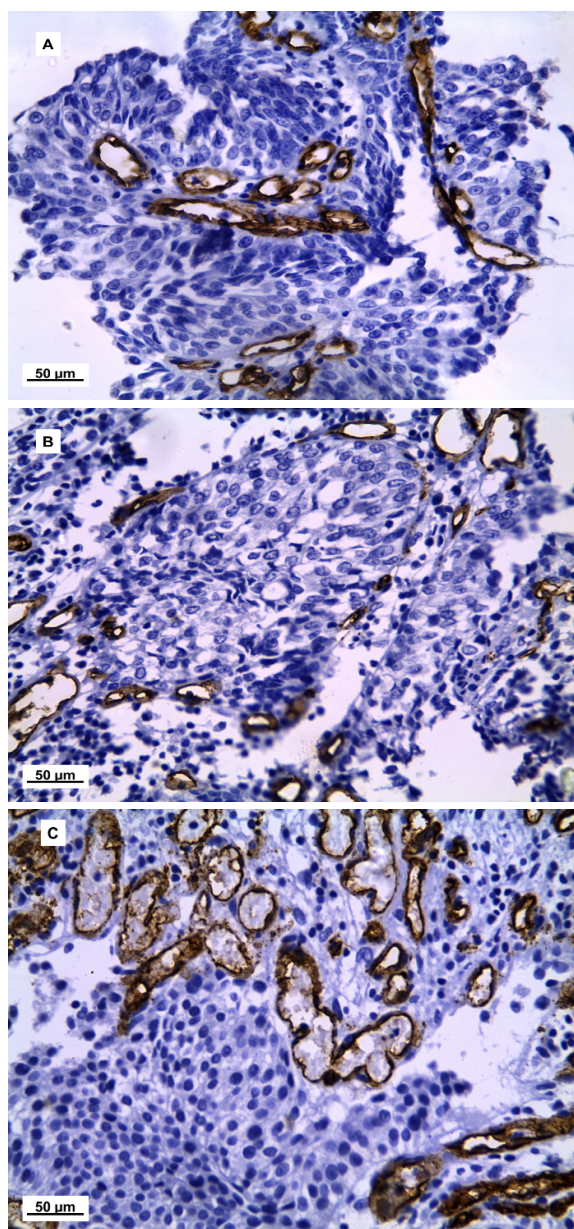


Figure 4. Expression of CD34 in Urothelial carcinoma studied cases: **(A)** Urothelial carcinoma invading core of papillae (T1) [x400]. **(B)** High grade urothelial carcinoma invading lamina propria (T1) [x400]. **(C)** Muscle-invasive urothelial carcinoma (T2) [x400].

One of the mechanisms that could explain the angiogenic effect of TAMs is hypoxia which is a very common process in malignant tumor tissues. Takeuchi et al. (2016) cleared this theory and supposed that the activation of angiogenic factor-secreting TAMs may be enforced by decreased oxygen level in tumor tissues, which accelerate tumor angiogenesis and invasion. Moreover, invasive bladder cancers promote the deep migration of TAMs into tumor tissues and the secretion of several angiogenic factors.

After several immunohistochemical and RNA sequencing studies, Xue et al. (2019) concluded that M2 phenotype macrophages in urothelial carcinoma expressed micro (mi)RNAs due to cancer-specific genomic alterations and were involved in bladder cancer muscle invasion and angiogenesis. Wu et al. (2020) stated that presence of TAMs in the tumor tissue microenvironment causes elevation of CXCL8 which in turn promotes MMP-9, E Cadherin and VEGF. This in turn alters the migration, invasion and pro- angiogenesis ability of urothelial carcinoma cells and promote tumor progression.

CONCLUSION

It can be concluded that TAMs have a role in promoting angiogenesis and invasion in the stroma of urothelial carcinoma which contribute to tumor progression suggesting that it could be used as a useful prognostic marker for urothelial carcinoma and may serve as a potential immunotherapy target in urothelial carcinoma cases.

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CONFLICTS OF INTEREST

All authors declared no conflicts of interest.

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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://jcbjournals.ekb.eg>) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

EACR Chairman,**Prof. Mohamed Labib Salem, PhD**

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GUIDE FOR AUTHORS

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