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Effect of propranolol on IL-10, visfatin, Hsp70, iNOS, TLR2, and survivin in amelioration of tumor progression and survival in Solid Ehrlich Carcinoma-bearing mice

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ABSTRACT

Background: β-Adrenergic signaling could contribute to initiation and progression of breast cancer. This research investigated some potential mechanisms of propranolol in amelioration of progression and survival in breast cancer.

Methods and results: Solid Ehrlich Carcinoma (SEC) xenograft model was induced in 30 mice divided into 3 groups; where group I served as untreated SEC group. In groups II and III, propranolol treatment i.p. in low (5 mg/kg) and high dose (10 mg/kg) caused significant increase in interleukin-10 (IL-10) and decrease in heat shock protein 70 (Hsp70) and inducible nitric oxide synthase (iNOS) activity with non significant change in visfatin in tumor tissues compared to untreated SEC. In untreated SEC, tumor volume (*V*) exhibited significant negative correlation with IL-10 levels and toll like receptor 2 (TLR2) expression with significant positive correlation with Hsp70 levels and iNOS activity. While propranolol in either doses caused reduction of tumor volume (*V*), and improved percentage tumor growth inhibition (% TGI) only its high dose exhibited significant impact on survival rate. Propranolol dose-dependent effect was evident for IL-10 and Hsp70, and even only the high dose significantly increased and decreased TLR2 and survivin, respectively. This comes in favor of recommending high dose of propranolol in cancer therapy. Nonetheless, use of low dose cannot be ignored when benefit to risk balance have to be considered.

Conclusions: Propranolol could provide palliative effects in progression and survival of breast cancer that are mainly mediated *via* direct immunomodulatory and apoptotic mechanisms and probably associated with indirect anti-angiogenic activity.

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide. About half the breast cancer cases and 60% of the deaths are estimated to occur in economically developing countries [1,2]. Epidemiological studies have associated psychosocial factors such as chronic stress with cancer progression and, to a lesser extent, cancer onset [3]. Sympathetic nervous system (SNS) regulation of cancer cell biology and tumor microenvironment has clarified the molecular basis for long-suspected relationships between stress and cancer progression and now suggest a highly leveraged target for therapeutic intervention [4]. This has led to hypothesis that β-blockers may favorably impact cancer progression [5]. The expression of the three subtypes of β-adrenergic receptor (β_1, β_2, and β_3) was found to reach maximal concentration even before the actual increase in tumor mass with higher β_2 concentrations (74%) than β_1 (36%) in tumor tissues [6–8]. β-Adrenergic signaling regulates multiple cellular processes that contribute to cancer initiation and progression, including inflammation, angiogenesis, apoptosis/anoikis, cell motility and trafficking, DNA damage repair,

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cellular immune response [4,9]. On molecular basis, cAMP is thought to mediate their effect on growth and differentiation of malignant cells [10]. Thus, β-blockade that has been routinely used for treatment of many cardiovascular disorders [11], could now provide an appropriate starting point in oncology [4]. In breast cancer, it has been observed that the optimal β-antagonist regimens were achieved by the non-selective β-blockers rather than β_1-selective agents [12,13]. In this context, propranolol as a prototype non-selective β-blocker that has no intrinsic sympathetic activity (ISA) would be expected to provide the broadest biological leverage and minimize the risk of missing an active β-receptor target [4]. Many emerging pre-clinical and clinical studies on breast cancer have focused only on impact of β-blockers in improving survival, progression and metastasis outcome [12,14–17], while the underlying mechanisms are still in need to be fully elucidated. Among these mechanisms, the cross-talk of immuno-apoptotic-angiogenic pathways is considered the corner stone in pathogenesis of breast cancer. Therefore, this research aimed to disclose some potential mechanisms of propranolol in low and high doses using biochemical markers as interleukin-10 (IL-10), visfatin, heat shock protein 70 (Hsp70), and inducible-nitric oxide synthase (iNOS) as well as immunohistochemical markers such as toll like receptor 2 (TLR2) and survivin that related to these pathogenic pathways and could contribute to amelioration of tumor progression and survival outcome in tumor-bearing mice using Solid Ehrlich Carcinoma (SEC) model.

Materials and methods

Drugs and chemicals

Propranolol (Mayestrotense, 1 mg/ml ampoule) was purchased as a product of Alex. Co. for Egypharma, Egypt. Other chemicals are of analytical gradient were obtained as products of Sigma–Aldrich Chemical Co., unless indicated otherwise.

Cell Line and induction of Solid Ehrlich Carcinoma (SEC) xenograft model

Ehrlich Ascites Carcinoma (EAC) cell line was obtained from the Pharmacology and Experimental Oncology Unit of the National Cancer Institute (NCI), Cairo University, Egypt. EAC cells are of mammary origin. The viability of the cells was 99% as judged by trypan blue exclusion assay. The xenograft model of Solid Ehrlich Carcinoma (SEC) was induced in male Swiss albino mice by viable EAC cells 2.5×10^6 in 0.2 ml isotonic saline implanted subcutaneously (s.c.) into the right thigh of the hind limb of each mouse. The tumor was developed in 100% of the mice with a palpable solid tumor mass ($\geq$100 mm^3) was achieved within 12 days post-implantation [18,19].

Animals groups and treatment protocol

Thirty adult male Swiss albino mice weighing 18–20 g, were allowed ad libitum to fed water and standard pellet chow (EL-Nasr Chemical Company, Cairo, Egypt) through the whole period of the experiment. Animals were housed and allowed to become acclimatized to laboratory conditions for 1 week prior to the experiment. The experiment was conducted in accordance to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All mice were rendered tumor-bearing and divided randomly into 3 equal groups; where group I served as untreated control group and received a vehicle of isotonic saline. The tumor-bearing mice in group II and group III were treated 5 days/week by intraperitoneal (i.p.) injection of propranolol in a dose of 5 mg and 10 mg/kg, respectively [20]. The treatment by either vehicle or propranolol was started from 12th day to 42nd day post-implantation. Based on its low oral bioavailability due to first pass hepatic metabolism, propranolol was administered parenteral to ensure high non fluctuating concentrations. The used doses are representative of ~3–6-folds the allowed intravenous dosage for human that is 0.15 mg/kg [21]. Nevertheless, these doses are still within tolerable limit away from intraperitoneal LD50 for mice (80 mg/kg).

Recording of survival rate

The day of implantation was considered zero point of the experiment for recording and analysis of the survival rate weekly for 6 weeks (by recording number of the survived mice in each group at the end of each week).

Tumor volume (V) and percentage tumor growth inhibition (% TGI)

Tumor volumes were recorded from the start point at 12th day post-implantation and thereafter every 4 days till the last record at 42nd day post-implantation just prior to scarification of the survived mice. Using a Vernier caliper, tumor volume (V) was calculated as $V(mm^3) = (a^2 \times b)/2$, where a (small diameter), and b (large diameter) are perpendicular, expressed in millimeters (mm).

Drug efficacy was expressed as the percentage tumor growth inhibition calculated as % TGI = $100 - (T/C \times 100)$, where T is the mean relative tumor volume (RTV) of the treated tumor and C is the mean RTV in the control group. RTV is defined as $Vx/V1$, where Vx is the tumor volume at the end point of the experiment before scarification of the animals and $V1$ is the tumor volume at the start point of the treatment [22].

Processing of tumor tissue samples

At the end point of the experiment (the 42nd day post-implantation), all the survived mice were sacrificed. The tumor was excised, washed immediately with ice-cold saline, blotted dry on a filter paper, and divided into two parts. One part was preserved in 4% buffered paraformaldehyde and further processed for histopathological and immunohistochemical examination. The other part was homogenized in 10 volumes of ice-cold 50 mM phosphate-buffered saline (PBS), pH 7.4 using a Potter–Elvenhjem tissue homogenizer. The resultant supernatant was frozen at -80°C for further assay of tissue levels of interleukin-10 (IL-10), visfatin, heat shock protein (Hsp70), and iNOS activity. Protein content in tumor tissue (mg/g wet tissue) was measured by method of Lowry et al. (1951) [23].

Enzyme-linked immunosorbent assay (ELISA) of visfatin, IL-10, and Hsp70 levels in tumor tissues

The supernatants were quantitatively assayed for visfatin, IL-10 and Hsp70 levels using ELISA kits supplied by (RayBiotech, Inc. Cat#: EIA-VIS-1), (BOSTER BIOLOGICAL TECHNOLOGY Co., Inc. Catalog No. EK0417), and (Uscn Life Science Inc. Wuhan Cat. No. E90873Bo), respectively, following the manufacture's protocol. The results were expressed as ng/mg protein, pg/mg protein and ng/mg protein for visfatin, IL-10 and Hsp70, respectively.

Measurement of inducible-nitric oxide synthase (iNOS) activity in tumor tissues

iNOS activity was measured according to the method of Ryoyama et al. [24], whereby L-arginine and molecular oxygen were catalyzed by NOS to generate nitric oxide (NO). The rate of NO production by NOS in 1 min was determined with the Griess

reaction. The iNOS activity was determined by spectrophotometric assay at 540 nm. iNOS activity is expressed as U/mg protein.

Histopathological examination

For histopathological characteristics of Solid Ehrlich Carcinoma, tumor specimens were fixed in 10% formalin, embedded in paraffin, and 5 μm sections were stained with hematoxylin and eosin (H&E). Sections prepared from different organs (liver, lung, brain, lymph nodes) and bone of the studied groups for detection of metastatic lesions.

Immunohistochemical expression and scoring of toll like receptor-2 (TLR-2) and survivin

For immunohistochemistry, 3 μm sections from tumor tissues were deparaffinized in xylene for 30 min and rehydrated with graded alcohol series. Sections were then processed for further immunohistochemical staining using the UltraVision Detection Kit (TP-015-HD, Lab Vision, USA) according to the manufacturer's protocol. Rabbit polyclonal anti-Toll-like Receptor 2 (TLR2/CD282) antibody (Cat. No. PA5-20020, Lab Vision, USA), in a dilution of 2 μg/ml, and rabbit polyclonal anti-survivin antibody (Cat. No. RB-9245-R7, Ready to use, Lab Vision, USA) were used for detection of TLR2 and survivin, respectively. As positive controls, sections from a case of prostatic carcinoma known to show over-expression of survivin were used, while sections from normal human spleen were used for TLR2. Negative controls were prepared by omission of the primary antibodies.

TLR2 positivity was indicated by cytoplasmic staining [25], while positivity for survivin was considered when distinct nuclear and/or diffuse cytoplasmic immunohistochemical reaction was found [26]. Quantification of TLR2 [27] and survivin [28] expression was performed on a 3-point scale, where 0 = no expression, (+) = weak expression, (++) = moderate expression, and (+++) = strong expression taking into account the percentage of positive epithelial cells and the intensity of expression.

Statistical analysis

Values of the measured parameters were expressed as mean ± SEM. One Way-ANOVA test (*F* value) was used to detect significance of the difference among more than two arithmetic means, followed by *post hoc* Scheffe test to detect the difference between each two means. Fisher's exact test was used to detect the difference between categorical data. The paired-samples *t*-test was applied to detect difference in the tumor volume monitored at the start point and at the end point of the experiment. The cumulative survival curve was plotted and the overall and pairwise comparisons of survival rate were analyzed by applying Breslow test (generalized Wilcoxon) using the Kaplan–Meier method. The difference was considered significant at values of $p < 0.05$. The statistical analysis was processed using the Statistical Program of Social Sciences (SPSS) for windows, version 14.0.

Results

Results of survival rate

In regard to survival rate (Table 1), the overall survival comparison among groups was significant ($p < 0.05$). The pairwise comparisons showed significant difference between the untreated group I and group III that treated by propranolol 10 mg/kg ($p < 0.05$). When group II treated by propranolol 5 mg/kg was compared to either the untreated group I or group III treated by propranolol 10 mg/kg, the pairwise comparisons showed non significant difference ($p > 0.05$). The cumulative survival function of the different studied groups was displayed in Fig. 1.

Table 1
Comparative statistics for survival rate in the studied groups at the end point of the experiment (the 42nd day post-implantation).

Groups	Survival rate (%)#	Survival duration (weeks; % confidence interval (CI), lower bound to upper bound)
Group I	50%	(5.3 ± 0.3 weeks; 95% CI, 4.74–5.93)
Group II	70%, P_1: NS	(5.9 ± 0.1 weeks; 95% CI, 5.67–6.13)
Group III	90%, $P_1 < 0.05$, P_2: NS	(6.0 ± 0.0 weeks; 95% CI, 6.00–6.00)

Values expressed as mean ± SEM; NS, non significant. Breslow test: #, significant overall survival comparison ($p < 0.05$). P_1: pairwise comparison of group I (untreated Solid Ehrlich Carcinoma, SEC) vs group II (SEC treated by propranolol 5 mg/kg) and group III (SEC treated by propranolol 10 mg/kg). P_2: pairwise comparison of group III (SEC treated by propranolol 10 mg/kg) vs group II (SEC treated by propranolol 5 mg/kg).

Results of tumor volume (V) and percentage tumor growth inhibition (% TGI)

Compared to the untreated group I, there was significant decrease in tumor volume of group III (treated by 10 mg/kg propranolol) at all recording points, while tumor volume of group II (treated by 5 mg/kg propranolol) exhibited significant decrease only at the recording points at the 16th and 20th days (Fig. 2). The % TGI was determined to be 14.9% in group II treated by propranolol 5 mg/kg, and 43.6% in group III treated by propranolol 10 mg/kg (Fig. 3).

Results of biochemical markers

Propranolol treatment of SEC either in a dose of 5 mg/kg or 10 mg/kg caused significant increase in IL-10 levels with significant decrease in Hsp70 and iNOS activity in tumor tissues compared to the untreated SEC. Treatment by propranolol 10 mg/kg resulted in significant higher levels of IL-10, significant decrease in Hsp70 levels, and non significant difference in iNOS activity when compared to treatment by propranolol 5 mg/kg. There was non significant difference in visfatin levels between all the studied groups (Table 2).

In the untreated SEC (group I), the tumor volume (*V*) exhibited significant negative correlation with IL-10 levels and significant positive correlation with Hsp70 levels and iNOS activity in tumor tissues, while visfatin levels showed non significant correlation (Table 3).

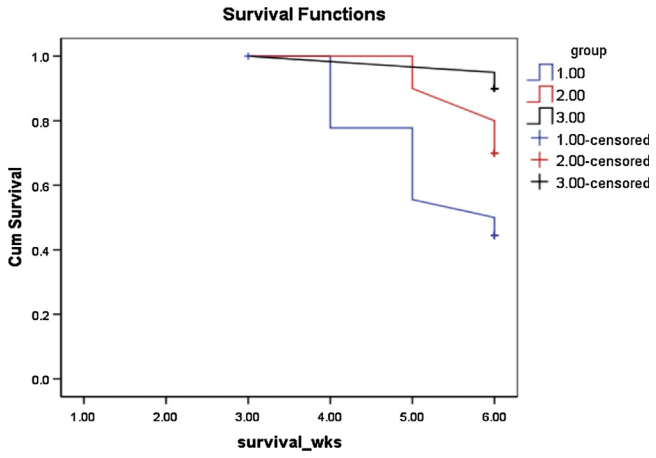

Fig. 1. The cumulative survival curve plotted by Kaplan–Meier method showing the censored cases (+) of each group recorded by the 6th week. 1: group I (untreated Solid Ehrlich Carcinoma, SEC). 2: group II (SEC treated by propranolol 5 mg/kg). 3: group III (SEC treated by propranolol 10 mg/kg).

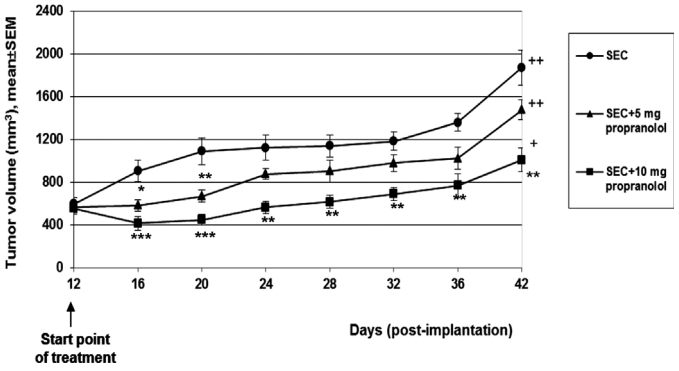

Fig. 2. Tumor volume of the studied groups at the recording points every 4 days from the 12th day (start point of propranolol treatment) to the last record at the 42nd day post-implantation. Paired-samples *t*-test: significant difference of tumor volume between start point and end point in each group, $^{*}p < 0.05$ and $^{**}p < 0.01$. Scheffe test: significant difference of group II (SEC treated by propranolol 5 mg/kg) or group III (SEC treated by propranolol 10 mg/kg) vs group I (untreated SEC), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. SEC; Solid Ehrlich Carcinoma.

Results of histopathological examination and immunohistochemical expression of toll like receptor 2 (TLR2) and survivin

Histopathological examination of the studied groups revealed the typical picture of Solid Ehrlich carcinoma (Fig. 4A and B). Examination of sections prepared from the organs (liver, lung, brain, lymph nodes) and bone of the studied groups revealed no metastatic spread of the primary tumors.

Positive TLR 2 expression was detected as diffuse cytoplasmic staining (Fig. 5A–C). Positive survivin expression was detected as diffuse cytoplasmic staining. None of the studied cases showed nuclear survivin expression (Fig. 6A–C). The score of TLR 2 and survivin expression was significantly higher and lower, respectively in group III compared to group I, while there was non significant difference for both in group II when compared to either group I or group III (Table 4).

In the untreated SEC (group I), the tumor volume (*V*) exhibited significant negative correlation with TLR2 expression and non significant correlation with survivin expression in tumor tissues (Table 3).

Discussion

β-Adrenergic signaling regulates multiple cellular processes that contribute to initiation and progression of breast cancer mainly immune responses [9], apoptosis [29,30], and angiogenesis [31,32]. However, there is limited information about the mechanisms by which β-blockers could impact the course and survival in cases of breast cancer. In this research work, SEC-bearing model in mice was established as a valid model that was frequently used to investigate chemotherapeutic strategies for breast cancer [33]. This model is typically of high virulence, quick development and infiltrative nature, reflecting its high-grade malignancy [34]. The tumor caused high lethality where survival rate was only 50% in the group with untreated SEC by the 6th week post-implantation. Treatment by propranolol resulted in a dose-dependent improvement in survival rate, reduction in tumor volume, and increase in the % TGI. These results confirmed the previously established impact of β-blockers in the clinical outcome of breast cancer [12,14–17]. The biomarkers that have been assessed in this study revealed that propranolol caused a dose-dependent increase in IL-10 with decrease in Hsp70 and iNOS, while there was non significant change in visfatin in tumor tissues compared to the untreated SEC. Such effects could be attributed to the β-blockade activity of propranolol based on the fact that β-adrenoceptors signaling plays an important role in regulation of tumor-directed immune responses. Among these responses is the immune-stimulated biosynthesis of NO, distinctive modulation of secretion of pro-inflammatory and anti-inflammatory cytokines such as IL-10 [35]. The data are complex regarding influence of IL-10 on cancer where it favors or inhibits tumor progression [36]. Inspit of this complexity, it is observable how well the present study provided negative correlation between tumor progression and IL-10 levels in tumor tissues. This finding indicates that increasing IL-10 levels by propranolol is one of its mechanisms in amelioration of breast cancer progression. The favor effects of IL-10 in cancer include suppression of angiogenesis either directly on tumor cells or indirectly enhances antitumor immunity by influencing infiltrating immune cells [37–39], modulation of apoptosis [40]. Accordingly, propranolol suppresses tumor growth by immuno-regulatory role mediated by the optimal level of IL-10 as supported by previous studies of Loppnow et al. [41] and Gage et al. [42]. In contrast, IL-10 may exert pro-tumorigenic effect *via* suppression of adaptive immune responses that lead to tumor escape from immune surveillance [43]. These opposing effects of IL-10 might depend on interactions with either cytokines or factors found in the tumor microenvironment. In this context, IL-10 has been reported to inhibit NO production and regulate the inducible NO synthase (iNOS) activation [44,45]. The iNOS-derived NO has been recognized as one of the most versatile players in immune system and pathogenesis of various diseases including cancer [46,47]. In consistent, the current results revealed a significant positive correlation of iNOS activity with ESC progression that reflects importance of inhibiting iNOS enzyme as a new target by beta blockers in alleviate stress associated tumors as reported by Powe et al. [15]. Visfatin is an adipocytokine that beside its biological role in many metabolic, energetic and stress responses, it

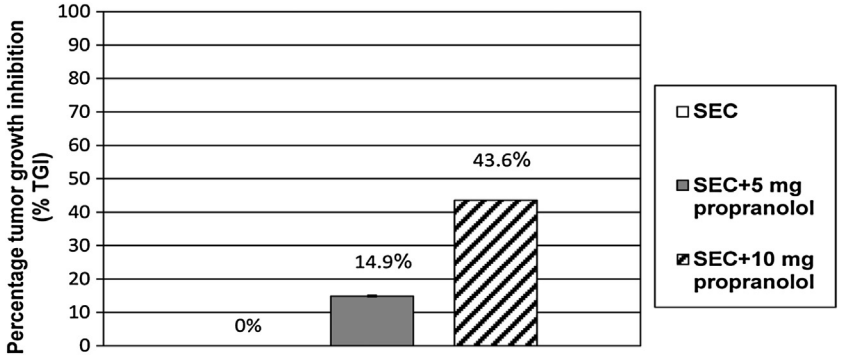

Fig. 3. Percentage tumor growth inhibition (% TGI) in group II (SEC treated by propranolol 5 mg/kg) and group III (SEC treated by propranolol 10 mg/kg) relative to group I (untreated SEC). SEC; Solid Ehrlich Carcinoma.

Table 2
Comparative statistics for levels of interleukin-10 (IL-10), visfatin, heat shock protein 70 (Hsp70), and inducible-nitric oxide synthase (iNOS) activity in tumor tissues from the survived mice of the studied groups at the end point of the experiment (the 42nd day post-implantation).

Parameter	Group I ($n=5$)	Group II ($n=7$)	Group III ($n=9$)	F value (p value)
IL-10 (pg/mg tissue protein)	36.3 ± 0.76	39.1 ± 0.41 $P_1 < 0.05$	41.7 ± 0.71 $P_1 < 0.001$ $P_2 < 0.05$	15.478 ($p < 0.001$)
Visfatin (ng/mg tissue protein)	13.8 ± 0.63	12.5 ± 0.23 P_1: NS	14.0 ± 0.47 P_1: NS P_2: NS	3.219 (p: NS)
Hsp70 (ng/mg tissue protein)	68.3 ± 1.2	57.4 ± 0.99 $P_1 < 0.01$	49.4 ± 1.8 $P_1 < 0.001$ $P_2 < 0.01$	33.320 ($p < 0.001$)
iNOS activity (U/mg tissue protein)	67.9 ± 1.31	40.4 ± 1.7 $P_1 < 0.001$	44.6 ± 0.99 $P_1 < 0.001$ P_2: NS	98.889 ($p < 0.001$)

Values expressed as mean ± SEM; n, number; NS, non significant. Scheffe test: P_1: group I (untreated Solid Ehrlich Carcinoma, SEC) vs group II (SEC treated by propranolol 5 mg/kg) and group III (SEC treated by propranolol 10 mg/kg). P_2: group III (SEC treated by propranolol 10 mg/kg) vs group II (SEC treated by propranolol 5 mg/kg).

Table 3
Correlation of tumor volume (V) with levels of interleukin-10 (IL-10), visfatin, heat shock protein 70 (Hsp70), inducible-nitric oxide synthase (iNOS) activity, and expression score of toll like receptor 2 (TLR2) and survivin in tumor tissues from the survived mice of untreated SEC group at the end point of the experiment (the 42nd day post-implantation).

Parameter	r value
IL-10 (pg/mg tissue protein)	-0.935^{*}
Visfatin (ng/mg tissue protein)	0.522^{NS}
Hsp70 (ng/mg tissue protein)	0.968^{**}
iNOS activity (U/mg tissue protein)	0.886^{*}
TLR2 expression	-0.894^{*}
Survivin expression	0.577^{NS}

NS, non significant; SEC, Solid Ehrlich Carcinoma.
* Significance at $p < 0.05$.
** Significance at $p < 0.01$.

exhibits proliferative, anti-apoptotic, pro-inflammatory, pro-angiogenic, and immunomodulating properties [48]. In contrast to previous studies [49,50], the present result showed non significant effect of propranolol on visfatin levels in tumor tissues denoting that its anti-angiogenic effect could be contributed to another indirect pathways such as immunomodulatory mechanism and arise a possibility that the effects of β-adrenoceptor signaling for angiogenesis in breast cancer may be independent of β-adrenoceptors expression by breast tumor cells. In context, other previous studies reported that propranolol mediates anti-angiogenic effect *via* inhibition of vascular endothelial growth factor (VEGF) [51]. Recently, β-adrenoceptors expression has been found to not correlate with the improved outcomes in breast cancer [52]. Thus, the effect of their agonists and antagonists may be paradoxical on cell proliferation and tumor growth [53]. Contrary to the present results, stimulation of β_2-adrenoceptors induced significant tumor growth suppression and tumor regression in mice bearing MDA-MB-231 human breast tumors [54]. However, this could be attributed to the nature of these cells that expressed high β-adrenoceptors levels, and when stimulated by an agonist, they evoked immediate and robust reductions in DNA synthesis [55]. In regard to the tumorigenic role of Hsp70 and its an anti-apoptotic capacity [56,57], the present findings showed that propranolol treatment decreased Hsp70 levels when compared to their allied untreated mice, reflecting role of propranolol in protein homeostasis under stress. Hsp70 expression is influenced by β-adrenergic receptor intermediates including cyclic AMP (cAMP) [58]. When over-expressed in cancers, Hsp70 is implicated in tumor cell proliferation, differentiation, metastasis, and recognition by the immune system [59]. In consistence, targeting Hsp70 may open new possibilities for treatment of resistance cancers [60,61]. Immune cells constitute a major cell population in tumor microenvironment and essential to provide signals for growth, anti-apoptosis, angiogenesis, and metastasis [62,63]. Among immune components, TLRs are critical in bridging innate and adaptive immune responses with significant role in many cancers immunosurveillance including breast cancer [64,65]. TLRs role in apoptosis is controversial, some studies suggested pro-apoptotic properties (especially TLR2 and TLR4) [65,66], while others suggested anti-apoptotic functions [67,68]. However, the current result favors the anti-tumorigenic role of TLR2 as its expression in tumor tissues exhibited significant negative correlation with tumor volume in the untreated SEC group. In addition, treatment by propranolol in high dose (10 mg/kg) caused significant increase

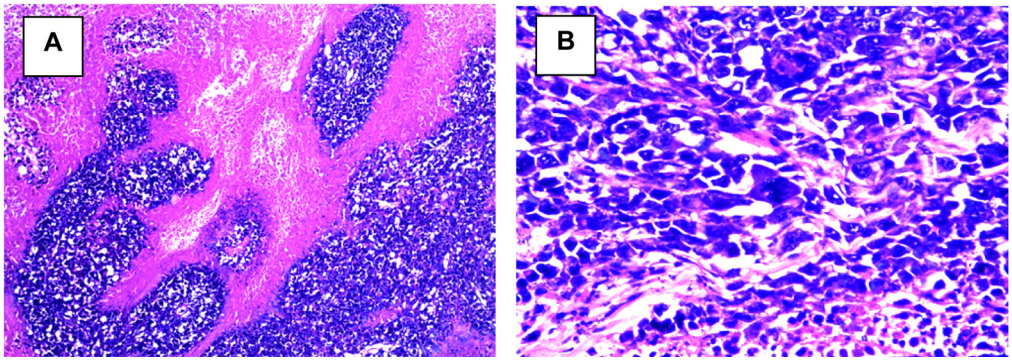

Fig. 4. Histopathological findings of untreated Solid Ehrlich Carcinoma (group I). [A] Tumor tissue composed of large necrotic centers surrounded by undifferentiated carcinomatous cells (H&E 100×). [B] Higher magnification of Solid Ehrlich Carcinoma showing the cellular details of the tumor, the cells are spherical in shape, containing relatively large, highly chromatophilic nuclei with one or more prominent nucleoli, giant forms are also seen (H&E 400×).

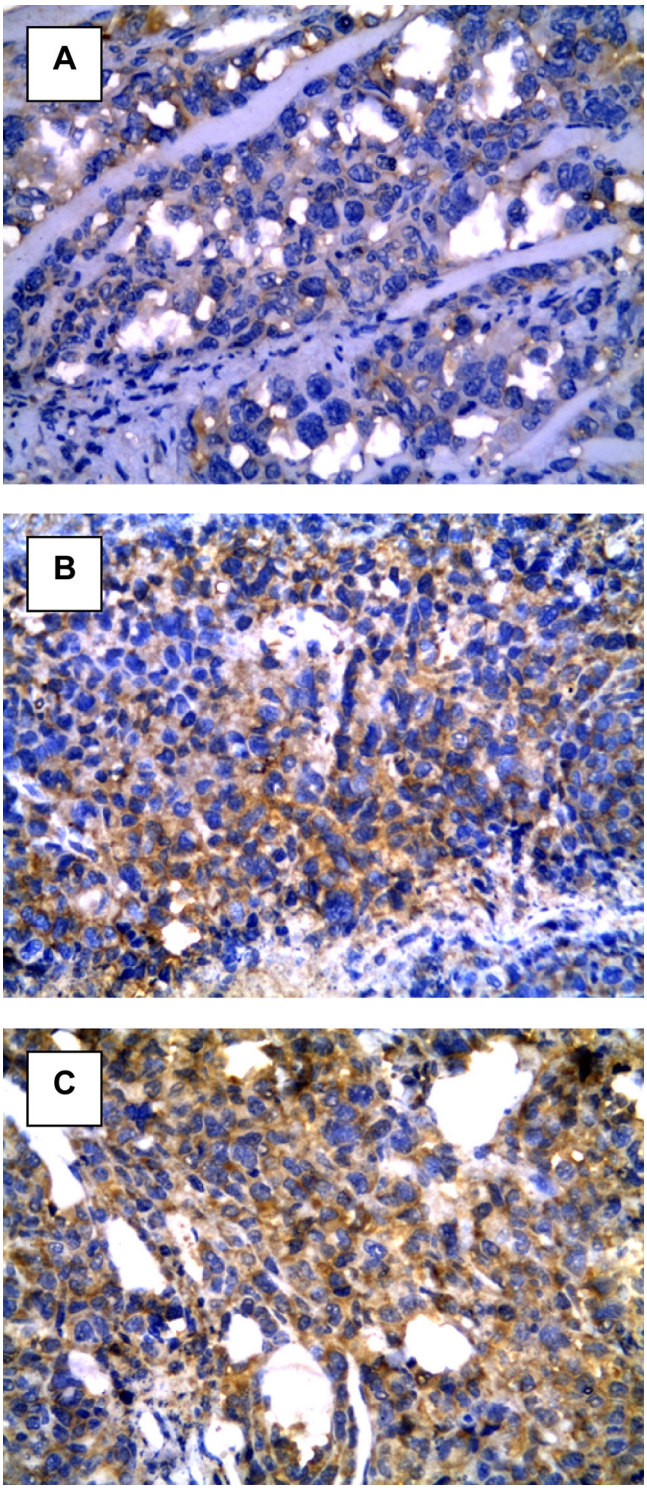

Fig. 5. Immunohistochemical expression of TLR2 in tumor tissues of the studied groups. [A] TLR2 staining of a section from group I (untreated Solid Ehrlich Carcinoma, SEC) showing weak (+) cytoplasmic expression (immunoperoxidase 400×). [B] TLR2 staining of a section from group II (SEC treated by propranolol 5 mg/kg) showing moderate (++) cytoplasmic expression (immunoperoxidase 400×). [C] TLR2 staining of a section from group III (SEC treated by propranolol 10 mg/kg) showing strong (+++) cytoplasmic expression (immunoperoxidase 400×).

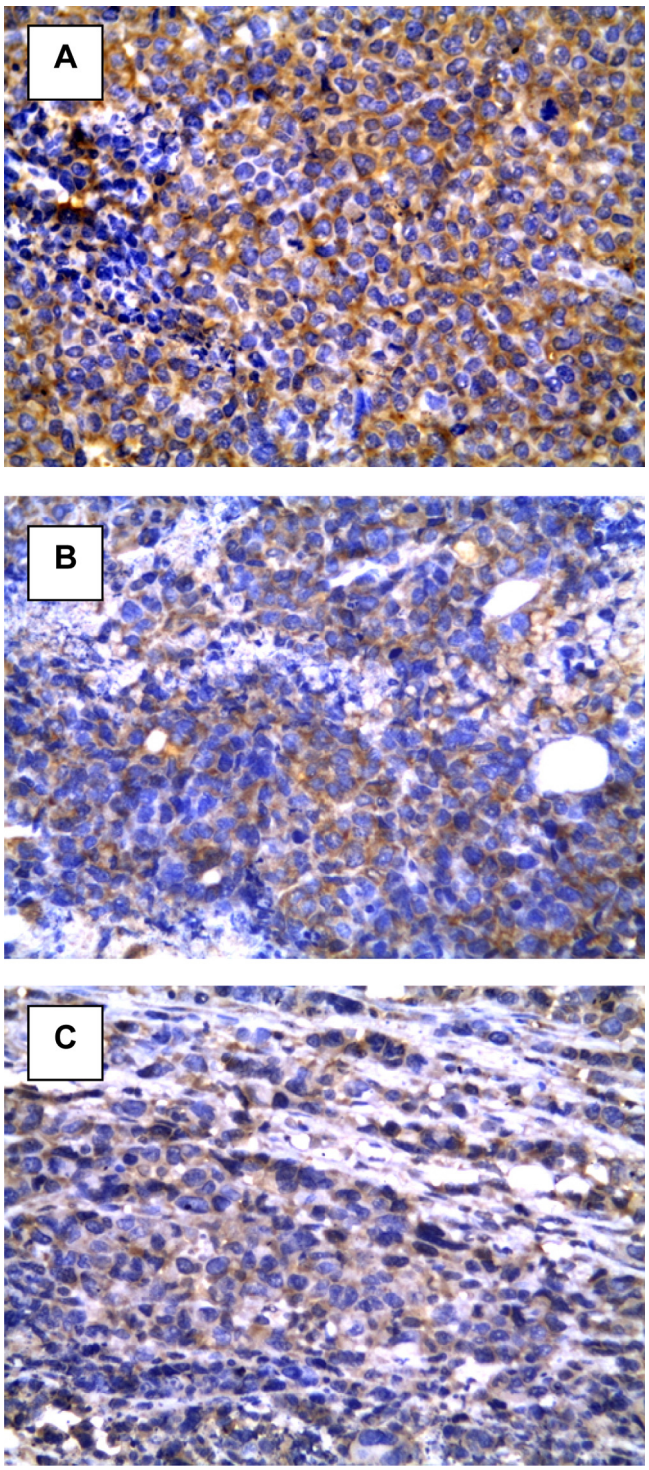

Fig. 6. Immunohistochemical expression of survivin in tumor tissues of the studied groups. [A] Survivin staining of a section from group I (untreated Solid Ehrlich Carcinoma, SEC) showing strong (+++) cytoplasmic expression (immunoperoxidase 400×). [B] Survivin staining of a section from group II (SEC treated by propranolol 5 mg/kg) showing moderate (++) cytoplasmic expression (immunoperoxidase 400×). [C] Survivin staining of a section from group III (SEC treated by propranolol 10 mg/kg) showing weak (+) cytoplasmic expression (immunoperoxidase 400×).

in immunohistochemical expression of TLR2 pointing to its role in amelioration of tumor progression. TLR2 or TLR4 agonists have been mentioned to stimulate the MyD88 signaling pathway in antigen presenting cells (APCs) with subsequent downstream activation of cytokines, chemokines, and their receptors, including IL-10 [66]. This comes in consistent with the anti-tumoral effect of β-blockers *via* improvement of the immune competence [69]. Survivin belongs to the inhibitor of apoptosis proteins (IAP) family

Table 4
Distribution (%) of toll like receptor 2 (TLR2) and survivin expression scores in tumor tissues from the survived mice of the studied groups at the end point of the experiment (the 42nd day post-implantation).

Groups	TLR2 expression, *n* (%)			Total	*p* value
	Weak (+)	Moderate (++)	Strong (+++)		
Group I (*n* = 5)	1 (20.0%)	1 (20%)	0 (0%)	2 (40%)	
Group II (*n* = 7)	1 (14.3%)	2 (28.6%)	1 (14.3%)	4 (57.2%)	P_1: NS
Group III (*n* = 9)	0 (0%)	3 (33.3%)	5 (55.6%)	8 (88.9%)	$P_1 < 0.05$, P_2: NS
Groups	Survivin expression, *n* (%)			Total	*p* value
	Weak (+)	Moderate (++)	Strong (+++)		
Group I (*n* = 5)	0 (0%)	2 (40%)	3 (60%)	5 (100%)	
Group II (*n* = 7)	2 (28.6%)	2 (28.6%)	1 (14.3%)	5 (71.5%)	P_1: NS
Group III (*n* = 9)	4 (44.4%)	0 (0%)	0 (0%)	4 (44.4%)	$P_1 < 0.01$, P_2: NS

n, number; NS, non significant. Fisher's exact test: P_1: group I (untreated Solid Ehrlich Carcinoma, SEC) vs group II (SEC treated by propranolol 5 mg/kg) and group III (SEC treated by propranolol 10 mg/kg). P_2: group III (SEC treated by propranolol 10 mg/kg) vs group II (SEC treated by propranolol 5 mg/kg).

and has been found to over-express in breast cancer and associated with more aggressive behavior and decreased survival [70] by enhancing cell proliferation, inhibition of apoptosis, and promotion of angiogenesis [26,28]. Thus, reduction in survivin expression in tumor tissues by propranolol in a high dose (10 mg/kg) confirms the beneficial effects of propranolol. Herein, the cytoplasmic survivin expression comes in consistent with the undifferentiated high grade nature of SEC, and is in line with the suggestion that cytoplasmic expression of survivin is associated with unfavorable prognosis in breast cancer, while nuclear expression is an indicator of good prognosis [26]. Considering efficacy of propranolol, low (5 mg/kg) and high (10 mg/kg) doses were adopted in this study. The dose-dependent effect of propranolol was evident for IL-10 and Hsp70, while the other biomarkers iNOS showed non significant difference between the low and the high dose, and even more only the high dose caused significant effect on TLR2 and survivin. In addition, treatment by propranolol in high dose exhibited higher impact in improvement of survival rate, tumor volume (*V*), and percentage tumor growth inhibition (% TGI) than its low dose. This comes in favor of recommending high dose of propranolol in cancer therapy. Nonetheless, use of the low dose cannot be ignored when the benefit to risk balance have to be considered.

Concluding these results, they indicate that propranolol could provide palliative effects in progression and survival of breast cancer that are mainly mediated *via* direct immunomodulatory and apoptotic mechanisms that probably associated with indirect anti-angiogenic activity.

Authors contribution

All the named authors participated sufficiently in this research work according to the specialty and expert of each one as follows: Dr. Amany A. Abdin designed the research protocol, conducted the handling of animals and treatment protocol of the research, recorded the clinical outcome such as survival rate, tumor volume and percentage tumor growth inhibition (% TGI), collected samples and participated in the assay of the biochemical parameters, conducted the statistical analysis, presented the results as tables or figures and participated in writing and revised the manuscript. Dr. Neama A. Soliman participated in the assay of the biochemical parameters, wrote and revised the manuscript. Dr. Eman M. Saied conducted the histopathological and immunohistochemical examination, participated in writing and revised the manuscript.

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Conflict of interest

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