RESEARCH ARTICLE

Significance of Tissue Expression and Serum Levels of Angiopoietin-like Protein 4 in Breast Cancer Progression: Link to NF-κB /P65 Activity and Pro-Inflammatory Cytokines

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Abstract

Background: The molecular mechanisms linking breast cancer progression and inflammation still remain obscure. The aim of the present study was to investigate the possible association of angiopoeitin like protein 4 (ANGPTL4) and its regulatory factor, hypoxia inducible factor-1 α (HIF-1α), with the inflammatory markers nuclear factor kappa B/p65 (NF-κB /P65) and interleukin-1 beta (IL-1β) in order to evaluate their role in inflammation associated breast cancer progression. Materials and Methods: Angiopoietin-like protein 4 (ANGPTL4) mRNA expressions were evaluated using quantitative real time PCR and its protein expression by immunohistochemistry. DNA binding activity of NF-κB /P65 was evaluated by transcription factor binding immunoassay. Serum levels of ANGPTL4, HIF-1α and IL-1β were immunoassayed. Tumor clinico-pathological features were investigated. Results: ANGPTL4 mRNA expressions and serum levels were significantly higher in high grade breast carcinoma (1.47±0.31 and 184.98±18.18, respectively) compared to low grade carcinoma (1.21±0.32 and 171.76±7.58, respectively) and controls (0.70±0.02 and 65.34±6.41, respectively), (p<0.05). Also, ANGPTL4 high/moderate protein expression was positively correlated with tumor clinico-pathological features. In addition, serum levels of HIF-1α and IL-1β as well as NF-κB /P65 DNA binding activity were significantly higher in high grade breast carcinoma (148.54±14.20, 0.79±0.03 and 247.13±44.35 respectively) than their values in low grade carcinoma (139.14±5.83, 0.34±0.02 and 184.23±37.75, respectively) and controls (33.95±3.11, 0.11±0.02 and 7.83±0.92, respectively), (p<0.001). Conclusion: ANGPTL4 high serum levels and tissue expressions in advanced grade breast cancer, in addition to its positive correlation with tumor clinico-pathological features and HIF-1α could highlight its role as one of the signaling factors involved in breast cancer progression. Moreover, novel correlations were found between ANGPTL4 and the inflammatory markers, IL-1β and NF-κB/p65, in breast cancer, which may emphasize the utility of these markers as potential tools for understanding interactions for axes of carcinogenesis and inflammation contributed for cancer progression. It is thus hoped that the findings reported here would assist in the development of new breast cancer management strategies that would promote patients' quality of life and ultimately improve clinical outcomes. However, large-scale studies are needed to verify these results.

Keywords: Breast cancer - angiopoietin –like protein -4 (ANGPTL4) - hypoxia inducible factor-1 alpha

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Introduction

Breast carcinoma is the most common cancer in females and is the principal cause of death among women globally (Taghavi et al., 2012). Hypoxia and inflammation associated cancer are important risk factors contributing to the breast cancer pathogenesis (Philip et al., 2004). Angiopoietin-like protein 4 (ANGPTL4) is a member of the angiopoietin family of proteins that have a similar structure. The native full-length ANGPTL4 exists in the form of dimeric or tetrameric complexes that can undergo proteolytic processing to generate the N-terminal coiled-coil fragment (nANGPTL4) and the COOH-terminal fibrinogen-like domain (cANGPTL4) (Feingold et al., 2012). It is highly expressed in adipose tissue, liver, placental tissue, and ischemic tissues (Santulli, 2014). The roles of ANGPTL4 in human cancers remain controversial. However, a novel role of ANGPTL4 in redox-mediated cancer progression has been postulated, hypothesized to be exhibited through its COOH-terminal fibrinogen-like domain (Feingold et al., 2012). Several lines of evidence have indicated that ANGPTL4 can be stimulated by inflammatory and hypoxic conditions (Santulli, 2014). Hypoxia observed in tumors has been noted to be the main cause of cell cycle arrest, apoptosis and angiogenesis (Xia et al., 2014). Cellular oxygen-signaling pathway requires the participation of hypoxia-inducible factors (HIFs), which exist in two types,

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oxygen-sensitive alpha subunit (HIF-α) and a constitutive beta subunit (HIF-β). Both subunits are heterodimeric, facilitating both oxygen delivery and adaptation to oxygen deprivation (Gaballah et al., 2014). HIF-1α is a transcription factor that binds to hypoxia responsive element (HRE), which in turn enhances the transcription of hypoxia-responsive genes. Thus, the survival of tumor cells is increased under hypoxic conditions (Zhang et al., 2013). A growing body of evidences highlights the role of HIF-1α as a regulator of ANGPTL4 (Khong et al., 2013). NF-kB is a sequence-specific transcription factor that plays a crucial role in linking inflammation and innate immunity to breast oncogenesis (Laere et al., 2006). However, cytoplasmic NF-ϰB is deactivated by binding of the inhibitory proteins IKB-α, IKB-β, IKB-ε, p105, and p100. NF-ϰB activity occurs by its translocation to the nucleus, binding to ϰB sites, and regulating target genes as a result of the phosphorylation and subsequent degradation of the inhibitory subunits (González-Ramos et al., 2012). Its constitutive activation is one of the early key events involved in breast cancer progression. Indeed, extant studies indicate that NF-ϰB signaling stimulates proliferation and prevents apoptosis (Biswas et al., 2003). The p65 subunit of NF-ϰB (NF-ϰB/p65) is responsible for most of NF-ϰB's transcriptional activity (van Loo and Beyaert, 2011). A cross-talk has been established between the NF-ϰB and the HIF pathways, as HIF-1 redox-sensitive induction might be due to binding at a distinct element in NF-ϰB proximal promoter. Moreover, activation of NF-ϰB pathway may be the main contributor of HIF-1 induction (Görlach and Bonello, 2008; Wang et al., 2015). The expression of important molecules in tumorigenesis such as chemokines, and inflammatory cytokines, all of which promote tumor cell invasion and angiogenesis—is regulated by NF-ϰB (Biswas et al., 2003). IL-1β is a member of IL-1 family that plays a central role in immune and inflammatory response regulation (Rider et al., 2011). IL-1β was found to be the primary mediator in context of cancer-associated chronic inflammation (Katanov et al., 2015). Interestingly, in cancer cells, HIF-1α was found to be activated by the proinflammatory cytokine IL-1β in a NF-ϰB dependent manner (Tewari et al., 2012). Thus it is necessary to focus research efforts on critical molecular phenomena that may link carcinogenesis and hypoxia to inflammation in tumor microenvironment. The ultimate goal of such studies is to propose new treatment strategies that could improve survival rates and patients' quality of life. Thus, The aim of present study was to investigate the possible association of angiopoeitin like protein 4 (ANGPTL4) and its regulatory factor, hypoxia inducible factor-1 α (HIF-1α) with nuclear factor kappa B/p65 (NF-ϰB /P65) and interleukin-1 beta (IL-1β) in order to evaluate their role in inflammation associated breast cancer progression.

Materials and Methods

Informed written consent was obtained from all patients prior to commencing the study. The study protocol was approved by the Local Research Ethics Committee, Tanta University and was in accordance with the principles of the Declaration of Helsinki II. This study included 74 women aged 40-56 years, who were admitted to the surgical department of Tanta University Hospitals for breast surgeries. All participants underwent clinical examination and routine laboratory investigations prior to being divided into two groups. Group I (n = 20) comprised of women who underwent surgical removal of benign breast lesions. As a part of this procedure, normal breast tissue samples were taken in the vicinity of the lesions, and served as controls. Group II (n = 54) consisted of female patients that had primary invasive breast carcinoma (confirmed by fine-needle aspiration) with no other primary cancers, all of whom underwent modified radical mastectomy or quadrantectomy with axillary clearance. Breast cancer biopsies were taken from the apparent lesion, processed by standard oncological procedures, studied and graded by a specialized pathologist. According to the histopathological grading, Group II was further subdivided into Group IIa consisting of 10 patients with low-grade cancer (Grade I) and Group IIb consisting of 44 patients with high-grade cancer (Grade II & III). None of the patients with invasive breast carcinoma had received any neoadjuvant therapy. Patients with other malignancies, any endocrinal disturbances, or systemic infections, those that received neoadjuvant therapy and smokers were excluded from the study. During the surgical procedures, tissue samples were obtained and processed by standard oncological procedures. These were subsequently divided into two portions, whereby one portion was kept in liquid nitrogen for ANGPTL4 mRNA gene expression investigations, while the other was kept in 10% formalin solution for histopathological and immunohistochemistry studies.

Biochemical and immunoassays:

All study participants followed overnight fasting protocol prior to the morning surgery. Immediately before the induction of general anesthesia, their early morning venous blood samples (10 ml in plain vacutainer tubes) were taken and transferred slowly into a dry sterile centrifuge tube. The samples were allowed to clot at room temperature, before being centrifuged at 2000 rpm for 10 minutes. Finally, serum was separated and stored at –70ºC for different estimations.

Enzyme-linked immunosorbent assays (ELISA) were used to detect the levels of serum ANGPTL4 (Cat # DY3485, R&D Systems, ANGPTL4 Duo kit, Minneapolis, USA), the levels of serum HIF-1α (R&D Systems, Minneapolis, MN 55413, USA) and serum levels of IL-1β (Cat # SEA563Hu, Cloud Clone Corp, USA). All ELISA assays were performed according to the manufacturer's instructions.

Preparation of peripheral blood mononuclear cells (PBMCs):

PMNCs were prepared using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) by means of density gradient centrifugation. First, 5 ml of Heparinised blood was layered on Ficoll and, after centrifugation for 30 minutes at 500 xg at room temperature, PBMC were harvested from the white interphase before being washed with phosphate buffered saline. The PBMCs samples

were stored at –80ºC until required for analysis of NFκB DNA-binding activity.

NFκB activation was examined by using transcription factor binding assay kit as described below:

1-Nuclear proteins isolation

Nuclear Extract kit (Cat # 40010, Active Motif, Carlsbad, CA, USA) was used to isolate nuclear proteins from PBMCs extract according to the manufacturer's instructions. Briefly, the kit provided ice-cold hypotonic buffer containing 10 mL KCL, 10 mmol/L HEPES (pH 7.9), 0.1 mmol/L EDTA, 0.1 mmol/L ethylene glycol tetraacetic acid, 1 mmol/L DTT; and Protease inhibitors. 0.8 mL of this ice-cold buffer was used for the samples. Incubation of homogenates on ice for 20 min, followed by adding 50 µL of 10% Nonidet P-40, then vortexed for 30 s and centrifuged for 2 min at 4 °C in an Eppendorf centrifuge. After supernatants were decanted, the nuclear pellets were suspended in an ice-cold hypertonic buffer. Then, a single wash with hypotonic buffer without Nonidet P-40 was performed. Next, they were incubated on ice for 20 min at 4 °C, mixed and centrifuged for 12 min at 4 °C. The supernatants were collected as nuclear extracts and stored at –80 °C until required (Gong et al., 2002). Concentrations of total proteins in the samples were determined according to the method described by Bradford (Bradford, 1976).

2-Determination of DNA-binding activity of NF-kB/p65

The ELISA-based TransAM™ NFκB p65 protein assay was used to evaluate NFκB/p65 DNA-binding activity (Cat # 40096, Active Motif, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly, the kit provided a duplexed NF-κB oligonucleotide containing a κB consensus sequence, which was attached to the surface of 96-well plates. Incubation with antibodies against the activated forms of NFκB/p65 was performed, allowing the NF-κB activated dimers in 20 µg nuclear extract bound to the attached oligonucleotide to be specifically and quantitatively detected. Then, incubation was performed with an enzyme-linked (horseradish peroxidase) secondary antibody, used for colorimetric scoring (Van Laere et al., 2006). The absorbance was measured on an ELISA reader at 450 nm with a reference wavelength of 655 nm.

Estimation of ANGPTL4 mRNA expression levels by real-time PCR

RNA extraction, cDNA synthesis and Real-time quantitative PCR: Total RNA was extracted from breast tissue samples using RN easy Mini Kit (Roche Diagnostics, GmbH, Mannheim, Germany) according to manufacturer's instructions. RNA was eluted, its concentration was measured spectrophotometrically and RNA samples were subsequently stored at –80°C until required. Each RNA sample was converted to cDNA using RT Superscript II (Cat # K1632, Thermo Scientific Fermentas, St. Leon-Ro, Germany), dNTP, and random primers (Roche, Mannheim, Germany) according to the manufacturer's instructions. cDNA corresponding to the RNA was used as a template for real-time PCR. PCR reactions were performed using Power SYBR Green PCR Master Mix and 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA). Sequence-specific primers were as follows: ANGPTL4: forward5'-ATTCTTTCCAGCGGCTTCTG-3', reverse: 5'-GAGGACTGGAGACGCGGAG-3' (According to gene bank accession No: NM_001039667), β-actin: forward: 5'- TGGCATTG CCGACAGGATGCAGAA-3', reverse: 5'-CTCGTCA TACTCCTGCTTGCTGAT-3' (According to gene bank accession No: NM_001101.3). β-actin primers were used as an internal control. Real-Time PCR was performed according to the manufacturer's instructions and comprised of denaturation at 95 ˚C for 10 s, followed by 40 cycles of 95 ˚C for 5 s and 60 ˚C for 20 s. Presence of the expected amplification fragments without unanticipated products and primers was confirmed by melting curve analysis. Comparative Ct (threshold cycle) method was used to determine relative product amounts, according to the Applied Biosystems instructions. All expression data were normalized by dividing the target amount by the amount of β-actin for each sample.

Histochemical and immunohistochemistry studies:

For histopathological study, breast tissue biopsies were embedded in paraffin. For this purpose 5 µm slices were stained with hematoxylin & eosin (H & E). For immunohistochemical staining, 4 mm thick sections were formed. The tissue sections were deparaffinized and rehydrated. Slides were incubated in 3% H2O2 for 10 minutes to reduce nonspecific background staining arising due to endogenous peroxides. For antigen retrieval, specimens were heated for 20 min in 10 mmol/l citrate buffer (pH 6.0) in a microwave oven (700 W). Following incubation with Ultra V Block (Lab Vision Corporation, Fremont, California, USA) for 7 min at room temperature to block background staining, slides were incubated with ER (rabbit polyclonal, ab37438), PR (rabbit monoclonal, ab16661), HER-2 (rabbit monoclonal, ab134182), KI67 (mouse monoclonal, ab15580), CK5/6 (mouse monoclonal, ab86974), EGFR (rabbit monoclonal, ab2430) and Mouse monoclonal to angiopoietin-like 4 (1:150 life science inc. Cat # MAB019Ra21) overnight at room temperature in a humid chamber. Antibody binding was detected using the Ultra Vision LP Detection System (Lab Vision Corporation) according to the manufacturer's recommendations. Color development was performed with 3, 30-diaminobenzidine and counterstained with hematoxylin. Internal adipose breast tissue served as positive control for ANGPTL4, whereas negative controls were obtained by replacing the primary antibody with non-immune immunoglobulin G. Immunostaining results for ER, PR, HER2/new, EGFR and CK5/6 were used for molecular subdivision of breast cancers (Holliday and Speirs, 2011).

Immunostaining results pertaining to ANGPTL4 were evaluated by image analysis (Q win Leica software). The percentage of positive cells was rated by assigning the 0 score to 0-5%, 1 score to 6-25%, a score of 2 to 26-50%, and 3 to more than 50%. Similarly, the staining intensity was rated by assigning 0 to no staining, 1 to weak staining, 2 to moderate staining, and 3 to strong staining. The percentage and intensity scores were added to an overall

score, whereby the ANGPTL4 protein expression with an overall score of 0-2 was designated as 'low/negative and that with an overall score of 3-6 was designated as 'high/moderate' (Yi et al., 2013).

Statistical analysis:

The data were analyzed using statistical package for the social science (SPSS) version 20.0 software (SPSS Inc., Chicago, IL, USA). Quantitative data expressed as mean and standard deviation. Categorical variables were compared using Chi-square test. Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Correlations were analyzed using the Pearson test. Receiver operating characteristics (ROC) analysis was used to identify the optimal threshold values of the studied parameters.

Results

A statistical comparison using ANOVA test followed by Tukey's test was performed between the studied groups with respect to the age and laboratory biochemical findings, as demonstrated in Table 1. No statistically significant differences were detected between the studied groups regarding age ($p > .05$). On the other hand, ANGPTL4 mRNA relative expression levels were significantly higher in Group IIb (1.47 ± 0.31) compared to Group IIa (1.21 ± 0.32). Once again, both groups had higher values than those measured in the controls (Group I) (0.7 ± 0.02), ($p < .001$). In addition, in high grade breast carcinoma patients (Group IIb), the serum levels of ANGPTL4, HIF-1α, NF-κβ/P65 and IL-1β (184.98 ± 18.18 ng/ml, 148.54 ± 14.20 μg/ml, 0.79 ± 0.03 and 247.13 ± 44.35 pg/ml, respectively) were significantly higher than those in low-grade cancer group (IIa) (171.76 ± 7.58 ng/ml, 139.14 ± 5.83 μg/ml, 0.34 ± 0.02 and 184.23 ± 37.75 pg/ml, respectively) and both were higher than those measured in the control group (65.34 ± 6.41 ng/ml, 33.95 ± 3.11 μg/ml, 0.11 ± 0.02 and 7.83 ± 0.92 pg/ml, respectively) ($p < .001$). Table (2) presents the immunohistochemical findings of cancer and control groups, by using chi-square test, significant differences in ANGPTL4 protein expression were revealed between control and study groups. More specifically, high/moderate ANGPTL4 expression was found in 86.4% (38/44) of the high-grade breast cancer group (Group IIb), and 40% (4/10) of low-grade breast cancer group (Group IIa), compared to only 25% (5/20) of the control cases. When the studied parameters in breast

Table 2. Immunohistochemical Results of ANGPTL4 in Control and Cancer groups

Group I (control) (n= 20)	Group IIa (low grade cancer) (n= 10)	Group IIb (high grade cancer) (n=44)
Low/negative ANGPTL4 expression		
15(75%)	6 (60%)	6 (13.6%)
High/moderate ANGPTL4 expression		
5(25%)	4(40%)	38 (86.4%)

ANGPTL4: angiopoietin like protein 4; P value was calculated by chi-square test; P was considered significant at <0.001; *Significant

Table 1. Demographic and Laboratory Findings of the Studied Groups

	Group I (control) (n= 20)	Group IIa (low grade cancer) (n=10)	Group IIb (high grade cancer) (n= 44)	ANOVA	
				f	P-value
Age (years)	49.1±5.3	47.3±5.3	49.9±5.3	1.054	0.354
ANGPTL-4 mRNA relative expression	0.70±0.02*#	1.21±0.32#	1.47±0.31	55.862	<0.001*
Serum ANGPTL-4 (ng/ml)	65.34±6.41*#	171.76±7.58#	184.98±18.18	462.282	<0.001*
Serum HIF-1α (μg/L)	33.95±3.11*#	139.14±5.83#	148.54±14.20	722.669	<0.001*
NFKB/p65 binding activity	0.13±0.03*#	0.36±0.04#	0.79±0.03	461.288	<0.001*
Serum IL-1β (pg/ml)	7.83±0.92*#	184.23±37.75#	247.13±44.35	287.345	<0.001*

ANGPTL4: angiopoietin like protein 4; HIF-1a: hypoxia inducible factor-1 alpha ; NFKB/p65: nuclear factor kappa B/p65 subunit ; IL-1β: interleukin-1beta . *Data are presented as the mean±SD; * statistically significant at $p<0.05$; * as compared to low grade breast carcinoma group; #as compared to high grade breast carcinoma group

Table 3. Pearson's Correlations between the Studied Parameters

		Age (years)	ANGPTL-4 mRNA relative expression	Serum ANGPTL-4 (ng/ml)	Serum HIF-1α (μg/L)	NFKB/p65 binding activity
ANGPTL-4 mRNA relative expression	r	0.047				
	P-value	0.738				
Serum ANGPTL-4 (ng/ml)	r	0.103	0.297			
	P-value	0.459	0.020*			
Serum HIF-1α (μg/L)	r	0.235	0.495	0.495		
	P-value	0.087	<0.001*	<0.001*		
NFKB/p65 binding activity	r	0.034	0.328	0.41	0.394	
	P-value	0.807	0.015*	0.002*	0.005*	
Serum IL-1β (pg/ml)	r	0.04	0.492	0.488	0.657	0.786
	P-value	0.772	<0.001*	<0.001*	<0.001*	<0.001*

ANGPTL4: angiopoietin like protein 4; HIF-1a: hypoxia inducible factor-1 alpha ; NFKB/p65: nuclear factor kappa B/p65 subunit ; IL-1β: interleukin-1beta .P was considered significant at <0.05; *Significant

Table 4. Association between ANGPTL4 Protein Expression and Clinico-pathological Features in Cancer Group

Group II (n=. 54)		Total		ANGPTL4 Low/negative expression		ANGPTL4 High/moderate expression		P value
		N	%	N	%	N	%	
Age (years)	<45	20	37	2	10	18	90	0.098
	>45	34	63	10	29.5	24	70.5	
Tumor size	T1	5	9.3	4	80	1	20	
	T2	21	38.9	3	14.3	18	85.7	0.011*
	T3	19	35.2	4	21.1	15	78.9	
	T4	9	16.7	1	11.1	8	88.9	
Lymph node metastasis	N0	10	18.5	7	70	3	30	
	N1	30	55.6	3	10	27	90	<0.001*
	N2	12	22.2	2	16.6	10	83.4	
	N3	2	3.7	0	0	2	100	
Grade	I	10	18.5	6	60	4	40	
	II	35	64.8	3	8.6	32	91.4	0.002*
	III	9	16.7	3	33.3	6	66.7	
Molecular type	Non basal type	15	27.8	6	40	9	60	0.047*
	Basal type	39	72.2	6	15.4	33	84.6	
Proliferation index	Negative	12	22.2	7	58.3	5	41.6	0.002*
	Positive	42	77.8	5	11.9	37	88.1	

ANGPTL4: angiopoietin like-4; P value was calculated by chi-square test; P was considered significant at <0.05; *Significant

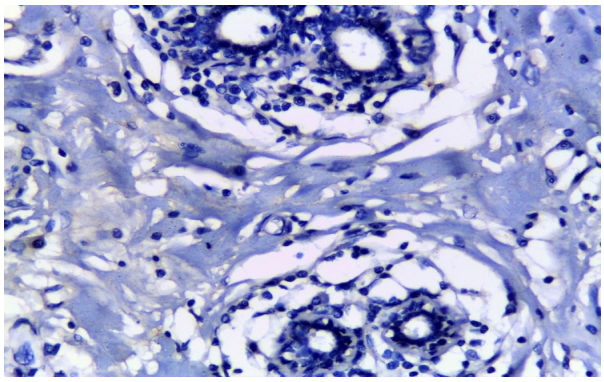

Figure 1.Immunohistochemical staining of ANGPTL4 in control case showed negative ANGPTL4 immunohistochemical expression in normal breast tissue (X400)

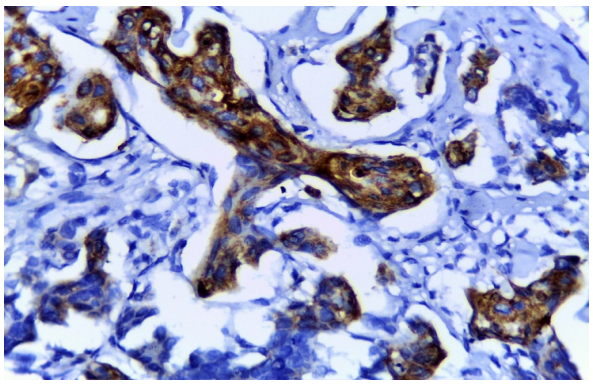

Figure 3. Immunohistochemical Staining of ANGPTL4 in Case of Invasive Breast Carcinoma Grade II Showed High ANGPTL4 Immunohistochemical Expression (X400)

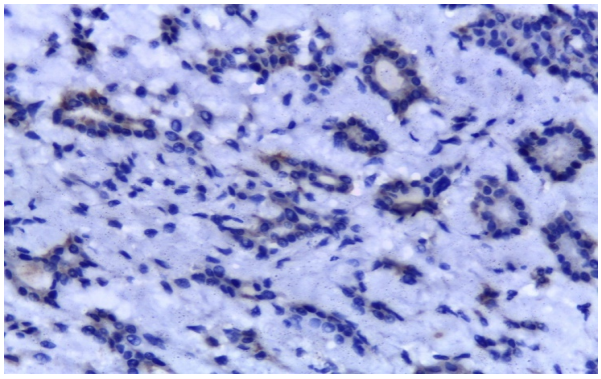

Figure 2. Immunohistochemical Staining of ANGPTL4 in case of Invasive Breast Carcinoma Grade I Showed Low ANGPTL4 Immunohistochemical Expression (X200)

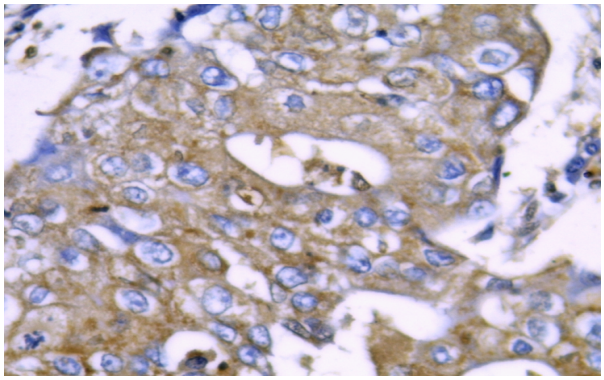

Figure 4. Immunohistochemical staining of ANGPTL4 in case of invasive breast carcinoma grade III showed moderate ANGPTL4 immunohistochemicalexpression (X400)

carcinoma were examined in table (3), no significant correlations were found between age and other studied parameters. Serum levels of ANGPTL4 exhibited a significant positive correlation with HIF-1α, NF-κβ/p65 DNA binding activity and IL-1β ($r = 0.495$, $r = 41$ and $r = 0.488$, respectively) ($p < .05$) and with relative mRNA expression ($r = 0.297$) ($p < .05$). In addition, serum levels of HIF-1α showed positive correlations with NF-κβ/p65 DNA binding activity and IL-1β, as well as relative mRNA expression ($r = 0.394$, $r = 0.657$ and $r = 0.495$, respectively)

($p < .05$). NF-κβ/p65 DNA binding activity exhibited positive correlations with IL-1β and with ANGPTL4 relative mRNA expression ($r = 0.786$ and $r = 0.328$) ($p < .05$). Finally, IL-1β showed positive correlations with ANGPTL4 relative mRNA expression ($r = 0.492$). Table (4) represents histopthological and immunohistochemical findings. As can be seen from the data, an elevated ANGPTL4 protein expression in breast carcinoma cases was significantly associated with lymph node metastasis, grade, tumor size and proliferation markers. However, there was no correlation between ANGPTL4 protein expression and patient's age. As 54 cases were diagnosed as invasive ductal carcinoma (Group II), 10/54 (18.5%) cases were classified as Grade I, 35/54 (64.8%) cases were diagnosed as Grade II, and 9/54 (16.7%) cases were of Grade III. Thus, patients in Group I were considered as having low-grade cancer, whereas those in Group II and III were considered as having high-grade cancer. With respect to the tumor size, 5/54 (9.3%) cases were classified as T1, 21/54 (38.9%) cases were T2, 19/54 (35.2%) cases were T3, and 9/54 (16.7%) cases were classified as T4. Moreover, 10/54 (18.5%) cases showed no lymph node metastasis, while 30/54 (55.6%) cases were N1, 12/54 (22.2%) cases were N2 and 2/54(3.7%) cases were N3. According to ER, PR, HER-2/new, CK5/6 and EGFR expression and the breast cancer cases could be further classified based on the molecular subtypes. According to this grouping, 39/54 (72.2%) cases were basal subtypes (as they showed ER-, PR-, HER2-, CK5/6+ and or EGFR+) and 15/54(27.8%) cases were of non-basal subtype, which were further divided into luminal A (ER+, PR+/-, HER2-) or luminal B (ER+, PR+/-, HER2+). Finally, with respect to KI-67 proliferation index, 12/54 (22.2%) cases were KI-67 negative and 42/54(77.8%) were KI-67 positive. These results are confirmed in Figures 1, 2 and 3.

Discussion

The novel adipocytokine, angiopoietin-like protein 4, is posited to play an important role in cancer progression and pathogenesis (Tan et al., 2012). Various types of human cancers were found to have altered expression of ANGPTL4 (Li et al., 2011; Kim et al., 2011). However, its role in breast carcinoma as well as its relevance to inflammation-related cancer progression remains controversial. The present study revealed that ANGPTL4 serum levels as well as its protein and mRNA expressions were significantly higher in cancerous breast tissue compared to normal breast tissue samples. In addition, increased tumor size, lymph node metastasis, high-grade breast carcinoma and proliferation index were associated with higher ANGPTL4 tissue expression, suggesting that ANGPTL4 plays a role in tumor progression and cancer cell differentiation. In line with these findings, increased mRNA and protein expressions of ANGPTL4 have been previously reported in other cancer types, such as oral Kaposi sarcoma and ovarian carcinoma (Hu et al., 2011; Brunckhorst et al., 2014). This phenomenon can be explained by the fact that tumor-derived ANGPTL4 facilitated disruption of vascular endothelial cell-cell junctions and tumor cell extravasation into other tissues, which ultimately leads to micro metastases (Padua et al., 2008). The same finding was reported by Zhang et al., 2012 who concluded that breast cancer metastasis was promoted by ANGPTL4. Similarly, Yi et al., 2013 reported that, in esophageal squamous cell carcinoma, higher expression levels of ANGPTL4 were correlated with more advanced tumor stages and more adverse clinical outcomes. In the present study, significantly higher protein expressions of ANGPTL4 in basal type of invasive duct carcinoma were noted relative to the non-basal type. This finding is in agreement with the results reported by Yotsumoto et al., 2013 who found that ANGPTL4 expression was involved in aggressive tumor metastasis to lungs and brain and in tumor development in triple negative breast cancer xenograft model. In the current study, there were significant differences between ANGPTL4 and proliferation index of breast cancer, thus concurring with Brunckhorst et al., 2014 who reported that ANGPTL4 significantly increased the number of proliferating ovarian cancer cells and KI-67. ANGPTL4-induced breast carcinoma proliferation may be achieved through tumor-derived ANGPTL4 interaction with integrins, which stimulate the prosurvival pathways, phosphoinositide 3-kinases/ protein kinase B (PI3K/ PKBα) and extracellular signal-regulated kinase (ERK) through NADPH oxidase-dependent production of O2- and proto-oncogene tyrosine-protein kinase Src, thus, promoting anoikis and tumor growth (Zhu et al., 2011). Further supporting the tumor induction role of ANGPTL4, Tan et al., 2012 reported that ANGPTL4 functions as a negative regulator of apoptosis. It is particularly noteworthy that, under tumor hypoxic conditions, ANGPTL4 is regulated by HIF-1α (Wagner et al., 2011). In addition, in microarray analysis, ANGPTL4 was found to be the only gene bound and highly induced by the two different regulators, peroxisome proliferators-activated receptor family (PPARβ/δ) and HIF-1α (Inoue et al., 2014). Contrary to the results of the current study, Ng et al., 2014 and Galaup et al., 2006 reported that ANGPTL4 suppressed growth, angiogenesis and metastasis in hepatocellular carcinoma and melanoma. Tumor progression and treatment response are highly affected by hypoxia (Li et al., 2015). The current study revealed a significant increase in serum HIF-1α levels in high grade breast cancer patients compared to low grade cancer and control subjects. This finding can be explained by the fact that protein translation and proteasome-dependent degradation are impaired by hypoxia, which affects the HIF-1α levels (Anad et al., 2011). Furthermore, these results are in line with the previous findings indicating that mature and functional HIF-1α are generated by cells in response to hypoxia in solid tumors (Kafshdooz et al., 2014) which plays a pivotal role in tumor progression, infiltration and metastasis (Unwith et al., 2015). The results yielded by this study also demonstrated that serum HIF-1α was significantly correlated with ANGPTL4 serum and expression levels. This finding is in keeping with the notion of an existing crosstalk or interplay between ANGPTL4 and HIF-1α, particularly in cancer progression. In agreement, Potente et al., 2011 reported that tumor angiogenesis is orchestrated by angiogenic

factors, including angiopoietin-like proteins and enhanced by HIF-1α. In addition, Inoue et al., 2014 found five HIF-1α binding sites, identified under hypoxia in the ANGPTL4 gene locus, suggesting that hypoxia is an activator of ANGPTL4 gene. The authors further asserted that pro-inflammatory cytokines in tumor microenvironment and the subsequent activation of NF-kB transcription factor play a role in tumor pathogenesis and progression. In this context, we further investigated the serum levels of IL-1β and DNA-binding capacity of NF-kB/p65 in breast cancer. The present study showed that the DNA binding capacity of the p65 subunit of NF-κB significantly increased in PMNCs from high-grade breast cancer patients compared to those with low-grade cancer and the control subjects. This finding is consistent with the results reported by Wang et al., 2013 who noted that, in human breast cancer cell line, NF-κB transcriptional activity was increased secondary to overexpression of NF-κB/p65. Consequently, cancer progression and metastasis can be promoted by active NF-κB signaling through induction of several cell cycle, anti-apoptotic and chemotactic regulatory genes that enhance tumor (Xia et al., 2014). It can thus be postulated that an existing interplay between NF-κB and HIF-1α particularly in carcinogenesis has been established. The present study revealed that serum HIF-1α was significantly correlated with NF-κB/P65 activity and that both played an important role in tumor progression. HIF-1α protein accumulation under hypoxia requires the participation of NF-κB, which is a critical transcriptional activator of HIF-1α (Taylor and Cummins, 2009). Moreover, authors of several studies focusing on different cancer types reported that hypoxia-inducible factor-1α is up-regulated by a hypoxia-dependent transcription by NF-κB (Yoshida et al., 2013), stimulating epithelial to mesenchymal transition (Cheng et al., 2014), thus promoting tumor progression and angiogenesis. NF-kB transcription factor plays a crucial role in tumor development through transcriptional regulation of genes associated with tumor growth, invasion and metastasis, including cytokines such as IL-1β (Tewari et al., 2012). The present study revealed that IL-1β serum levels were significantly increased in patients with high-grade breast cancer compared to those with low-grade cancer and to the control subjects. At the cellular level, IL-1β exerts its effects through binding of IL-1 receptor I (IL-1RI) and recruitment of the co-receptor, interleukin-1 receptor accessory protein, thus inducing cellular changes and signal transduction (Aggarwal et al., 2006). In cancer cells, several authors observed that IL-1β acts via autocrine and/or paracrine mechanisms, positing that it may regulate the expression of angiogenic factors, such as IL-8, thus promoting angiogenesis and tumor progression (Katanov et al., 2015). In addition, IL-1β contributes to the progression of tumors through stimulation of cell growth and differentiation and the inhibition of apoptosis of altered cells at the inflammatory site (Snoussi et al., 2005). The results yielded by the present study are in good agreement with those reported by Saijo et al. 2002, who demonstrated that interleukin IL-1β autocrine production in pancreatic carcinoma cell lines induced tumor growth and confers chemoresistance. With respect to the positive correlation between IL-1β and HIF-1α, IL-1β and TNF-α have been shown to increase HIF-1α activity in breast cancer through activation of NF-κβ pathway (Tewari et al., 2012). In addition, IL-1β mediated up-regulation of HIF-1α via an NF-kB/Cyclooxygenase-2 pathway was reported to identify HIF-1 as a critical link between inflammation and oncogenesis progression (Jung et al., 2003). Nevertheless, in this study, strong positive correlations between ANGPTL4 levels and HIF-1α, NF-κB/p65 activity and IL-1β in breast cancer patients were noted, reflecting ANGPTL4 involvement in inflammatory pathways associated with breast cancer progression. Collectively, serum levels of ANGPTL4, HIF-1α, and IL-1β and NF-κB/p65 activity were significantly associated with breast carcinoma pathogenesis and progression.

In conclusion, ANGPTL4 high serum levels and tissue expressions in advanced grade breast cancer, in addition to its positive correlation with tumor clinico-pathological features and HIF-1α could highlight its role as one of the signaling factors involved in breast cancer progression. Moreover, novel correlations were found between ANGPTL4 and the inflammatory markers, IL-1β and NF-κB/p65, in breast cancer, which may emphasize the utility of these markers as potential tools for understanding interactions for axes of carcinogenesis and inflammation contributed for cancer progression. It is thus hoped that the findings reported here would assist in the development of new breast cancer management strategies that would promote patients' quality of life and ultimately improve clinical outcomes. However, large-scale studies are needed to verify these results.

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