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Effect of Interleukin-6 on expression of CD44 and CD24 in Breast Carcinomas (*Ex vivo* study)

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ABSTRACT

Breast cancer is the most frequently diagnosed cancer and is the leading cause of cancer death among women. Cancer stem cells (CSCs) display stem cell properties and may contribute to metastasis and treatment resistance**.** CSCs are regulated by cellular interactions in their microenvironment. These interactions involve inflammatory cytokines including IL-1, IL-6, IL-8, and TGF-β. There are some putative stem cell markers that are in major use for identification and isolation of CSCs**,** the CSCs phentype is CD44+/CD24−/low CSC phenotype. The aim of the present study is to investigate the effect of different IL-6 concentrations on CD44 and CD24 expression as breast cancer stem cell markers in primary breast cancer tumor microenvironment. This study was conducted on 30 radically mastectomized breast cancer patients. CD44 and CD24 expression level were immunohistochemically evaluated in breast cancer patients` tumor tissues cultured without and with different concentrations of IL-6. Results of the current study revealed that CD24 expression level is found to be higher significantly in tumor tissue samples incubated with IL-6 (10 µg/50 mm^3 tissue) (p=0.033) and insignificantly higher in samples incubated with IL-6 (conc. 25, 50, 100µg/50 mm^3tissue) (p=0.272, 0.588, 0.086 respectively) than those incubated without IL-6. CD44 expression levels are higher in tumor tissue samples incubated with IL-6 than those incubated without IL-6. The statistical difference is significant in samples incubated with IL-6 (25, 50, 100µg/50 mm^3 tissue) (p=0.0233, 0.011, 0.0233 respectively) while it was insignificant in samples incubated with IL-6 (10 µg/50 mm^3 tissue) (p=0.065). Regarding IL-6 as a corner stone in dynamic equilibrium between CD24 and CD44 expressions, we can conclude that Il-6 and its receptors may form striking targets for promising breast cancer immunotherapeutic approach.

Key words: Breast cancer, Tumor microenvironment, Cancer stem cells, IL-6, CD24, CD44.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and is the leading cause of cancer death among women [(1)].

Improved early detection and new treatments have led to a decline in the overall mortality due to breast cancer, but the survival rates for patients with metastatic disease have not improved significantly [(2)]. There is a substantial evidence that many human cancers are driven by a subpopulation of cells that display stem cell properties and may contribute to metastasis and treatment resistance, these cells named cancer stem cells(CSCs)[(3)]. Just as normal stem cells, CSCs are regulated by their microenvironment or “niche” which interact with and in turn are regulated by cells in the tumor microenvironment (TME). These interactions involve inflammatory cytokines including IL-1, IL-6, IL-8, and TGF-β, which in turn activate STAT3/NF-κβ pathways in both tumor and stromal cells [(4)]. These cytokines are frequently elevated in breast tumors, generating a positive feedback loop which stimulates the tumor stem cell components accelerating metastasis and therapeutic resistance [(5)].

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CSCs are self-renewal under non-differentiation conditions, and also have the ability to differentiate into non-stem cancer cells (NSCCs)[6]. It is often thought that CSCs are precursors of differentiated cancer cells (NSCCs), but it is also possible that CSCs are derived from NSCCs or arise independently, but the basis of this phenomenon is unknown [5].It is suggested that CSCs which are derived from NSCCs is required for tumor formation. Once formed, CSCs self-renew, continuously generate NSCCs via differentiation, and convert some NSCCs back into CSCs by secreting extracellular signals such as IL-6 within the confines of the tumor [5].

There are some putative stem cell markers that are in major use for identification and isolation of CSCs from different solid tumors [7,8]. Prevalence of CSC markers is heterogeneous among breast cancer special histological types [9]. Although low-grade and luminal type, tubular carcinomas display the phenotype CD44+CD24−/low, metaplastic and medullary carcinomas, which are high-grade and basal-like, are the two special types, enriched in the CD44+/CD24−/low CSC phenotype. CD44 is considered a potential CSC marker [10]. It has been referred to CD44 as a commonly expressed surface marker. The majority of cancer cell lines express high levels of CD44. CD24 is another important marker whose prognostic value and significance remains controversy [10].

The aim of the present study is to investigate the effect of different IL-6 concentrations on the level of CD44 and CD24 expression as breast cancer stem cell markers in primary breast cancer TME.

SUBJECTS AND METHODS

Subjects:

Thirty Egyptian females undergoing modified radical mastectomy for cytologically proved breast cancer were recruited from the Department of Surgery, Medical Research Institute, Alexandria University**.** They were all subjected to full history taking and clinical examination.

Methods:

Tumor Tissue preparation:

Fresh sterile tissue samples from each patient primary breast tumor larger than 0.2 cm was obtained immediately after surgical resection. Each tumor sample was divided into two parts; one part for the routine histopathological studies and the other part was maintained in organ transportation medium on ice until used for the tissue culture.

Tissue Culture:

Equal volumes of each tumor tissue sample were submerged in complete RPMI medium and incubated at 37^{o}c in a constant atmosphere of 5% CO2 for 24 hours in absence and presence of appropriate different concentrations of IL-6 (Recombinant Human Interleukin-6 Active) (HIL6A-50) (Immunostep). By the end of the incubation period, samples were fixed in 10% phosphate-buffered formalin PH 7.4 for 24 hours then processed for preparation of microscopic slides for immunohistochemical detection of CD44 & CD24 expression. [10]

Assessment of CD44 & CD24 expression:

It was achieved by semi-quantitative detection of CD44 & CD24 expression using:

1-Rabbit polyclonal IgG(ProSci-INCORPORATED) as a primary antibody and using a labeled streptavidin-biotin immunoenzymatic antigen detection system, (Ultra Vision Detection System, AntiPolyvalent, HRP/DAB) according to the manufacturers' manual.

2-CD44 Std. / HCAM Ab-4 (Clone 156-3C11) (Thermo Fisher Scientific, UK).

3-CD24 (GPI-linked surface mucin) Ab-2 (Clone SN3b) (Thermo Fisher Scientific, UK).

The DAB chromogen yielded brown color reaction end point at the site of target antigen. Visual evaluation of immuno-staining was performed with a light microscope using a semi-quantitative scale according to the intensity of staining as follows: No staining (-ve), Weak staining (+), Intermediate staining (++) and Strong staining (+++) [11].

Statistical analysis

All data were presented using SPSS statistical SPSS software package. The following statistical tests were used: Chi-Square test: It determines whether the observed frequencies differ significantly from expected frequencies.

RESULTS

The descriptive analysis of the clinicopathological parameters of the subjects is illustrated in table-1.

A-CD24 expression:

CD24 expression level is found to be higher significantly in tumor tissue samples incubated with IL-6 (10 µg/50 mm^3 tissue) (p=0.033) and insignificantly higher in samples incubated with IL-6 (concentrations: 25, 50, 100µg/50 mm^3 tissue) (p=0.272, 0.588, 0.086 respectively) than those incubated without IL-6. Table-2. Fig.1-3.

B-CD44 expression:

CD44 expression levels are higher in tumor tissue samples incubated with IL-6 (concentrations: 10, 25, 50, 100µg/50 mm^3 tissue) than those incubated without IL-6. The statistical significant difference in samples incubated with IL-6 (concentrations: 25, 50, 100µg/50 mm^3 tissue)(p=0.0233, 0.011, 0.0233 respectively) while it was insignificant in IL-6 (10µg/50 mm^3 tissue) (p=0.065). Table-3. Fig-4-6.

Table-1. The clinicopathological parameters of the patients enrolled in the study:

Clinicopathological parameters	No.	%	
Age			Range 37-68 Mean±SD 50.6±7.9
< 50	16	53.3	
> 50	14	46.7	
Histologic grade			
I	25	83.3	
II	4	13.3	
Vascular invasion			
Negative	3	10	
Positive	27	90	
L.N invasion			
Negative L.N	14	46.7	
Positive L.N	13	43.3	
Hormonal status			
ER			
Negative	3	21.4	
Positive	11	78.6	
+1	4	28.6	
+2	3	21.4	
+3	4	28.6	
PR			
Negative	3	21.4	
Positive	11	78.6	
+1	2	14.3	
+2	4	28.6	
+3	5	35.7	

Figure-1. Comparison of CD24 expression in breast cancer tumor tissues incubated with different IL-6 concentrations

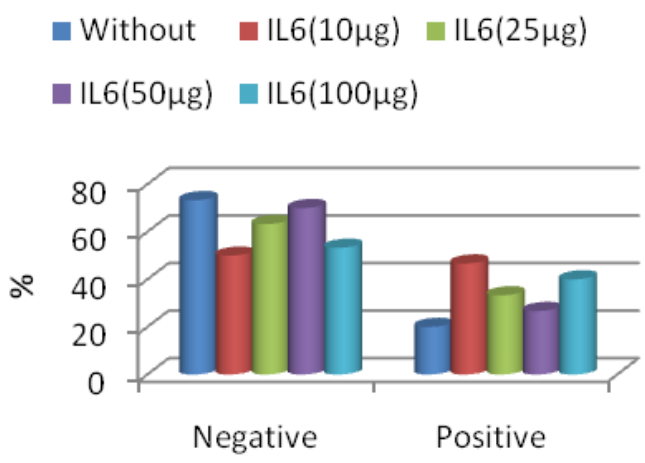


Figure-2. Invasive ductal carcinomas (IDC) tissue, incubated with IL-6 (10µg/50 mm³ tissue) showing weakly immunostained cells (+) for CD 24 (IHC X400)

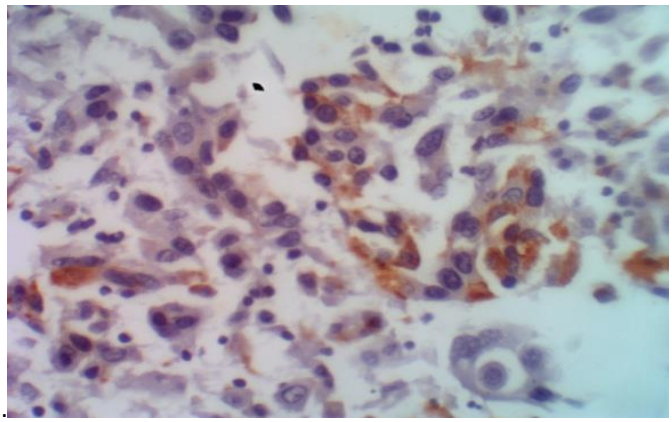


Figure-3. IDC tissue, incubated with IL-6 (100µg/ 50 mm³ tissue) showing negatively immunostained cells for CD 24 (IHC X400)

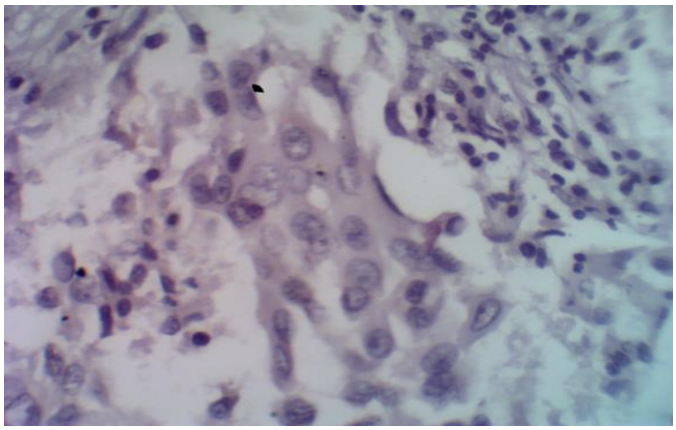


Figure-4. Comparison of CD44 expression in breast cancer tumor tissue incubated with different IL-6 concentrations.

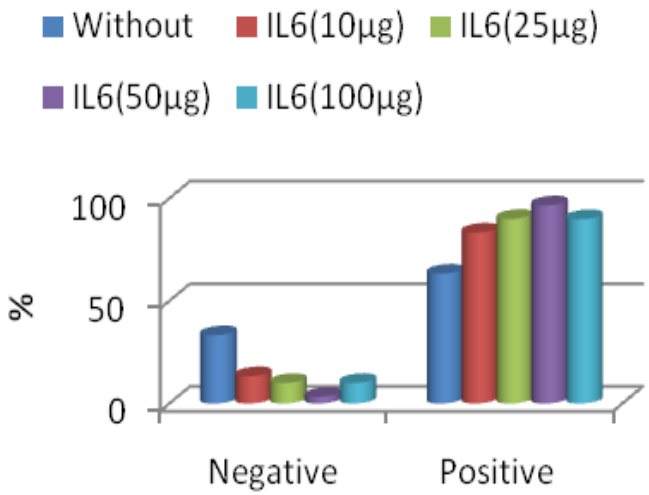
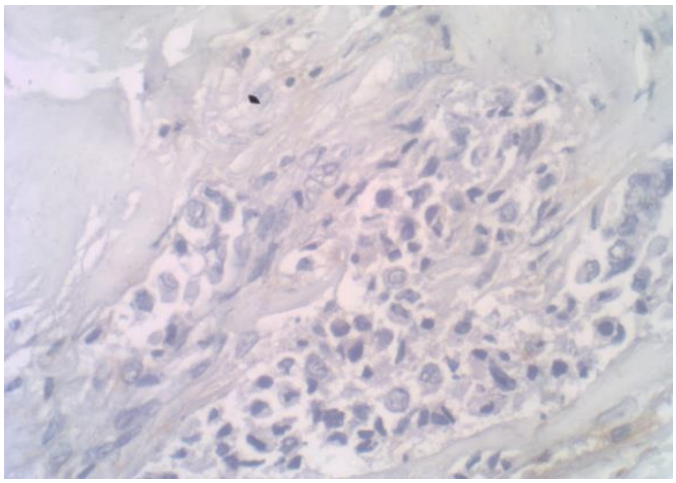


Figure-5. IDC tissue, incubated without IL-6 showing negatively immunostained cells for CD 44 (IHCX400)



DISCUSSION

The dynamic interaction between cancer cells and the tumor microenvironment is increasingly considered as an important regulator of malignant progression. In this regard, tumor cells secrete cytokines, and growth factors that are able to recruit and activate a variety of non-transformed stromal cells. In turn, stromal cells provide signals that promote the ability of tumor cells to invade and metastasize [(12)].

It was shown that breast cancer cells with a CD44+&CD24−/low cell surface profile were capable of driving tumor development and were proposed as CSCs [(13)].

IL-6 has been shown to be a direct regulator of breast cancer stem cell self-renewal [(14)], a process mediated by the IL6R/GP130 complex through activation of STAT 3[(15)].

So we aimed in the current study to investigate the effect of different IL-6 concentrations on CD44 and CD24 expression as breast cancer stem cell markers in primary breast cancer tumor microenvironment. CD44 and CD24 expression levels were immunohistochemically (IHC) evaluated in breast cancer patients` in tumor tissues cultured without and with different concentrations of IL-6.

Results of this study revealed that**:** CD24 expression level is found to be higher significantly in tumor tissue samples incubated with IL-6 (10 µg/50 mm^3 tissue) (p=0.033) and insignificantly higher in samples incubated with IL-6 (concentrations: 25, 50, 100 µg/50 mm^3 tissue) (p=0.272, 0.588, 0.086 respectively) than those incubated without IL-6.

Meanwhile CD44 expression levels are higher in tumor tissue samples incubated with IL-6 (concentrations: 10, 25, 50, 100 µg/50 mm^3 tissue) than those incubated without IL-6, the statistical significant difference in samples incubated with IL-6 (concentrations: 25, 50, 100 µg/50 mm^3 tissue) (p=0.0233, 0.011, 0.0233 respectively) while it was insignificant in samples incubated with IL-6 (10µg/50 mm^3tissue) (p=0.065).

In accordance with these finding, Iliopoulos et al (2011) [(16)], demonstrated that addition of IL-6 to culture media increased the proportion of cancer stem cells in

Table-2. Comparison of CD24 expression in breast cancer tumor tissues incubated with different IL-6 concentrations.

	Without IL-6		IL-6(10µg)		IL-6(25µg)		IL-6(50µg)		IL-6(100µg)	
	No. (28)	%	No. (29)	%	No. (29)	%	No. (29)	%	No. (28)	%
Negative	22	73.3	15	50.0	19	63.3	21	70.0	16	53.3
Positive	6	20	14	46.7	10	33.3	8	26.7	12	40
P1			0.033*		0.272		0.588		0.086	

*: p value for t Chi-Square test **: Statistically significant at p ≤ 0.05

P1: Comparison between samples incubated without and with different concentrations of IL-6

Table-3. Comparison of CD44 expression in breast cancer tumor tissue incubated with different IL-6 concentrations.

	Without IL-6		IL-6(10µg)		IL-6(25µg)		IL-6(50µg)		IL-6(100µg)	
	No (29)	%	No. (29)	%	No. (30)	%	No. (30)	%	No. (30)	%
Negative	10	33.3	4	13.3	3	10.0	1	3.3	3	10.0
Positive	19	63.3	25	83.3	27	90	29	96.6	27	89.9
P1			0.065		0.0233*		0.011*		0.0233*	

*: p value for t Chi-Square test **: Statistically significant at p ≤ 0.05

P1: Comparison between samples incubated without and with different concentrations of IL-6

breast cancer cell lines as well as in primary cells isolated from tumors. Both breast cancer stem cells and mesenchymal breast cancer cells secrete up to 1000-fold more IL-6 than non-stem epithelial breast cancer cells, indicating the presence of an autocrine positive feedback loop [17-19].

These results are in concomitant with those of Liu et al(2011)[20] who reported that mesenchymal stem cells (MSCs) have been shown to induce CSC growth, and IL-6 has been demonstrated to be the key player mediating the interaction between MSCs and CSCs.
Acoordingly it has been suggested that IL-6 might facilitate the generation of breast cancer cells with stem cell-like properties [21]. Taking into consideration that IL-6 is able to induce epithelial mesenchymal transition (EMT) which has been implicated in generation of stem cell phenotype and promoted the invasiveness of breast cancer cells [16].

Hence, we can suggest that IL-6 is a corner stone in dynamic equilibrium regarding the expression of CD44, CD24.This suggestion is in agreement with those of many previous studies [22-24].

In accordance with our finding, it has been demonstrated that CD44+CD24–/low cells have been associated with stem cell maintenance, cancer cell survival, or poor breast cancer prognosis. In particular, IL-6 is implicated in the maintenance of CSCs [22,23], and its downstream effector Stat3 enforces the undifferentiated state [24]. This process identifying IL-6 and its receptor as attractive therapeutic targets [14].
In this regard, when an antibody against IL6 is added to the medium [6], the conversion of NSCCs to CSCs is largely blocked, suggesting that secreted IL6 is important for generating CSCs from NSCCs [5].

CONCLUSION

We can conclude that Il-6 and its receptors may form striking targets for promising breast cancer immunotherapeutic approach.

Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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