Original Research Papers

Diagnostic significance of Beclin-1 and Transforming growth factor β in Breast Cancer

ABSTRACT

Background: The main cause of cancer deaths amongst women breast cancer remains a clinical and social challenge, and a serious public health problem. On a worldwide level, it continues to be a devastating disorder.BECN1 is a tumor suppressor gene implicated in the initiation of autophagy. It encodes beclin-1 protein that inhibits cancer growth. There is wide disputation concerning its role in initiation, promotion of tumor and predictive importance of autophagic molecules. Transforming growth factor β (TGF-β) induces process of epithelial-mesenchymal transition (EMT) keeping, epithelial cells more motile and invasive resulting in cancer progression and metastasis.
Aim: Detection of beclin-1 expression level in metastatic and non-metastatic breast cancer patients and study its role in tumorigenesis of breast cancer through attainable association with the inflammatory cytokine, TGF-β.
Methods: Expression levels of beclin-1 and TGF-β were assessed in 70 breast cancer female patients and 20 controls using quantitative real-time PCR.
Results: Beclin-1 expression levels as well as TGF-β were significantly higher in metastatic breast cancer patients and non-metastatic patients compared to controls. Positive correlation was found between beclin-1 expression level and TGF-β expression level in breast cancer patients.
Conclusion: Our results indicated that over-expression of both beclin-1 and TGF-β was associated with aggressive clinical outcomes of breast cancer patients and tumor growth. These findings suggest that beclin-1 and TGF-β are associated with tumorigenesis of breast cancer.

Keywords: autophagy, BECN1, TGF-β, breast cancer.

1. INTRODUCTION

Breast carcinoma is the y main cancer that influences females accounting for 37.7% of their overall malignancies in Egypt. Moreover, it is important reason of cancer related mortality in Egypt, constituting about 29.1% of all most cancer associated mortality [1]. Autophagy is a cellular degradation or “self-eating” pathway extremely preserved throughout all existence kingdoms. [2]. Considering the truth that autophagy is implicated in numerous cellular processes, and keeping in mind the ~~complexness~~ complexity of the molecular mechanisms of tumor initiation and development, it is no longer shocking that the interruption of autophagy plays role in tumor ~~inatition~~ initiation and progression. Indeed, cancer was the primary disease allied with disturbed autophagy besides, the first for which medical trials in humans were performed [3].
Beclin-1 (BECN1) is a rate-limiting element of autophagy and a haplosufficient cancer suppressor that is fundamental for embryonic development. Moreover, this protein is a core factor essential for autophagosome formation [4].Transforming growth factor-beta (TGF-β) is a protein that is essential regulator in homeostasis in various organs [5]. ~~(~~As a signaling molecule, TGF-β has a variety of biological function and participates in several physiological and pathological processes [6].

2. MATERIAL AND METHODS

2.1 Ethical approval

The study was approved by the Institutional Review Board (IRB) of the NCI, Cairo University and was conducted according to the rules of Helsinki declaration for human studies. A Written informed consent was obtained from all study subjects.

2.2. Subjects

The study included 70 Egyptian women with breast cancer at distinctive stages, their age ranged from (23-76 years). They were collected from Kasr Alainy hospital. Patients were diagnosed by clinical examination and affirmed by mammography and surgical biopsies. Twenty clinically normal adjoining specimens were moreover collected from adjacent healthy breast tissue of non-metastatic breast cancer cases. All specimens were subjected to estimation of expression levels of beclin-1 and TGF-β in breast tissue.

The studied subjects were separated into three groups as ~~following~~ follows:

Group I: (n=26) metastatic breast cancer female patients with distant metastasis (bone, liver, and lung).

Group II: (n=44) non-metastatic breast cancer female patients.

Group III: (n=20) healthy adjacent normal tissues of non-metastatic breast cancer cases as a control group.

2.3. Tissue sampling and RT PCR analysis

Total RNA was extracted from breast tissues using Qiagen tissue extraction kit (Qiagen, USA) according to instructions of manufacture. The first strand cDNA was derived from total RNA (0.5µg) and oligo (dT) using the Reverse Transcription System (The QuantiTect Reverse Transcription Kit). For real-time quantitative RT-PCR, gene specific primers listed in (Table 1) and QuantiTect SYBR Green PCR Kit were used. GAPDH was used to normalize the quantity of specific mRNA. The amplification efficiency determined for both target and housekeeping genes was equal. Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

Table 1: List of primers sequences applied in real time-PCR.

Genes	Forward primer	Reverse primer
GAPDH	CAATGACCCCTTCATTGACC	TTGATTTGGAGGGATCTCG
Beclin-1	GGCTGAGAGACTGGATCGG	CTGCGTCTGGGCATAACG
TGF-β	ACATTGACTTCCGCAAGCAC	GTCCAGGCTCCAAATGTAGG

2.4. Statistical analysis:

Statistical analysis of data was performed by Sigma Plot version 12.5 and Graphpad Prism 5. Data were illustrated as mean ± standard deviation. Differences among groups were analyzed by Shapiro-Wilk test. Post-hoc testing was performed by the Tukey test to compare the difference among the groups. Simple linear correlation (Pearson correlation coefficient test) (r) was also done to test for linear relations between beclin-1 and TGF-β with other variables. P-value is

considered significant if < 0.05. Receiver operating characteristics curves (ROC curves) were utilized to assess the diagnostic performance of the studied parameters.

3. RESULTS

3.1 Expression levels of beclin-1 and TGF-β in different clinicopathological factors in patients with breast cancer

The associations between the levels of beclin-1 and TGF-β with clinicopathological factors were analyzed. The levels of beclin-1 and TGF-β were increased with TNM stage ($p < 0.01$) but showed no significant correlation with the other factors (Table 2).

Table (2): Correlation between expression levels of beclin-1 and TGF-β with clinicopathological factors in the malignant groups

Clinicopathological Factors	Malignant patients (70)	Beclin-1 P value	TGF-β P value
Metastasis			
Metastatic	26 (37.1 %)	>0.05	>0.05
Non-metastatic	44 (62.9%)		
Family history			
No family history	54 (77.1%)	>0.05	>0.05
Family history	16 (22.9%)		
Menopause			
Pre-menopause	47 (67.1%)	0.05>	0.05>
Post-menopause	23 (32.9%)		
Site			
Right	35 (50%)	>0.05	>0.05
Left	32 (45.7%)		
Bilateral	3 (4.3%)		
Pathology			
IDC II	58 (82.8%)		
Medullary	1 (1.4%)		
IDC III	6 (8.6%)	>0.05	>0.05
IDC + ILC	3 (4.3%)		
ILC	2 (2.9 %)		
ER sensitivity			
Positive	49 (70%)	>0.05	>0.05
Negative	21 (30%)		
PR sensitivity			
Positive	42 (60%)	>0.05	>0.05
Negative	28 (40%)		
HER sensitivity			
Positive	29 (41.4%)	>0.05	>0.05
Negative	41 (58.6%)		
Molecular diagnosis			
HER 2 enriched	29 (41.4%)	>0.05	>0.05

Triple negative Luminal B Luminal A	11 (15.7 %) 26 (37.15%) 4 (5.75%)		
Stage Stage II Stage III Stage IV	 18 (25.7%) 26 (37.15%) 26 (37.15%)	<0.05**	<0.05**

a* significant from stage II.
ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor 2.

3.2 Expression levels of beclin-1 and TGF-β in patients with breast cancer compared to control group

Expression Level of beclin-1 was highly significant in metastatic malignant group (11±3.6) and non-metastatic malignant group (9.3±4.5) compared to the control group (1.2±.34) with (p value <0.05), as well as expression Level of TGF-β was highly significant in metastatic malignant group (9.5±7.9) and non-metastatic malignant group (9.3±7.7) compared to the control group (1.1±30) with (p value <0.05) (Figure 1).

a* significant from controls

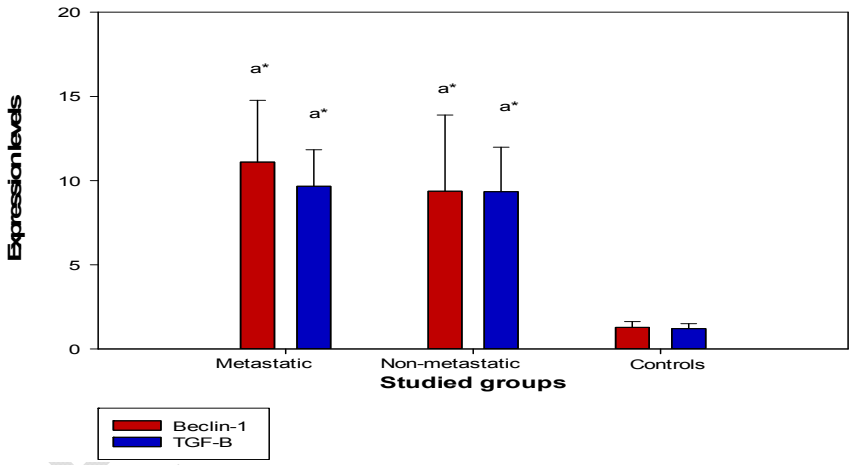

Figure (1): Mean ± SD of beclin-1 and TGF-β expression levels for the studied groups.

3.3 Receiver operating characteristics (ROC) curves

Receiver operating characteristic curves were carried out to assess the diagnostic performance of beclin-1 and TGF-β and their sensitivity and specificity independently or in combination. The best cut-off value for beclin-1 in malignant patients was >1.9 (*P* <0.0001) with 95 % sensitivity and 98.5% specificity producing area under the curve (AUC) 0.997 (Figure 2). For tissue expression of TGF-β in malignant patients; the best cut-off point was >3.98 (*P* <0.001) with 100% sensitivity and 98.5% specificity producing AUC=1 (Figure 3). On the other hand, the best cut-off point for both beclin-1 and TGF-β in combination was >1.9 (*P* <0.001) with 97.5 % sensitivity and 99.2 % specificity and AUC= 0.99 (Figure 4)

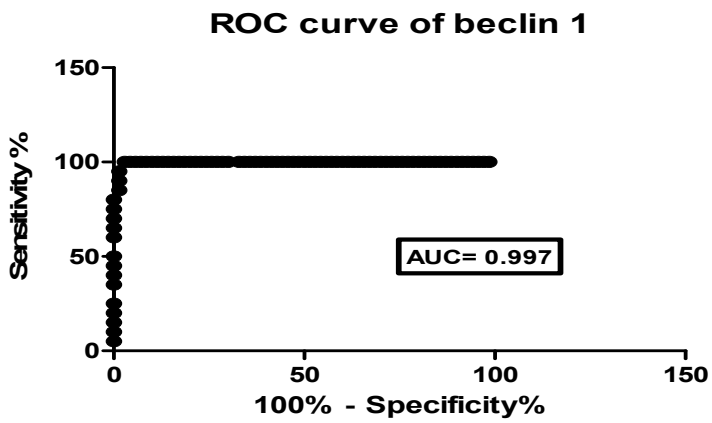

Figure (2): ROC curve of beclin-1.

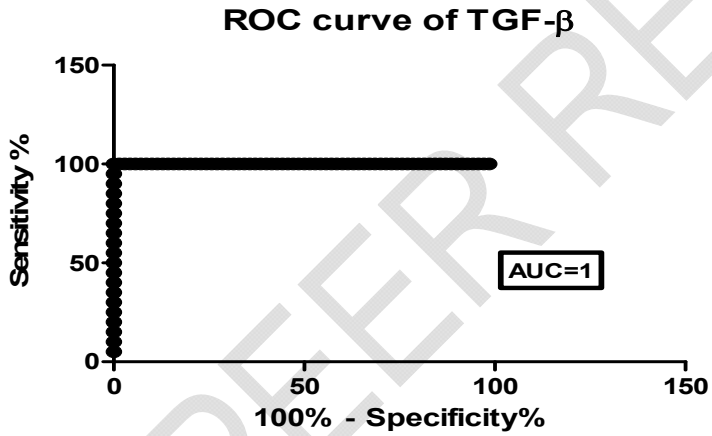

Figure (3): ROC curve of TGF-β.

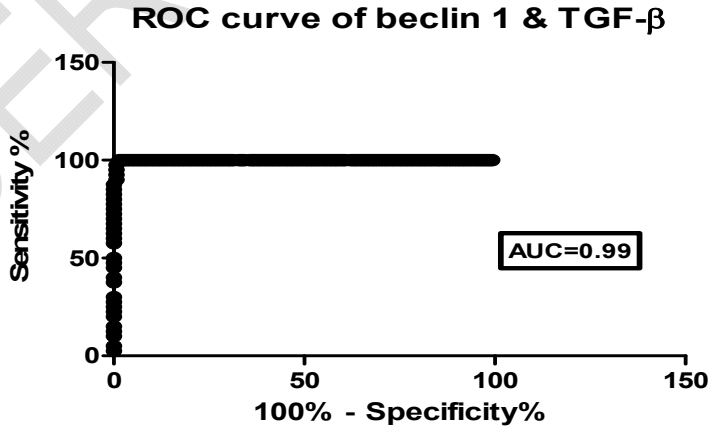

Figure (4): ROC curve of beclin1 and TGF-β.

3.4 Pearson's correlation analysis

There was a significant positive association between expression level of beclin-1 in breast cancer patients and TGF-β (r= 0.241 and *P* < 0.05) (Figure 5).

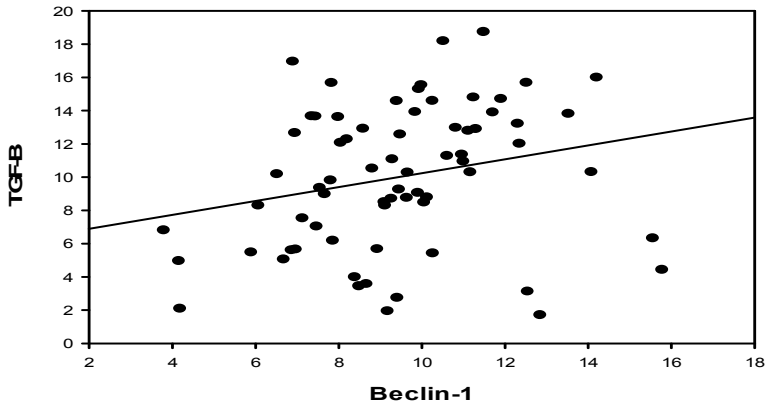

Figure (5): Correlation between beclin-1 and TGF-β expression levels.

4. DISSCUSSION

Autophagy is utilized by ordinary and cancer cells. However, the mechanism of autophagy in different cancers including breast cancer remains not clear in oncogenic/protumorigenic and tumor-suppressor feature [7]. The purpose of our study was to assess expression levels of beclin-1 and TGF-β in breast carcinoma Egyptian women compared ~~with~~ to normal controls and to determine the potential value of beclin-1 and TGF-β as molecular biomarkers for diagnosis of breast cancer in Egyptian women. We found, compared ~~with~~ to the expression in normal breast tissue, that beclin-1 and TGF-β were over-expressed in tumor tissue. Moreover, the over-expression of beclin-1 and TGF-β in breast cancer was considerably associated with TNM stage. These findings may suggest that beclin-1 and TGF-β have direct influence on development and progression of breast carcinoma. In the current study, beclin-1 level was significantly increased in breast cancer groups in comparison to healthy subjects (P value < 0.01). Our results are supported with different previous studies presented by **Hamurcu, et al.,** [8] who have studied the relative concentrations of beclin-1 in the tissue of patients with breast cancer, metastatic disease and healthy women and stated that expression levels of beclin-1 was higher in highly aggressive, metastatic and noninvasive cancer cells compared to non-tumorigenic normal human breast cells. The function of autophagy is exist different opinion in solid cancers including breast cancer [8]. In another study **Wang, et al.,** [9] founded a significant increase of beclin-1 expression in eighteen breast carcinoma patients which was associated with tumor progression. Also, **Park, et al.,** [10] revealed that the expression of beclin-1 was increased in tumor cells relative to normal-appearing and adjacent colonic mucosa in all cases. Similarly, **Chen, et al.,** [11] reported an increased expression of beclin-1 in tumor tissue compared with under-expression in normal gallbladder specimen (p < 0.05). The concept that autophagy represents a mechanism that promotes tumor growth is based on the need of tumor cells to adjust to ischemia in the surroundings that are hypoxic, besides growth factors and supplemental deprivation. Consistent with this aspect, autophagy is activated in hypoxic environment of tumors [12]. Beclin-1 was firstly found to have tumor suppressor role. However, elevated expression of beclin-1 was related with cancer progression in some

carcinoma [13]. In contrast to our study, decreased beclin-1 expression was observed in seventy percent of the breast tumors, and the protein levels were co-related to the mRNA levels [14]. However, **Claude-Taupin, et al.,** [15] reported that no major difference in beclin-1 mRNA expression between tumor and healthy adjacent tissues. The current study revealed that TGF-β was significantly increased in breast carcinoma patients compared to control group (P value< 0.001) and this finding ~~are~~ is affirmed by **Ciftci, et al.,** [16] who have studied the expression level of TGF-β and established that the mean serum TGF-β level of breast cancer patients was considerably higher than controls. There was no considerable difference according to known disease-related clinicopathological parameters. Our finding is supported by **El-Aziz et al.,** [17] who revealed that the level of TGF-β was considerably higher in malignant groups than normal control group with p value (<0.0001). The results of the present study was supported by **Scherer et al.,** [18] who detected the level of TGF-β in breast cancer patients; he found that its level was increased. In the current study, ROC curve of beclin-1 showed 95% sensitivity and 98.5% specificity and this is supported by **Harb, et al.,** [19] who reported that the sensitivity of beclin-1 as a predictor for advanced stage of IDC was 85.5% and the specificity was 98.5%. Also, ROC curve of TGF-β showed 100% sensitivity and 98.5% specificity and this is supported by **El Husseini, et al.,** [20] who reported that 98.2% sensitivity and 100% specificity demonstrating the diagnostic power of this studied marker in differentiating between breast cancer patients and controls. TGF-β is connected with expanded cancer progression, higher cell movement, cancer invasiveness, and metastasis. Furthermore, it is included in cancer surrounding medium alteration and advancement of migration and invasiveness [21]. TGF-β elevated the mRNA levels of beclin-1, and other protein kinase implicated in death process. Moreover, TGF-β evoked autophagy in some mammary carcinoma cell. These findings illustrate that TGF-β signaling pathway activates autophagy in certain human cancer cells and that induction of autophagy is a novel aspect of biological role of TGF-β [22]. Concerning clinicopathological factors, the over-expression of beclin-1 was considerably related with TNM stage (p< 0.05) but it showed no noteworthy association with the other factors. The current results were similar to **Chen, et al.,** [12] **work** who declared that over expression of beclin-1 was essentially related with TNM stage but had no vital relationship with age, sex, lymphatic metastasis, or tumor differentiation. Also, the affiliation between hormonal status and TGF-β expression was examined for both Estrogen receptors (ER) and Progesterone receptors (PR) by **El-Aziz, et al.,** [17] concluding that no noteworthy difference of TGF-β expression neither between ER positive versus ER negative tumors, nor between PR positive and PR negative tumors and this is similar to our data. Concerning the relation between tissue tumor subtypes and TGF-β level, **El-Aziz et al.,** [17] reported no critical difference between TGF-β level and tissue tumor subtypes as we found.

5. CONCLUSION

This study affirmed overexpression of beclin-1 in breast carcinoma tissues and demonstrated that beclin-1 and TGF-β high expression levels were associated with forceful clinical outcomes of breast cancer patients. Also, their overexpression contributed to the tumor development. The present study has a limitation: the sample size was relatively small. Consequently, the present study should be done on large number of breast cancer population to emphasize our results and clarify the diagnostic importance of beclin1 and TGF-β in the process of tumorigenesis.

Ethical approval

The study was approved by the Institutional Review Board (IRB) of the NCI, Cairo University and was conducted according to the rules of Helsinki declaration for human studies.

Consent: A Written informed consent was obtained from all study subjects.

REFER/ENCES

1. Morsy M M, Raafat A, Mohamed M, Fayed A. (2018). Prognostic Value of Percentage of Positive to Total Excised Axillary Lymph Nodes in Egypt with Triple Negative Breast Cancer : Multiple-Centers Experience. Int. J. of Life Sciences, 6(3), 719–732.
2. Arakawa S, Honda H, Yamaguchu H, Shimizu S. (2017). Atg12 Conventional autophagy ATG5-ATG12 LC3-PE Beclin-1 Alternative autophagy Rab-9. Proceedings of the Japan Academy, Series B, 93(6), 378–385.
3. Abraham NM, Kirubel, MM, and Abraham DA. (2018). Autophagy as a Possible Target for Cancer Therapy. Journal of Orthopedic Oncology, 04(01), 1–9. https://doi.org/10.4172/2472-016X.1000124.
4. Morris DH, Yip CK, Shi Y, Chait BT, Wang QJ. (2015). BECLIN 1-VPS34 COMPLEX ARCHITECTURE: UNDERSTANDING THE NUTS AND BOLTS OF THERAPEUTIC TARGETS. Front. Biol. (Beijing, 10(5), 398–426. https://doi.org/10.1038/s41556-018-0061-z.
5. Furler RL, Nixon DF, Brantner CA, Popratiloff A, Uittenbogaart CH. (2018a). TGF-β sustains tumor progression through biochemical and mechanical signal transduction. Cancers, 10(6), 1–18. https://doi.org/10.3390/cancers10060199.
6. Wang ., Gong X, Xu J, Xie R. (2018). The Role of TGF-β in Gastrointestinal Cancers. Journal of Cancer Science & Therapy, 10(11), 345–350. https://doi.org/10.4172/1948-5956.1000566.
7. Mizushima N. (2017). The exponential growth of autophagy-related research: from the humble yeast to the Nobel Prize. FEBS Letters, 591(5), 681–689. https://doi.org/10.1002/1873-3468.12594.
8. Hamurcu Z, Delibaşı N, Geçene S, Şener EF, Dönmez-Altuntaş H, Özkul Y,et al, (2018). Targeting LC3 and Beclin-1 autophagy genes suppresses proliferation, survival, migration and invasion by inhibition of Cyclin-D1 and uPAR/Integrin β1/ Src signaling in triple negative breast cancer cells. Journal of Cancer Research and Clinical Oncology, 144(3), 415–430. https://doi.org/10.1007/s00432-017-2557-5.
9. Wang MC, Wu AG, Huang Y, Shao, GL, Ji SF, Wang RW, et al, (2015). Autophagic regulation of cell growth by altered expression of Beclin 1 in triple-negative breast cancer. International Journal of Clinical and Experimental Medicine, 8(5), 7049–7058.
10. Park JM, Huang S, Wu TT, Foster NR, Sinicrope FA (2013). Prognostic impact of Beclin 1, p62/sequestosome 1 and LC3 protein expression in colon carcinomas from patients receiving 5-fluorouracil as adjuvant chemotherapy. Cancer Biology and Therapy, 14(2), 100–107. https://doi.org/10.4161/cbt.22954.
11. Chen Y, Yan J, Yu S, Wang X, Zheng Q. (2014). Over-expression of beclin-1 in gallbladder carcinoma and its relationship with prognosis. Wspolczesna Onkologia, 18(3), 171–176. https://doi.org/10.5114/wo.2014.41395.
12. Ávalos Y, Canales J, Bravo-Sagua R, Criollo A, Lavandero S, Quest AFG,et al, (2014). Tumor Suppression and Promotion by Autophagy. BioMed Research International, 2014, 1–15. https://doi.org/10.1155/2014/603980.
13. Parkhitko A, Myachina F, Morrison TA, Hindi KM, Auricchio N, Karbowniczek M, Henske EP. (2011). Tumorigenesis in tuberous sclerosis complex is autophagy and p62/sequestosome 1 (SQSTM1)-dependent. Proceedings of the National Academy of Sciences, 108(30), 12455–12460. https://doi.org/10.1073/pnas.1104361108.
14. Li Z, Chen B, Wu Y, Jin F, Xia Y, Liu, X. (2010). Genetic and epigenetic silencing of the beclin 1 gene in sporadic breast tumors. BMC Cancer, 10(98), 1–12. https://doi.org/10.1186/1471-2407-10-98.
15. Claude-Taupin A, Fonderflick L, Gauthier T, Mansi L, Pallandre JR, Borg et al, (2018). ATG9A Is Overexpressed in Triple Negative Breast Cancer and Its In Vitro Extinction Leads to the Inhibition of Pro-Cancer Phenotypes. Cells, 7(12), 1–17. https://doi.org/10.3390/cells7120248.
16. Ciftci R, Tas F, Yasasever CT, Aksit E, Karabulut S, Sen F, et al, (2014). Clinical significance of serum transforming growth factor beta 1 (TGFB1) level in breast cancer. Journal of Clinical Oncology, 32(15), e11526. https://doi.org/10.1200/jco.2014.32.15.
17. El-Aziz G, Kamel MM, Alkaffas M, Abdelhady EG, Rashed LA. (2018). Can Transforming Growth Factor Beta Affect Breast Cancer by Targeting MicroRNA 195? J Mol Cell Biochem, 2(1), 1–4. Retrieved from http://www.imedpub.com/journal-molecular-cellular-biochemistry.
18. Scherer SD, Bauer J, Schmaus A, Neumaier C, Herskind C, Veldwijk MR, Sleeman JP. (2016). TGF-β1 is present at high levels in wound fluid from breast cancer patients immediately post-surgery, and is not increased by intraoperative radiation therapy (IORT). PLoS ONE, 11(9), 1–14. https://doi.org/10.1371/journal.pone.0162221.
19. Harb OA, Salem AA., Haggag R, El-shorbagy S, Gertallah LM. (2016). Immunohistochemical expressions of

SQSTM1 / p62 , Beclin-1 , and SOX4 in infiltrating duct carcinoma of the breast. Egyptian Journal of Pathology, 62, 95–103. https://doi.org/10.1097/01.XEJ.0000484380.44411.44.

20. EL-Husseini M, Hussein F, Bassily N, Abdelghany B. (2013). Clinical significance of TGF alpha , TGF beta1 and VEGF in Sera of Egyptian patients with breast cancer Clinical Significance of TGF Alpha , TGF Beta1 and VEGF in Sera of Egyptian Patients with Breast Cancer Abstract : The Egyptian Journal of Hospital Medicine July, 52, 555–565. https://doi.org/10.12816/0000592.
21. Zarzynska J. (2014). Two faces of TGF-beta1 in breast cancer. Mediators of Inflammation, 2014, 141747. https://doi.org/10.1155/2014/14174.
22. Kiyono K, Suzuki H, Matsuyama H, Morishita Y, Komuro A, Kano MR, et al, (2009). Autophagy is activated by TGF-beta and potentiates TGF-beta-mediated growth inhibition in human hepatocellular carcinoma cells. Cancer Reseach, 69(23), 8844–8852.