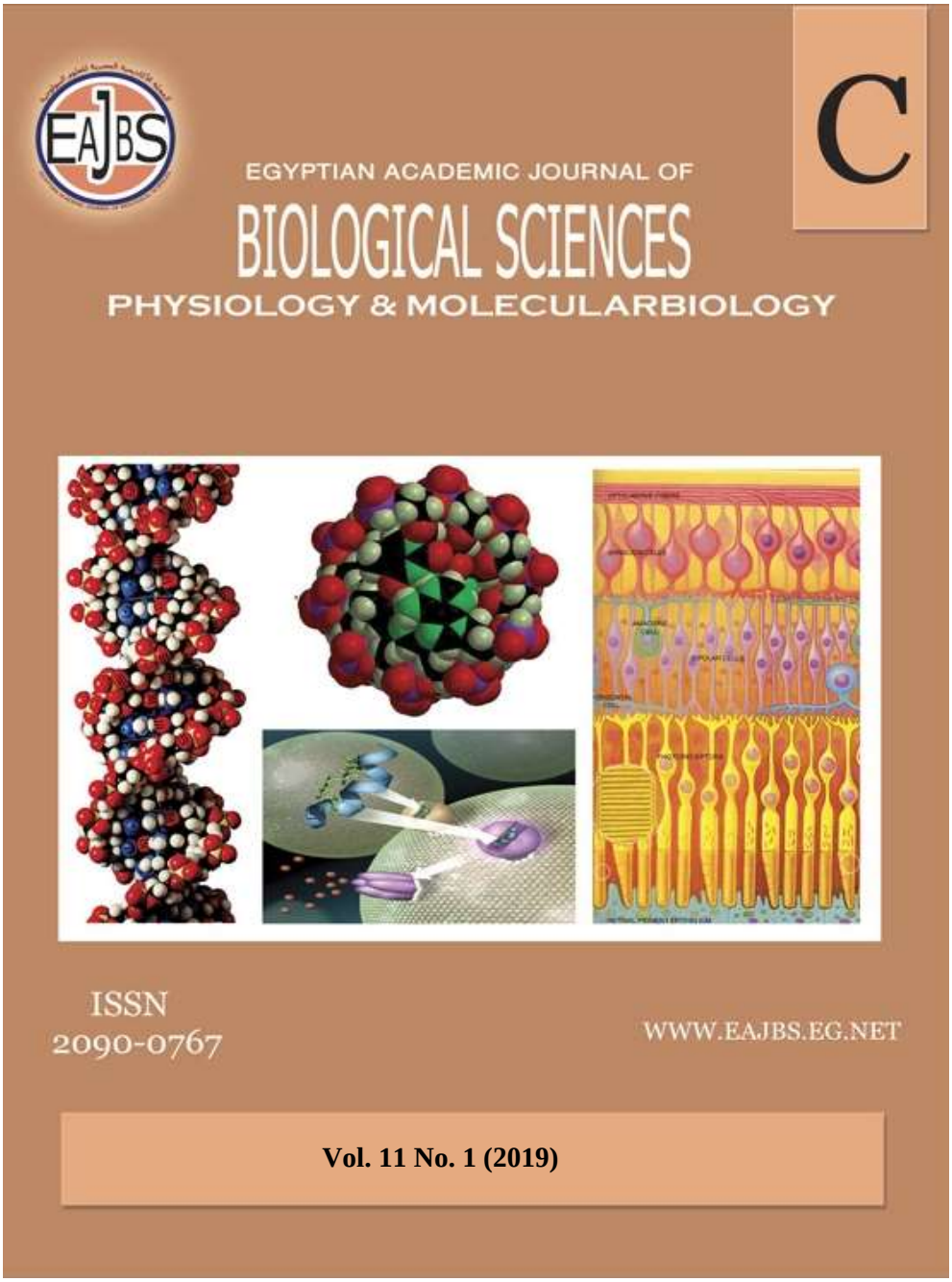

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Cathepsin L is A Potential Marker for Triple-Negative Breast Cancer

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ABSTRACT

. Breast cancer is the second cancer-related death among women worldwide. Triple-negative breast cancer (TNBC) is the most aggressive and lethal subtype, which is associated with high metastasis and poor prognosis. Cathepsins, a family of lysosomal cysteine proteases, such as cathepsin L (*CTSL*), are involved in cancer invasion and metastasis. Thus, *CTSL* may emerge as a marker for TNBC. So, we characterized the expression of *CTSL* mRNA in tissue specimen of TNBC (n = 10) and non-TNBC (non-TN) (n = 10) using quantitative real-time PCR. Data were statistically analyzed using Mann-Whitney U-test. Our data demonstrate that *CTSL* mRNA expression was up-regulated in TN BC vs. non-TN BC patients. In conclusion, the high expression of *CTSL* may represent a marker for TNBC and its targeting could have therapeutic implications.

Introduction

Breast cancer is the second leading cause of cancer-related death among females worldwide (DeSantis, Ma et al. 2014). Approximately 1.7 million of diagnosed breast cancer resulted in corresponding 522,000 death (Tao, Shi et al. 2015). In Egypt, Breast cancer makes up 32% of all newly diagnosed cancer among females (Ibrahim, Khaled et al. 2014). Notably, breast cancer has different molecular subtypes based on the expression of the hormone receptors (HR), such as the estrogen receptor (ER) and progesterone receptor (PR), and the human epidermal growth factor receptor (HER2) (Li, Gonzalez-Angulo et al. 2011, Biswas, Efird et al. 2016). There are four main subtypes of breast cancer, which are defined as: luminal A (ER-positive and/or PR-positive and HER2-negative), luminal B (ER-positive and/or PR-positive and HER2-positive), HER2-positive (ER-negative, PR-negative and HER2-positive), and triple-negative (ER-negative, PR-negative and HER2-negative) (Lambertini, Santoro et al. 2016).

Triple-negative breast cancer (TNBC) represent 11.3% among Egyptian women with breast cancer (Salhia, Tapia et al. 2011). TNBC is the most aggressive subtype (Salhia, Tapia et al. 2011), because it presents with a higher histological grade and proliferation rate than other subtypes. It is associated with poor prognosis (earlier and higher risk of relapse and decreased overall survival) and shows a higher prevalence of germline BRCA mutation (Chikarmane, Tirumani et al. 2015, Sharma 2018). Moreover, we and others showed enrichment of TNBC with the cancer stem cell properties, thereby leading to cancer metastasis and relapse (Ibrahim, Gadalla et al. 2017). In spite of the extensive studies to understand the etiology of breast cancer (Duke, Jahed et al. 2010), the molecular mechanisms underlying the aggressiveness of TNBC is yet fully unexplored. Owing to lacking of therapeutic targets, TNBC is the most complex and deadly subtype of breast cancer, whereby chemotherapy and/or radiotherapy are the only the available treatment options (Lee and Djamgoz 2018). Therefore, deciphering new molecular targets for TNBC is highly warranted.

One of the molecular factors that are implicated in metastasis and invasion of tumor cells are proteases (Goel and Chauhan 1997). Cathepsins are a ubiquitously lysosomal cysteine proteinase belongs to the papain subfamily of cysteine proteases1 and exhibits endopeptidase activity (Mohamed and Sloane 2006). There are 16 members of cathepsins in humans (Sun, Jiang et al. 2016). Under normal physiological conditions, it plays an important role in degradation, processing and turnover of intracellular and secreted proteins (Cui, Wang et al. 2016). Their expression has been shown to be upregulated in different types of human cancers (Jedeszko and Sloane 2004). Elevated levels of Cathepsin L (*CTSL*) expression are reported in ovarian cancer (Sui, Shi et al. 2016) and breast carcinoma (Sudhan and Siemann 2015). Overexpression of *CTSL* occurs not only in tumor cells but also in tumor-associated cells such as macrophages and endothelial cells (Mohamed and Sloane 2006). *CTSL* participates in tumor metastasis and invasion through its activation of latent pro-forms of other key metastasis-related enzymes such as Heparanase (Sudhan and Siemann 2015). There is one study showed increased levels of nuclear *CTSL* as a novel biomarker for subsets of TNBC patients (Grotsky, Gonzalez-Suarez et al. 2013). The aim of the present study was to determine the expression levels of *CTSL* mRNA in carcinoma tissues of TNBC vs. non-TNBC patients by qPCR.

MATERIAL AND METHODS

Patients' Samples:

For patient recruitment, Institutional Review Board (IRB) approval was obtained from the ethics committee of Ain-Shams University, Cairo, Egypt. Dr. Mohamed Shinawi (co-author) enrolled 20 breast cancer patients from the breast clinic of Ain Shams university hospitals. According to molecular subtypes, breast cancer patients were divided into 2 subgroups; triple negative (TN) and non-triple negative (non-TN) patients. One part of the fresh carcinoma tissues was fixed in 10% PBS-formalin buffered solution for immunohistochemical staining and the other part was lysed for isolation of the total RNA.

Quantitative Real-Time PCR:

According to the manufacturer instructions, total RNA was isolated using GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA) and 1 µg RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific,, CA, USA). Quantitative real-time PCR and melting curve analysis of *CTSL mRNA expression* gene was performed using SYBR™ Green PCR Master Mix (Applied Biosystems, USA) and StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA). Each sample was initially denatured at 95˚C for 10 min, then subjected to 40 cycles of the following: Denaturation at 95˚C for 15 sec, annealing at 55 ˚C for 30 sec and extension at 60˚C for 30 min. Melting curve analysis and 2% agarose gel electrophoresis of the PCR products was used to verify the product specificity. After normalization to GAPDH,, the $2^{-\Delta\Delta Ct}$ method was used to determine relative gene transcript levels (Livak and Schmittgen 2001). *CTSL* and GAPDH primers were purchased from Qiagen (Hilden, Germany).

Statistical Analysis:

Results were analyzed using SPSS (SPSS, Chicago, IL, USA), version 15.0. Differences among variables were evaluated using Fischer's exact tests. Mann-Whitney U-test (for non-normally distributed data) was used for two group comparisons. Unless otherwise stated, all data are expressed as mean ± SEM. The level of significance was set at $p < 0.05$.

RESULTS

Clinical and Pathological Characteristics of Patients:

Clinical and pathological characteristics of patients enrolled in the present study are described in Table 1. TNBC patients (n =10) were with an average age of 46 ± 11.85 years (ranging from 27 to 65 years) and non-TNBC (n =10) patients with an average age of 56.6 ± 11.95 years (ranging from 38 to 73 years). Measurements of the tumor size showed that 44.44% of TNBC patients had a tumor size ≤ 4 cm and 55.56% had a tumor > 4 cm, while in the non-TNBC group 71.43% of the patients had a tumor size ≤ 4 and 28.57% had a tumor > 4. The histological tumor grade revealed that 100% were diagnosed as tumor grade 2 (G2) in TNBC and were classified as 12.5% were tumor grade 1 (G1), 75% were tumor grade 2 (G2) and 12.5% were tumor grade 3 (G3) in non-TNBC. All TNBC patients who underwent surgery were lymph nodes metastasis positive: 88.89% had <4 positive lymph nodes and 11.11% had ≥ 4 positive metastatic lymph nodes while in non-TNBC patients, 42.86% had < 4 lymph nodes and 57.14% had ≥ 4 positive lymph nodes. Pathological examination of tissue sections revealed that lymphovascular invasion was positive in 10% of TNBC and in 42.86% of non-TNBC patients.

Table 1. Clinical and pathological data of IBC and non-IBC patients

Characteristic	TN BC (*N* = 10)	Non-TN BC (*N* = 10)	*P* value
Age (years)			
Range	27-65	38-73	0.06[a]
Mean ± SD	46 ± 11.85	56.6 ± 11.95	
Tumor size (cm)			
≤ 4	4 (44.44%)	5 (71.43%)	0.36[b]
> 4	5 (55.56%)	2 (28.57%)	
NA	1	3	
Lymph node status, n (%)			
< 4	8 (88.89%)	3 (42.86%)	0.11[b]
≥ 4	1 (11.11%)	4 (57.14%)	
NA	1	3	
Tumor grade, n (%)			
G1	0	1 (12.5%)	0.18[b]
G2	10 (100%)	6 (75%)	
G3	0	1 (12.5%)	
NA	0	2	
Lymphovascular invasion,n (%)			
Negative	9 (90%)	4 (57.14%)	0.25[b]
Positive	1 (10%)	3 (42.86%)	
NA	0	3	
ER, n (%)			
Negative	10 (100%)	0	0.000*[b]
Positive	0	10 (100%)	
PR, n (%)			
Negative	10 (100%)	3 (30%)	0.000*[b]
Positive	0	7 (70%)	
HER-2, n (%)			
Negative	10 (100%)	8 (80%)	0.000*[b]
Positive	0	2 (20%)	

Data are expressed as mean ± SD
NA Data not available
*significant P value calculated by [a]Student's t-test or [b]Pearson Chi-Square

A higher Expression of *CTSL* mRNA in Tissues of TNBC Than in Non-TNBC Patients:

The mRNA expression level of *CTSL* in carcinoma tissues of TNBC (n = 10) vs. non-TNBC (n = 10) patients was assessed using qRT-PCR. We found that there was a significant (*P* = 0.02) increase of *CTSL* mRNA expression by 7.2-fold in carcinoma tissues of TNBC than in non-TNBC patients (Fig. 1, and Fig. 2a & 2b). However, no significant correlation between *CTSL* expression and patients' clinic-pathological data has been detected

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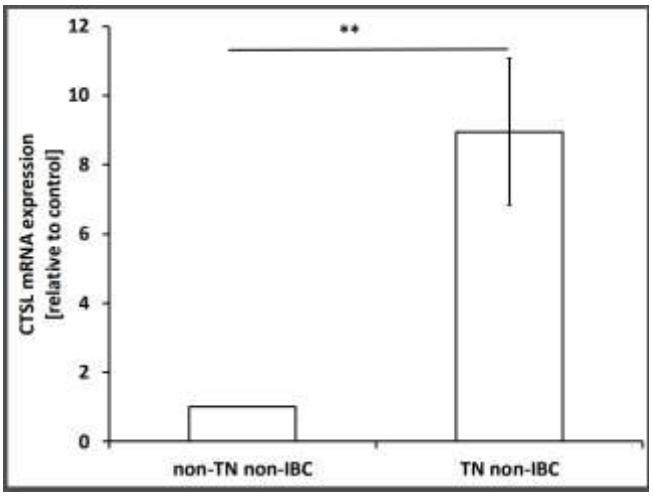

Fig.1 Expression of CTSL mRNA in carcinoma tissues of TN BC *vs.* non-TN BC patients by using qPCR. The expression of CTSL was up-regulated in TN (n = 10) relative to non-TN patients (n = 10) (8.95 fold). ** P < 0.01 as determined by Mann-Whitney U-test.

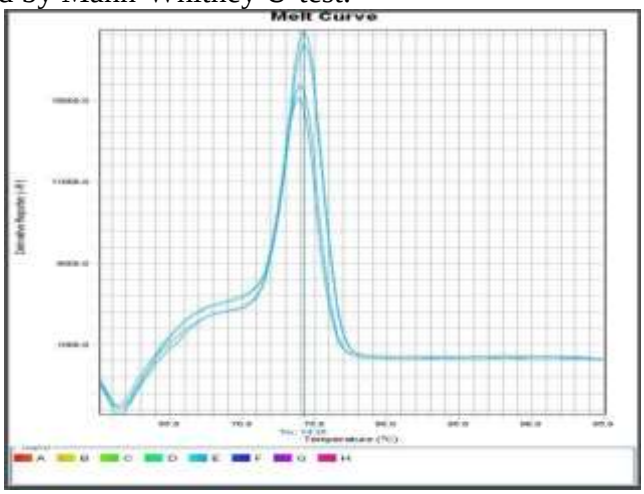

Fig.2a Representative graph of the raw data analysis showing CTSL amplification plots of BC patients (n = 4).

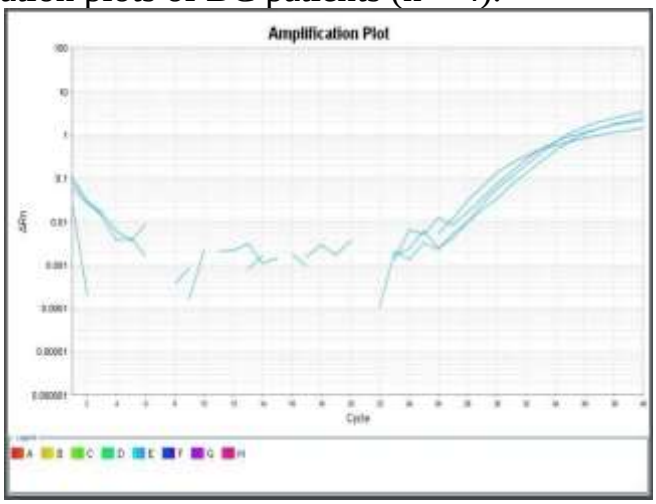

Fig.2b Representative graph of the raw data analysis showing CTSL melt curves of BC patients (n = 4).

DISCUSSION

In this study, we demonstrate a higher expression of *CTSL* mRNA level in carcinoma tissues of TNBC vs. non-TNBC. The high expression of CTSL has been assigned in different tumors whereby it modulates cancer invasiveness and metastasis (Mohamed and Sloane 2006). Therefore, *CTSL* may emerge as a biomarker and therapeutic target for TNBC. Our findings are in agreement with a study showed that nuclear *CTSL* is considered as a positive biomarker for TNBC (Grotsky, Gonzalez-Suarez et al. 2013) and CTSL level in serum may be a marker for invasion and metastasis in ovarian cancer (Sui, Shi et al. 2016). It is well established that TNBC is characterized by frequent somatic alterations of BRCA1 function and BRCA1-deficient cells activate *CTSL* through alterations in chromatin structure that either enhances the transcription of *CTSL* and/or alter the stability of *CTSL* mRNAs. This ultimately leads to bypass growth arrest via degradation of the DNA repair factor 53BP1 (Grotsky, Gonzalez-Suarez et al. 2013). The mesenchymal protein expression, namely Snail, vimentin and *CTSL*, was elevated in the TNBC compared to non-TNBC cells and that expression was functionally associated with the higher proliferative and migratory phenotype (Burton, Hawsawi et al. 2018). This observation was further confirmed in the clinical specimen of TNBC tissue but not in luminal A cancer tissue of African Americans as compared to Caucasian Americans patients (Burton, Hawsawi et al. 2018). Moreover, overexpression of Snail in the ER-positive MCF-7 cells resulted in enhanced migration and invasion via increased CTSL activity-mediated STAT-3 signaling (Burton, Smith et al. 2015).Of note, inhibition of nuclear localization of both *CTSL* and its substrate the transcription factor CCAAT-displacement protein/cut homeobox transcription factor (Cux1) was observed upon Snail and importin β1 silencing in MCF-7 cells (Burton, Henderson et al. 2017). Interestingly, treatment of the mesenchymal cells with the CTSL inhibitor Z-FY-CHO resulted in relocalization of *CTSL* from nucleus to cytoplasm, transition into epithelial cells, and decreased cell migration/invasion (Burton, Dougan et al. 2017, Burton, Henderson et al. 2017). In an immunohistochemical study of 188 breast cancer tissues, expression of *CTSL* has been shown to be associated with advanced cancer stages (Sun, Jiang et al. 2016). However, this correlation was not observed in our study. This could be attributed to the difference in characteristics of our collectives and to the low number of the enrolled patients. Overall, we suggest that the high expression of *CTSL* may act as a biomarker for TNBC patients and its targeting may represent a therapeutic strategy. However, our findings should be verified in a large group of breast cancer patients.

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ARABIC SUMMERY

كاثبسين إل يُعد دلالة محتملة لسرطان الثدي السالب الثلاثي

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إن سرطان الثدي يعد أكثر ثاني أنواع السرطان المسببة للموت بين النساء في العالم بينما سرطان الثدي السالب الثلاثي هو أخطر أنواع سرطان الثدي وأكثرها عدوانية حيث إنه يمتاز بالانتشار السريع بين أنسجة الجسم. إن من أهم الإنزيمات التي لها دور فعال في انتشار الخلايا السرطانية هي عائلة الكاثبسينات ومنها كاثبسين إل الذي نفترض أنه يمكن أن يكون له دلالة مميزة لسرطان الثدي السالب الثلاثي لذلك قمنا بقياس تعبير الكاثبسين إل على مستوى الحمض النووي الريبوزي الرسول في سرطان الثدي السالب الثلاثي مقارنة بمثيله غير السالب الثلاثي وذلك باستخدام تفاعل البلمرة المتسلسل اللحظي ووجدنا أن تعبير الكاثبسين إل أعلى ف سرطان الثدي السالب الثلاثي عن غير السالب الثلاثي على مستوى الحمض النووي الريبوزي الرسول لذلك تعبير الكاثبسين إل المرتفع ف سرطان الثدي السالب الثلاثي يعتبر علامة مميزة له ويمكن استخدامه في التطبيقات العلاجية.