



## Characterization of The Surgical Leakage Collected After Breast Cancer Surgery and Studying Their Effect on Breast Cancer Cell Line

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### ABSTRACT

Globally, breast cancer is the most common cancer in women and the second leading cause of death. The choice of breast cancer treatment is based on its clinical staging. Although there is a development in the adjuvant of systemic treatments, surgery is a primary choice for treatment. One of those complications is surgical leakage which is suggested to have many cell growth factors, cytokines, and chemokines in order to tissue repair process. Many studies reported that surgical leakage may have a role in tumor progression and local recurrence by inducing the lasting cancer cells. Invasion and metastasis are one of several cellular mechanisms in cancer development and progression through secretions of many inflammatory mediators. For example, TNF- $\alpha$  is one of the inflammatory mediators that up-regulates the expression and secretion of proteases (such as matrix metalloproteinases MMPs family) which are involved in carcinoma cell motility, invasion, and metastasis. Indeed the role of TNF- $\alpha$  in cancer progression and metastasis is depending on its ability to produce and stimulate (MMP) which have a role in tumor angiogenesis, invasion and metastasis process. Also one of TNF- $\alpha$  function is to induce epithelial-mesenchymal transition (EMT) process which known to occur through embryogenesis, metastasis. Previous studies by the authors showed that TNF- $\alpha$  is highly expressed and secreted by tumor microenvironment by Egyptian cancer patients. Using different concentrations of recombinant TNF- $\alpha$ , we test the morphological changes of cancer cells from a round shape to spindle shape (migratory cells), in addition, we studied how different concentrations of TNF- $\alpha$  induce invasion and migration on MDA-MB-231 breast cancer cell line using Matrigel Invasion chambers.

### INTRODUCTION

Breast cancer is considered the most common cancer among women and the second leading cause of death worldwide after lung cancer (Momenimovahed and Salehiniya 2019). According to the 2018 World Health Organization (WHO) estimates, breast cancer accounts for 23% of all cancer cases and about 627,000 women died which represents approximately 15% of all cancer deaths among women(Tran *et al.* 2019).

The choice of breast cancer treatment is based on its clinical staging (Stoyanov *et al.* 2017). Although there is a development in the adjuvant of systemic treatments, surgery is a primary choice for treatment. There are pieces of evidence suggest that surgery may change the tumor microenvironment due to complications resulted after surgery. One of those complications is surgical leakage. This surgical leakage is suggested to have many cell growth factors, cytokines, and chemokines in order to tissue repair process (Ramolu *et al.*, 2014). Many studies reported that surgical leakage may have a role in tumor progression and local recurrence by inducing the lasting cancer cells (Belletti *et al.*, 2008). Invasion and metastasis are one of several cellular actions in cancer development and progression (Wolczyk *et al.*, 2016). Cancer cells change from a round shape to spindle shape before motility and invasion. Many inflammatory cytokines are included in the initiate on of invasion and metastasis process and regulate the secretion of various proteolytic enzymes such as cCysteine cathepsins and matrix metalloproteases (Mohamed *et al.*, 2014). Those proteolytic enzymes also are known as proteases that digest the components of the extracellular matrix (ECM) and the cell surface proteins (Oxford, Reeck, and Hardy 2019). One of the inflammatory cytokines that upregulate the production of proteases that are involved in the invasion and metastasis in cancer is TNF- $\alpha$ . (Ji *et al.*, 2014) TNF- $\alpha$  is an inflammatory cytokine produced by immune cells such as macrophages and lymphocytes and by non-immune cells such as tumor cells. According to the TNF name, the tumor necrosis factor has the ability to lyse certain types of cells especially tumor cells (Granger *et al.*, 1969). TNF- $\alpha$  has different cellular functions involving apoptotic activity and stimulates

survival, proliferation, angiogenesis, invasion, and migration of cancer cells. TNF- $\alpha$  has two receptors TNFR1 and TNFR2 through binding with them; it can regulate the signaling of cellular responses (Sirotković-Skerlev, Čačev, and Kapitanovic 2006). TNFR1 is more distributed on mammalian cells than TNFR2 and considered as the major functional receptor for TNF- $\alpha$  (Sirotković-Skerlev, Čačev, and Kapitanovic 2006). The role of TNF- $\alpha$  in cancer progression and metastasis is depending on its ability to produce and stimulate MMPs which have a role in tumor angiogenesis, invasion and metastasis process through regulate cell-matrix composition. Numerous MMPs such as MMP-9 and -2 plays an important role in breast cancer invasion and are associated with low survival and prognosis (Hagemann *et al.*, 2004). They also have the ability to degrade the basement membrane components such as Laminin, IV collagen and elastin. Another role of TNF- $\alpha$  is to induce the epithelial-mesenchymal transition (EMT) process which known to occur through embryogenesis, metastasis (Yu *et al.*, 2014). EMT cells have been known to change their morphology from epithelial to mesenchyme shape and digest the ECM to be able to invade to distance tissues through regulation of various cytokines, suggesting that different cytokines may have a role to induce EMT (Liao *et al.* 2019). To test whether TNF- $\alpha$  causes morphological changes and invasion of breast cancer cells; we stimulated MDA-MB-231 breast cancer cells with TNF- $\alpha$  and assessed changes in cell migration afterward.

#### **MATERIALS AND METHOD**

Unless otherwise stated, all tissue culture reagents (media and FBS) and supplies were from (Lonza, A.A., Maryland, USA).

#### **Patients and Samples Collection:**

A total of 10 patients who underwent breast cancer surgery was

enrolled in the present study. Institutional Review Board (IRB) approval was obtained (IRB#0006379) and the protocol was approved by the ethics committee of Ain Shams University (Cairo, Egypt). Patient enrollment in our study was agreed to publish the data and was determined by the breast cancer multidisciplinary team at Ain Shams University as patients were diagnosed by clinical examination, ultrasound, mammography and confirmed by tru-cut biopsy. The surgical leakage in the site of surgery was collected within the first 24 hrs of the surgery in sterile tubes, filtered, and stored at  $-80^{\circ}\text{C}$  for further experiments.

#### **Cytokine Profiling Of The Leakage Collected After Breast Cancer Surgery:**

RayBio™ human cytokine antibody array 3 (AAH-CYT-3) that simultaneously detects 42 cytokines was used to characterize the composition of the surgical leakage collected after breast cancer surgery. Methods were conducted according to the manual guidelines. Briefly, a blocking buffer was added to the arrays with shackling gently for two hrs at room temperature. The leakage arrays were incubated overnight with the leakage at  $4^{\circ}\text{C}$  with gentle shaking. After overnight incubation, the membranes signals were detected by using the chemiluminescence detection reagent provided with the kit. Signal intensity values representing detected cytokines were subtracted from the background and normalized to positive controls on the same membrane using ImageJ software (National Institutes of Health, MD, USA) as described by the authors before (El-Ghonaimy *et al.*, 2016).

#### **Cell Culture:**

MDA-MB-231 breast cancer cell lines represent triple negative cell lines were a gift from Professor B.F. Sloane (Wayne State University, Detroit, MI, USA). MDA-MB-231 cells were cultured in Dulbecco's modified

Eagle's medium (DMEM) supplemented with 10% FBS, 1% glutamine and 1% antibiotics (penicillin/streptomycin) in a humidified atmosphere of 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

#### **Cell Growth and Stimulation with TNF- $\alpha$ :**

MDA-MB-231 cells were seeded in 2D culture using DMEM and 10% FBS in T75 flasks. When cells reached confluence of 80%, the media was removed, and the cells washed with warm PBS. After that, the cells were seeded in DMEM media supplemented with 1% FBS for control cells or with different concentrations of TNF- $\alpha$  10 and 20 ng/ml. after 48 hrs of incubation, a microscopic examination was conducted to examine the effect of TNF- $\alpha$  on the morphology of breast cancer cells.

#### **Invasion Assay:**

To test whether media conditioned by different concentrations of TNF- $\alpha$  promotes the invasion of MDA-MB-231, we conduct invasion assay using BD Bio-Coat Matrigel™ Invasion Chambers (Becton Dickinson Labware, Franklin Lakes, NJ, USA). MDA-MB-231 cells were seeded in the upper chamber with DMEM serum-free media at a density of  $3 \times 10^3$  cells. In the lower chambers, DMEM media was conditioned with TNF- $\alpha$  with a concentration of 10 ng/ml and 20 ng/ml and 1% FBS and incubated for 24 hr. After incubation, cells were fixed (cold 10% methanol) and non-invasive cells settled in the upper side of the filter were removed with cotton swabs. The migrated cells at the lower side of the filter were stained with stained Diff-Quik Diagnostic Diffusion Kit (DDK- Italia, vigevano, Italy) and counted using a Zeiss Axiovert microscope (Zeiss, Jena, Germany).

## **RESULTS**

### **Patient Clinical and Pathological Characteristics:**

Clinical and pathological characterizations of the breast cancer

patients who contributed to the study are: age of the patients ranged from 29-63. Tumor size valuation showed that patients who had tumor sizes  $\leq 4$  cm were 6 and who had tumor sizes  $>4$  cm were 3 and 1 is unavailable. A number of lymph nodes showed that patients had lymph nodes  $\leq 4$  were 6 and 4 patients  $>4$ . Tumor grade showed that 7 patients were diagnosed as tumor grade II, 2 patients diagnosed as tumor grade III and one was unavailable. 4 patients diagnosed as positive presences of tumor emboli and 1 was unavailable.

Hormonal and HER2 studies showed that patients diagnosed with ER-negative were 2 and 8 were diagnosed with ER-positive. In contrast, patients diagnosed with PR negative were 2 and 8 were diagnosis with PR. Finally, HER2 expression study showed that 6 patients were diagnosed with HER2 negative and 4 were diagnosed with HER2 positive.

#### **The Panel of The Cytokines of Collected from Surgical Leakage After Breast Cancer Surgery:**

The surgical leakage collected from patients who underwent breast cancer surgery was subjected to profiling using RayBio™ human cytokine antibody array 3 that detects 42 cytokines. Differences in the secreted cytokines/chemokines were assessed (Fig. 1A). The results showed that cytokines, chemokines and growth factors secreted with low level were: MDC, IL-13, SDF-1, GM-CSF, VEGF-A, IL-12, INF gamma, IL-3,

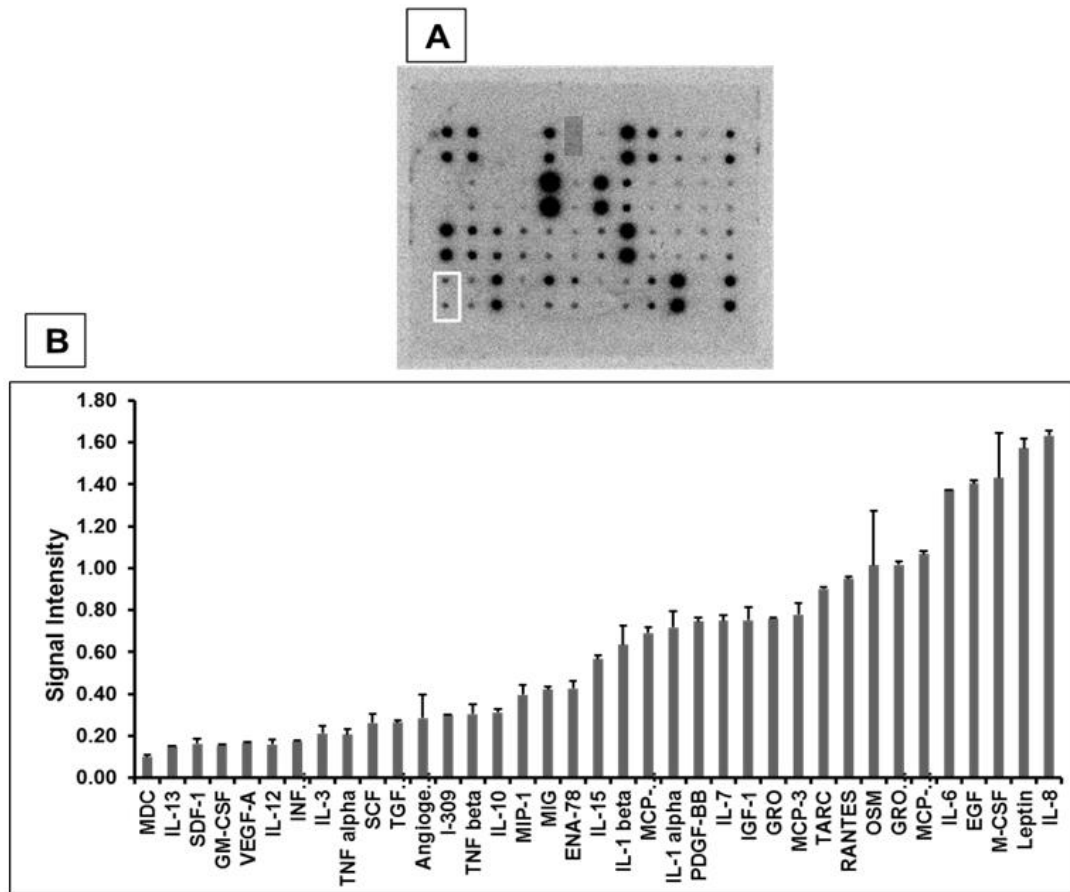
TNF alpha, SCF, TGF beta 1, Angiogenin, I-309, TNF beta, IL-10, MIP-1, MIG, ENA-78. Cytokines, chemokines and growth factors secreted with moderate levels were: IL-15, IL-1 beta, MCP-2(CCL8), IL-1 alpha, PDGF-BB, IL-7, IGF-1, GRO, MCP-3. While cytokines and chemokine secreted with high level were: TARC, RANTES, OSM, GRO alpha, MCP-1(CCL2), IL-6, EGF, M-CSF, Leptin, IL-8 (Fig. 2A).

#### **TNF- $\alpha$ One of The Detected Cytokine in The Surgical Leakage Changes the Morphology of MDA-MB-231 Cells:**

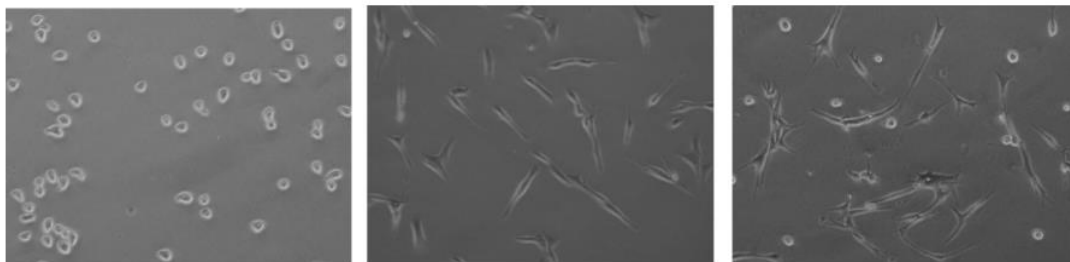
We found that the morphology of MDA-MB-231 cells stimulated with different concentrations of TNF- $\alpha$  (10 mg/ml and 20mg/ml) was changed to be mesenchymal-spindel shape compared to control cells (Fig.2).

#### **TNF- $\alpha$ Enhances the Invasion of MDA-MB-231 Cells by Using BD-Invasion Chambers:**

BD-invasion chambers coated with the reconstituted basement membrane that represents the in-vivo tumor microenvironment were used to test whether TNF- $\alpha$  may enhance the invasion of breast cancer cells. The present results showed that the invasion of MDA-MB-231 cells treated without/with different concentrations of TNF- $\alpha$  (10 mg/ml and 20mg/ml) is significantly increased (by  $P < 0.009$ ,  $P < 0.005$  respectively) compared to control cells (Figs.3 A and B).

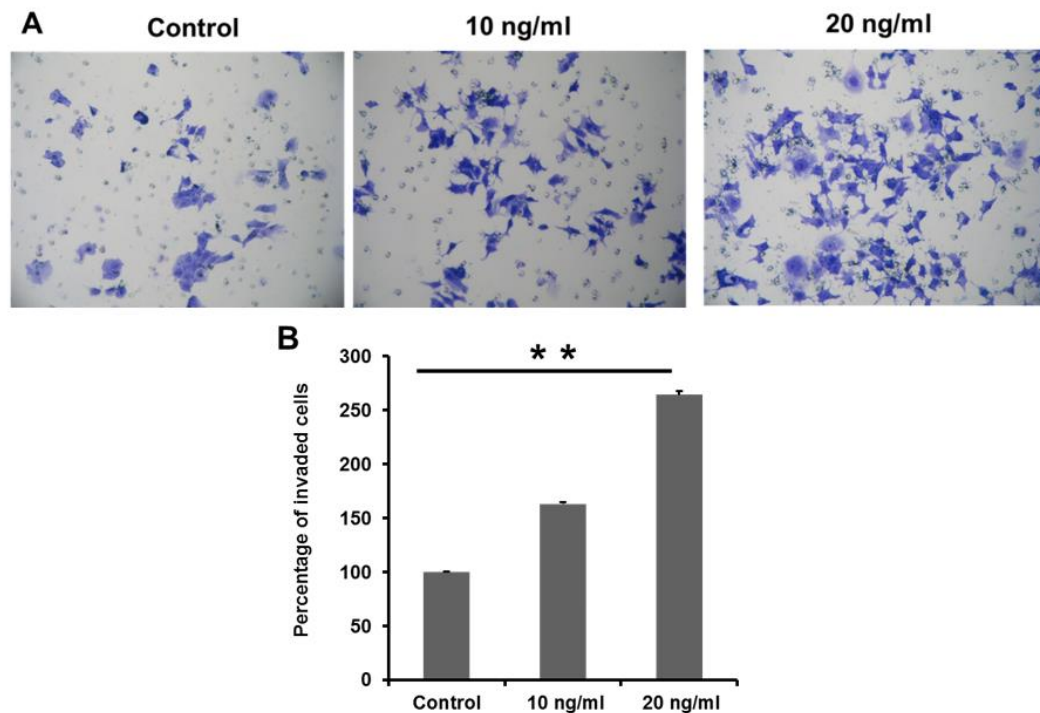


**Fig. 1 Cytokine profile of the surgical leakage collected from patients undergone breast cancer surgery.** A) Representative of Human Cytokine Array™ assessed 42 cytokines, chemokines and growth factors. (B) Densitometric quantification of the signal intensity of each cytokine collected from the drainage of patients undergone breast cancer surgery (n = 10). Data represent mean  $\pm$  SEM.



**Fig. 2 morphological changes in breast cancer cells line stimulated by different concentrations of TNF- $\alpha$ .** The morphology of MDA-MB-231 cells seeded in media conditioned with 10 and 20 ng/ml of TNF- $\alpha$  changed from round to mesenchymal-spindle shape in comparison with control cells.





**Fig. 3 TNF- $\alpha$  induces invasion of MDA-MB-231 cells.** (A) quantification of invaded MDA-MB-231 cells (purple color) were stimulated with different concentrations of TNF- $\alpha$  10 and 20 ng/mL using BD invasion chambers in comparing to control cells seeded in culture media containing 1% FBS . (B) Representative images of the stained Matrigel filters. Results are representative of three independent experiments. Data are expressed as mean  $\pm$  SEM. \*\* represents  $P < 0.01$  as determined by Student's t-test.

### DISCUSSION

The surgical leakage collected from breast cancer is one of the postoperative events which contain inflammatory mediators involved in healing responses (Kuroi *et al.*, 2005). The healing process includes the release of various pro-inflammatory cytokines such as TNF- $\alpha$  play an important role in the cellular events such as angiogenesis and extracellular matrix formation (Yilmaz *et al.*, 2011).

TNF- $\alpha$ , inflammatory cytokine is known to play a role in acquired and innate immunity. It can represent antitumor through regulate cell death and pro-tumor by inducing tumor growth, angiogenesis and metastasis (Waters, Poher, and Bradley 2013). Moreover, TNF- $\alpha$  is found to be expressed in the blood serum of patients who have been diagnosed with advanced prognostic factors such as

tumor stage and number of metastatic lymph nodes (Takahashi *et al.*, 2010).

In this study, we investigate whether TNF- $\alpha$  one of the surgical leakage components alters the morphology of breast cancer cells and induces invasion. There are previous studies indicated that TNF $\alpha$  induces EMT process and leads to tumor invasion and migration (Takahashi *et al.*, 2010). In the agreement with those previous studies, we found that TNF- $\alpha$  changed the morphology of MDA-MB-231 cells to be mesenchymal-spindel shape, which is a characteristic of EMT process.

The mechanisms of EMT involve epithelial cells loss their cell-cell adhesion by degrading cell to cell junctions. in this regard, the invading cells take the mesenchymal-spindel shape and detached from the basement membrane to invade and migrate to

distant organs (Scioli *et al.* 2019). Furthermore, previous studies support our findings that TNF- $\alpha$  enhances the invasion of breast cancer cells (Wolczyk *et al.*, 2016).

We also showed that low and high concentrations of TNF- $\alpha$  affect the invasion potential of breast cancer cell lines. This observation is confirmed by other reports that low levels of TNF- $\alpha$  is related to increase tumor growth, invasion, and metastasis (Waters, Pober, and Bradley 2013). In conclusion, we propose that TNF- $\alpha$  that is one of the inflammatory cytokine in the surgical leakage enhanced the EMT morphological changes and increase the invasion and migration for MDA-MB-231 breast cancer cells by stimulating them to lyse the extracellular matrix constituents through release proteolytic enzymes. Thus TNF can be considered as a therapeutic target in breast cancer clinical trials.

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## ARABIC SUMMARY

تحديد خصائص التسريب الجراحي الذي يتم جمعه بعد جراحة سرطان الثدي ودراسة تأثيرها على خط خلايا سرطان الثدي

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عالمياً، سرطان الثدي هو السرطان الأكثر شيوعاً بين النساء والسبب الثاني الرئيسي للوفاة وفقاً لتقرير منظمة الصحة العالمية لعام ٢٠١٨. ومع تطور الطرق العلاجية لهذا المرض إلا ان الجراحة هي الاختيار الرئيسي للعلاج وينتج عنها مضاعفات. واحد هذه المضاعفات، التسريب الجراحي والذي يحوى العديد من السيتوكينات التى تساهم فى عملية التئام الانسجة المتهتكه بعد الجراحه. وهناك اراء على ان هذه السيتوكينات قد يكون لها دور فعال فى تنشيط الخلايا السرطانية المتبقية بعد الجراحه ومن ثم تساعد على تطوير وانتشار الورم مرة اخرى. ويعد غزو وانتشار الورم واحد من الاليات الخلوية فى تطور وتقدم السرطان وذلك من خلال السيتوكينات الالتهابيه والتي تعمل على تنشيط وتنظيم عملية انتشار الورم من خلال افراز بعض الانزيمات البروتينيه والتي من بينها تى ان اف الفا. ينظم التى ان اف الفا العمليات الخلوية الحيوية عن طريق مستقبلات على سطح الخلايا، وهم مستقبل ١ ومستقبل ٢ ولكنه ينظم اغلب العمليات الخلوية بواسطة مستقبل ١.

ومن بين الادوار الفعاله ل التى ان اف الفا تنظيم افراز بعض الانزيمات التى تقوم بتكسير الغشاء القاعدى للخلايا ومن امثلتها ماتريكس ميتالوبروتياز والتي لها دور فى غزو وانتشار الورم. وقد اظهرت ايضا بعض الدراسات ان تى ان اف الفا له دور فى حركة الخلايا الساكنة الى الحركة الوسطية المتحركة لتتمكن الخلايا من الحركة واختراق الانسجه المحيطة عن طريق تغيير شكل الخلية الدائرى الى الشكل المغزلى والهروب من الغشاء القاعدى للخلايا. ولذلك فان الهدف من الدراسه الحالية هو دراسة تأثير تركيزات مختلفه من تى ان اف الفا على التغيير الشكلى لخلايا الثدي السرطانيه بالاضافة الى دراسة تأثيره على تحفيز غزو وانتشار الورم باستخدام اوعيه مغطاة بغشاء قاعدى اصطناعى.