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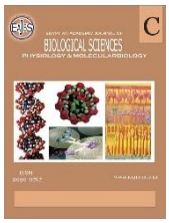
Citation: *Egypt.Acad.J.Biolog.Sci. (C.Physiology and Molecular biology) Vol. 16(2) pp157-179 (2024)*
DOI: 10.21608/EAJBSC.2024.379480

Egypt. Acad. J. Biolog. Sci., 16(2):157-179 (2024)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology
ISSN 2090-0767
www.eajbsc.journals.ekb.eg



Absence of Caveolin-1 P132L Mutation in Egyptian Breast Cancer Patients

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ARTICLE INFO
Article History
Received:9/8/2024
Accepted:12/9/2024
Available:16/9/2024

Keywords:
Caveolae, Cav-1, P132L, mutation and tumor suppressor.

ABSTRACT

Understanding the distinctive immunomodulatory processes of the breast cancer microenvironment will be crucial for the creation of novel therapeutic approaches. Cav-1's role in BC is still debated because it has been demonstrated to have a dual opposing role, functioning as both an oncogene and a tumor suppressor. Various studies reported the occurrence of a somatic mutation in Cav-1 in patients' primary breast tumors leading to the substitution of a proline amino acid-to-leucine at 132 (P132L) location. Therefore, the current study aims to examine the expression of Cav-1 in tissue samples of patients with breast cancer and assess the incidence of p132L point mutation of the Cav-1 gene in patients with breast cancer. The current study enrolled 50 patients with breast cancer, and the levels of expression of Cav-1 protein were measured by immunohistochemistry. Sequencing of amplified Cav-1 gene in sample groups was done to assess the incidence of a spontaneous mutation (P132L) inside the human Cav-1 in breast cancer patients. The current results demonstrated the absence of P132L target point mutation in all Cav-1 downregulated Egyptian breast cancer patients (90%). Accordingly, the alteration in the expression level of Cav-1 in tissues of Egyptian patients with breast cancer is not due to the P132L target point mutation, but other factors may contribute to this down-expression.

INTRODUCTION

Globally, breast cancer (BC) represents the second leading cause of morbidity for women (Siegel *et al.*, 2021). Over 521,900 fatalities globally have been attributed to 1.7 million women diagnosed with BC (De Silva *et al.*, 2019; Fletcher *et al.*, 2017). With over 95% of new cases being diagnosed in patients over 40, the incidence of BC-related deaths is predicted to increase by 20% (Saeg *et al.*, 2018). Compared to patients in Western nations, Egyptian BC patients present at a younger age and exhibit more aggressive characteristics (Harwansh *et al.*, 2020; Schlichting *et al.*, 2015). Clinical professionals could find novel diagnostic and treatment approaches to predict clinical outcomes and responses to treatment plans with molecular biomarkers for BC (Yamashiro *et al.*, 2008). However, the prevalence of this disease is rising annually, and the process through which BC develops is still undefined (Saeg *et al.*, 2018).

Understanding the distinctive immunomodulatory processes of the breast cancer micro-environment will be crucial for the creation of novel therapeutic approaches (Hanamura *et al.,* 2023).

Potential BC biomarkers include caveolae that invade plasma membranes are rich in proteins and involved in developing various human disorders (Mercier *et al.*, 2009). In addition to lymphocytes and neurons of the central nervous system, fat cells, endothelial cells, fibroblasts, pneumocytes, and muscle cells are abundant in caveolae (Razani *et al.,* 2002).

The caveolin proteins have a pro-vital role in the progress and preservation of caveolae. Caveolin-1 (Cav-1), Caveolin-2, and Caveolin-3 are the three members of the caveolin protein relatives. While Cav-3 is only found in some types of muscles, Cav-1 is broadly expressed in many tissues (Totta *et al.,* 2016; Wang *et al.,* 2017). Furthermore, Cav-2 co-expresses with Cav-1 and needs Cav-1 for stability and localization of cell membrane (Totta *et al.,* 2016; Parolini *et al.,* 1999).

Cav-1 is involved in numerous physiological functions, including signal transduction, molecular transport, and cell adhesion (Chai *et al.,* 2019; Shaul and Anderson 1998; Sternberg and Schmid 1999). Cav-1's role in BC is still debated because it has been demonstrated to have a dual opposing role, functioning as both an oncogene and a tumor suppressor (Ren *et al.,* 2021; Patani *et al.,* 2012a; Mercier *et al.,* 2012; Fu *et al.,* 2017). Three exons make up the Cav-1 gene, which maps to chromosome 7's long arm (7q31.1) near the fragile site (FRA7G), which is commonly found deleted in cancer. This finding supports the theory that Cav-1 is purpose as a tumor suppressor gene (Ren *et al.,* 2021; Patani *et al.,* 2012a). Recent research has revealed decreased levels of the Cav-1 protein in several human malignancies, including breast, ovarian, colon, lung, and sarcoma cancers. This indicates that Cav-1 has a negative regulatory function in tumor growth (Ren et al., 2021; Racine *et al.,* 1999; Bender *et al.,* 2000; Weichen *et al.,* 2001a; Weichen *et al.,* 2001b; Lee *et al.,* 1998; Razani *et al.,* 2001c; Williams *et al.,* 2003; Sagara *et al.,* 2004).

Caveolae need the caveolin-scaffolding domain (CSD) To interact with a range of signalling molecules, such as SRC tyrosine kinases (i.e., c-SRC/FYN), protein kinase C (PKC), the epidermal growth factor receptor, ERB-B2 receptor tyrosine kinase 2 (also known as HER2/neu), H-ras, and Neu and extracellular signal-regulated kinase (ERK) proteins (Lisanti *et al.,* 2012). CSD domain is hypothesized to perform a tumor suppressor role to Cav-1 by attaching signalling proteins in a restricted shape through consensus caveolin-binding motif, hence adversely regulating numerous kinases (Ren *et al.,* 2021; Patani *et al.,* 2012b; Razani *et al.,* 2002; Lisanti *et al.,* 1994; Williams *et al.,* 2004a).

Several breast cell lines showed decreased expression levels of Cav-1, such as MCF-7 and T47D (Bernatchez *et al.,* 2020; Lisanti *et al.,* 2012; Chen *et al.,* 2019; Fridolfsson *et al.,* 2014; Wu *et al.,* 2008; Wu *et al.,* 2007). Human Cav-1 overexpression reduces the transformed phenotype by decreasing anchorage-independent colony growth on soft agar, matrix invasion, and MMP-2 collagenolytic activity (Bernatchez *et al.,* 2020; Fiucci *et al.,* 2002; Engelman *et al.,* 1997). Several investigations discovered that Cav-1 serves as a suppressor of BC metastasis by modulating the activity of metallopeptidases (MMPs), which can increase tumor invasiveness and metastasis formation (Ren *et al.,* 2021; Williams *et al.,* 2004b; Sloan 2004; Coussens *et al.,* 1996). Generally, the expression of Cav-1 is linked to improved cell adhesion and decreased cell motility (Volonte *et al.,* 2001; Zhang *et al.,* 2000). Transfection of murine Cav-1 up-regulates BRCA1 in MCF7 cells via a p53-dependent mechanism (Glait *et al.,* 2006). Phosphorylation of serine or tyrosine, which directs Cav-1 for secretion, has been linked to function loss (Lee *et al.,* 2000; Schlegel *et al.,* 2001). The loss of Cav-1 expression in

BCAFs has been linked to worse outcomes, including accelerated tumor growth, local metastasis, and ER negativity (Yeong *et al.,* 2018; WitkiewiczA *et al.,* 2009c; El-Gendi *et al.,* 2012; Kalluri *et al.,* 2006; Tlsty *et al.,* 2011; Qian *et al.,* 2011).

Cav-1 has also been linked to the regulation of PTEN (phosphatase-possessing tumor suppressor functions) (Caselli *et al.,* 2002). The loss of both Cav-1 and the INK4a locus, which encodes p16INK4a and p19ARF cell cycle regulators, is enough to cause immortalization (William *et al.,* 2004c). It was found that in NIH/3T3 cells transformed by different oncogenes, such as v-Abl, Bcr-abl, H-RasG12V, and polyomavirus middle T antigen (PyMT), there is a significant reduction in the levels of Cav-1 mRNA and protein expression (Koleske *et al.,* 1995). It has been suggested that Cav-1 inhibits Wnt/β-catenin/Tcf/Lef-1 signalling by retaining β-catenin in the cell membrane and blocking the transcription of genes, such as cyclin D1 (Hulit *et al.,* 2000). Cav-1 over-expression in a metastatic mammary adenocarcinoma cell line (MTLn3) caused the cell to revert to a non-motile phenotype, suggesting that Cav-1 can stop the growth and invasion of cells with metastatic properties (Lisanti *et al.,* 2012).

It has been determined that stromal Cav-1 reduction in the cancer-associated fibroblast compartment is the only independent forecaster of clinical prognosis (Mercier *et al.,* 2008; Witkiewicz *et al.,* 2009a, Witkiewicz *et al.,* 2009b; Witkiewicz *et al.,* 2010; Witkiewicz *et al.,* 2009c; Sloan *et al.,* 2009a). For example, in patients with breast cancer, stromal Cav-1 depletion predicts tamoxifen resistance, lymph node metastases, and early tumor recurrence. Therefore, a "fatal" tumor microenvironment can be indicated by the decreased stromal Cav-1 (Lisanti *et al.,* 2010). Cav-1 overexpression causes apoptotic cell death by inhibiting PI3-kinase and activating caspase-3, suggesting that Cav-1 may play a pro-apoptotic role (Zundel *et al.,* 2000; Liu *et al.,* 2001). According to previous findings, the lack of stromal Cav-1 results in oxidative stress and autophagy in the tumor microenvironment, which in turn promotes the manufacture of recycled high-energy nutrients locally, which cancer cells can utilize to "fuel" their anabolic growth (Martinez-Outschoorn *et al.,* 2010a; Martinez-Outschoorn *et al.,* 2010b; Martinez-Outschoorn *et al.,* 2010c).

Various studies reported the occurrence of a somatic mutation in Cav-1 in patients' primary breast tumors leading to the substitution of a proline-to-leucine at 132 (P132L) position (Lisanti *et al.,* 2012). In a non-transformed human mammary epithelial cell line, it was demonstrated that the Cav-1 P132L mutant mis-localizes and retains wild-type endogenous Cav-1 intracellularly, functioning as a dominant-negative mutant (Li *et al.,* 2006). Therefore, in the setting of mammary epithelial cells, this heterozygous mutation results in the total functional suppression of the Cav-1 protein (Li et *al.,* 2006).

The response to chemotherapy and radiation therapy used to treat BC is influenced by Cav-1. Previous research demonstrated that stromal Cav-1 level has a pro-vital role in determining BC outcomes and that its modification in response to oxidative stress may help choose the optimum therapy (Martinez-Outschoorn *et al.,* 2014; Sloan *et al.,* 2009a). Another factor that may contribute to trastuzumab resistance in BC cells is Cav-1 (Pucci *et al.,* 2015). As a result, the lack of Cav-1 and caveolae may be a prognostic or predictive indicator of trastuzumab therapeutic response (Pucci *et al.,* 2015). On the other side, numerous studies hypothesized that Cav-1 might contribute to the emergence of tamoxifen resistance (Pucci *et al.,* 2015). According to recent research, phosphorylation of Cav-1 may have a key role in switching on a survival mechanism for cancer cells. This finding may inspire the development of new cancer treatment strategies (Jiang *et al.,* 2022a). Therefore, the current study aims to examine the expression of Cav-1 in tissue samples of patients with breast cancer and assess the incidence of p132L point mutation of the Cav-1 gene in patients with breast cancer.

MATERIALS AND METHODS

1-Patients' Selection:

This current study was established on 50 patients who were diagnosed with breast cancer at Ain Shams University Hospitals. Each patient was approved by the Institutional Review Board of Ain Shams University Hospitals Ethics Committee. Patients' ages ranged from 33-67 years, with mean SE=38.8 ±1.9, who were diagnosed at stages II and III.

2-Samples:

Tissue samples were collected from conservative breast surgery or modified radical mastectomy and ten ml peripheral blood was collected from patients before surgical operation.

3-Immunohistochemistry (IHC) for Cav-1:

3.1-Preparation of Paraffin Blocks:

First, fixation took place overnight in neutral buffered formalin, pH=6.8, (6.5 g sodium phosphate dibasic, 4 g sodium phosphate monobasic and 100 ml Formaldehyde, 37% dissolved in 900 ml distilled water) and infiltrated in paraffin.

3.2-Tissue Section Preparation and Immunohistochemistry (IHC):

The tissue sections of 4 µm in thickness were cut from the paraffin-embedded blocks using a microtome. After staining tissue sections with hematoxylin and eosin, they were mounted on positively charged slides and allowed to air dry for an entire night. Sections were mounted and then hydrated by immersing them three times in xylene for five minutes, followed by a decreasing sequence of alcohol (100%, 95%, 80%, and 50%) for three minutes. Positive slides were incubated for an hour at 99ºC in a water bath with citrate buffer, pH=6 (2.1 g of citric acid dissolved in 1 L of distilled water). The slides were then allowed to come to room temperature before being immersed in two different concentrations of Tris-buffered saline TBS (0.05 mol/L Tris-HCl, pH 7.6, 0.15 mol/L NaCl, and 0.05% tween 20) for five minutes each for washing. The slides were cleaned with TBS after being blocked for 10 minutes with 3% hydrogen peroxide (Dual Endogenous Enzyme block, Dako K4065). After that, they were incubated with a monoclonal primary antibody against Cav-1 for an entire night at room temperature. The slides underwent two 5-minute TBS rinses, a 45-minute room-temperature incubation with 100 µl of peroxidase-labeled polymer rabbit/mouse, and a final 5-minute washing in TBS. After applying substrate and chromogen to the slides and letting them sit for five to ten minutes, the slides were cleaned with distilled water. The slides were counterstained by adding Mayer's hematoxylin. After rinsing the slides with tap water, increasing the alcohol concentration to dehydrate them, and eventually immersing them in xylene, mounting material was used to cover them (Fisher Scientific).

4-PCR and Sequencing:

DNA extracted from tissue and blood were performed by using QIAamp DNA Mini kit (QIAGEN, Germany). The extracted DNA was subjected to amplification of the target trans-membrane domain and flanking sequences of Cav-1 gene with accession number (-NM001753-) by Polymerase Chain Reaction (PCR). In PCR, the current study used two primers to amplify 210 bp from (39226 - 39435) DNA fragments corresponding to 70 amino acids which include the whole trans-membrane domain (amino acids 102 to 134) of Cav-1.

The Sequence For Forward and Revere Primers Was:

5' CCAGCTTCACCACCTTCACT 3' and 5' CACAGACGGTGTGGACGTAG 3'. The PCR program was modified in this way: first denaturation for five minutes at 95 ºC, followed by thirty-five cycles of one minute at 95 ºC, one minute at 55 ºC, and one minute at 72 ºC for extension. Every sample underwent a 10-minute exposure to the final extension at 72 ºC before being stored at 4 ºC.

A gel purification kit (k0691) was used to purify the PCR product. Each sample was sequenced twice using the forward and reverse PCR primers in order to verify any potential mutations. The identical forward and reverse primers used in the PCR were used for sequencing 20 µl of each PCR product, 20 µl of the forward primer (10 pmole/µl), and 20 µl of the reverse primer (10

pmole/µl). The Cav-1 PCR results from blood and breast cancer tissue samples were sent for sequencing to look for the presence of the P132L mutation.

5-Statistical Analysis:

The data was analyzed using SPSS software version 18.0. Data were expressed as mean ± standard deviation and correlations between categorical variables were assessed using the Spearman correlations test.

RESULTS

1-Clinical and Pathological Characteristics Of Patients:

Clinical and pathological characteristics are viewed in Table (1), including age, tumor grade, tumor size, lymph node metastasis, lymph vascular invasion and expression of estrogen receptor (ER), progesterone receptor (PR) and HER-2 as explained below:

The present study applied to patients with tumor sizes ranging from 2-10 cm (mean size 4.8). Tumor grade documented that 62% of patients were with tumors of grade II, 24% were with tumors of grade III and 14% of tumor samples were grade I.

About 60% of patients were classified as ≤ 4, while 40% of patients were classified as > 4. Among patients, 64% were negative lymph vascular invasion and 36% were positive lymph vascular invasion.

Hormonal receptors analysis: Thirty-eight percent of patients were positive ER, while 62% of patients had negative ER. About 36% of patients were positive PR, while 64% of patients were negative PR. Thirty-eight percent of patients were positive for HER-2, while 62 % of patients were negative for HER-2.

Table 1: Patients and tumor characteristics**.**

Characteristics	N%
Age (years)	
Mean± SD	38.8± 9.825
Range	33-67
Tumor size	
Mean	4.8
Range	2-10
Tumor grade	
Grade I	7 (14)
Grade II	31 (62)
Grade III	12 (24)
No. of metastatic lymph nodes	
≤4	30 (60)
>4	20 (40)
Lymph vascular invasion	
Positive	18 (36)
Negative	32 (64)
Estrogen receptor	
Positive	19 (38)
Negative	31 (62)
Progesterone receptor	
Positive	18 (36)
Negative	32 (64)
HER-2	
Positive	19 (38)
Negative	31 (62)

2-Expression of Caveolin-1 Protein by Using Immunohistochemistry:

Immunohistochemistry results revealed that cytoplasmic and cellular membrane Cav-1 protein was expressed in five (10%) breast cancer patient samples, while it was down-regulated in forty-five (90%) samples (Fig. 1).

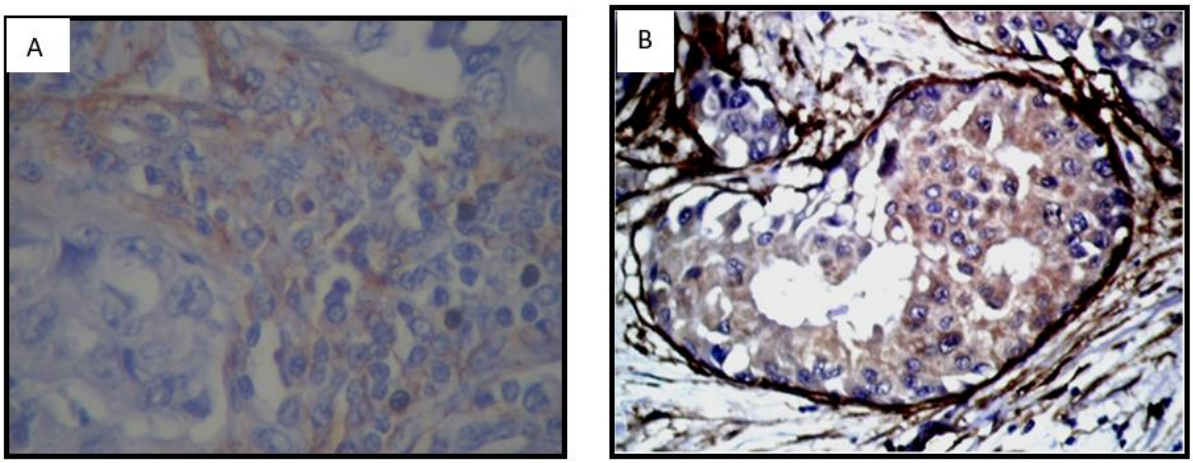

Fig 1: Photomicrographs represent the IHC staining of Cav-1. (A) showing low protein expression, (B) showing strong staining of cellular membrane and cytoplasmic staining for Cav-1 (over expression of Cav-1 protein) (magnification X400).

3-Lack of P132L Mutation In Breast Cancer Samples:

Sequencing results revealed the absence of the targeted point P132L mutation in all Cav-1 downregulated breast cancer samples (90%) at position 143-145 from the beginning of the forward primer of Cav-1 where CTA replaces CCA which, when present leads to amino acid leucine replacing amino acid proline in position 132 of the amino acid sequence starting from the beginning of the open reading frame (Figs. 2 & 3).

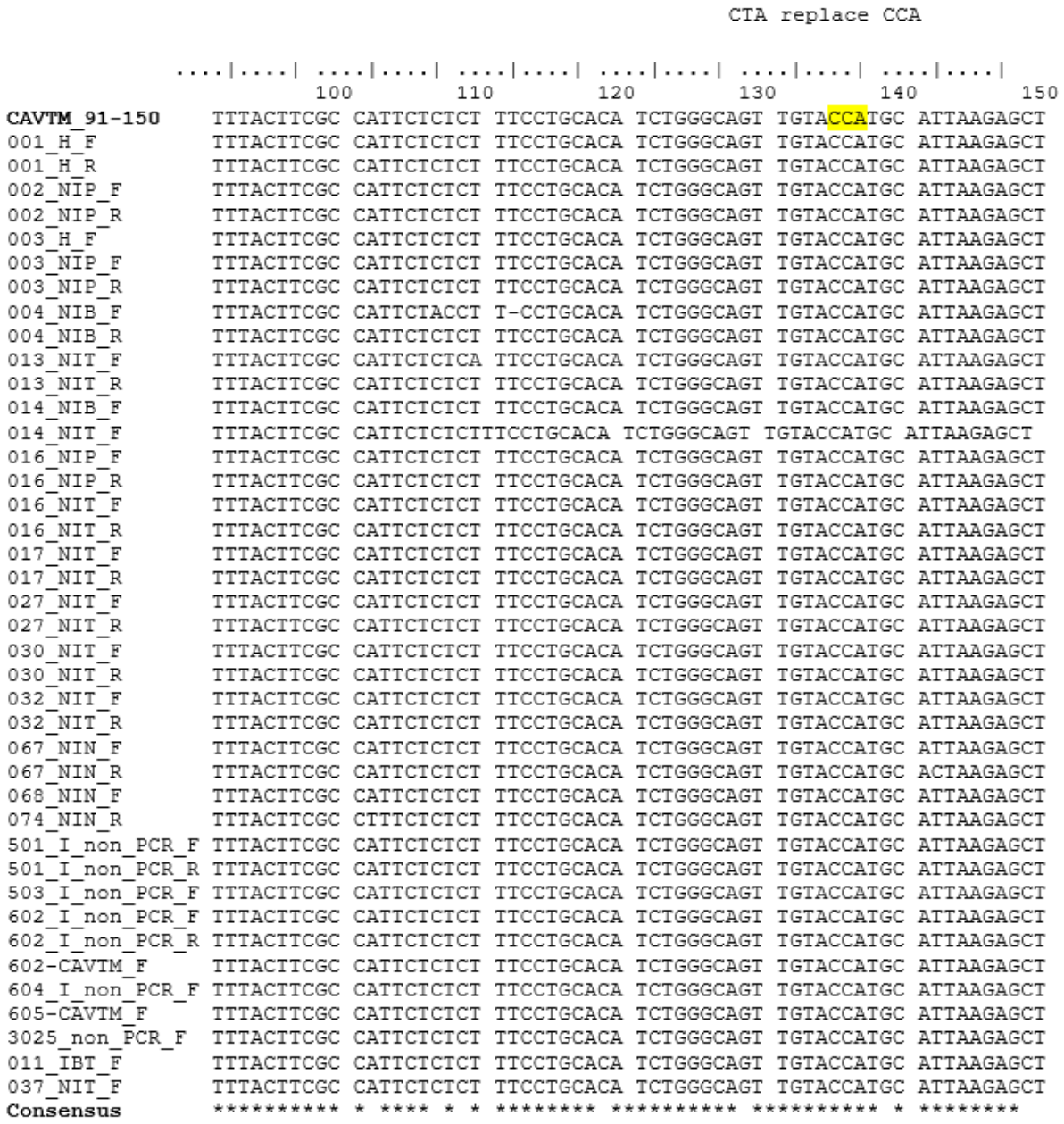

Fig 2: Alignment of sequence of cav-1 gene of breast cancer patients and their normal tissues.

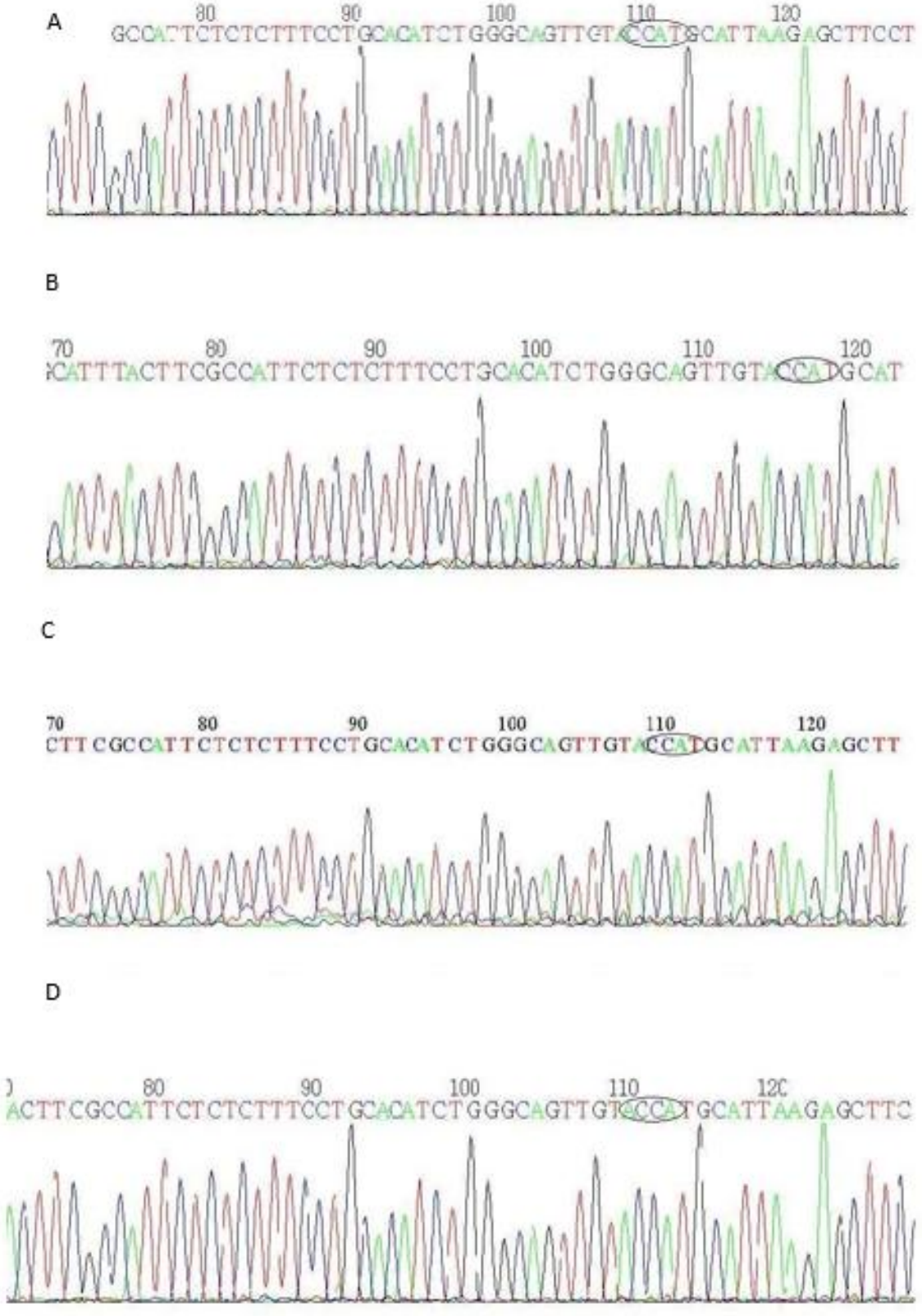

Fig 3: DNA sequence of cav-1 gene using ABI PRISM model 3730XL analyzer software in (A) normal tissues, (B) breast cancer patient paraffin tissue, (C) breast cancer patient tissue, (D) blood of breast cancer patients.

DISCUSSION

The current study documented the absence of P132L target point mutation in all Cav-1 downregulated Egyptian patients with breast cancer (90%). Furthermore, the alteration in the expression level of Cav-1 in Egyptian tissues of patients with breast cancer is not due to the P132L target point mutation, but other factors may contribute to this down-expression.

Now, the most serious malignant tumor endangering women's lives is breast cancer (BC) (Siegel *et al.,* 2021). BC is a very diverse category of tumors in terms of both molecular makeup and clinical presentation. A complicated interaction between growth factors, hormones, oncogene activation, and tumor suppressor gene inactivation occurs throughout normal breast development and breast carcinogenesis (Park *et al.,* 2005). The control of breast cancer is to be inadequate in less developed and developed countries (Ezzat *et al.,* 1999). Genetic screening of breasts plays a vital role in breast cancer prediction.

Furthermore, premenopausal Egyptian women have high peripheral lymphocyte DNA damage levels and urinary estrogen metabolites, which are biomarkers for breast cancer prediction in Egyptian patients (Soliman *et al.,* 2004). Because of several subtypes of BC, selecting a single therapy that applies to all patients is impossible. Several current therapeutic approaches are based on receptor status and tumor stage (Bravatà *et al.,* 2013; Yamashiro *et al.,* 2008). Over the past few decades, there has been a considerable change in the treatment strategy for BC patients, and there has also been a notable fall in the death rate among them.

Caveolae are cave-like plasma membrane structures implicated in controlling various cellular functions (Chidlow *et al.,* 2010). Caveolae act as the site for cell surface protease compartmentalization (Cavallo-Medved *et al.,* 2003). Caveolae consist of caveolins and associated cell surface proteases such as CTSB, a lysosomal cysteine protease, and uPA (a serine protease) (Cavallo-Medved *et al.,* 2005). Besides three isoforms of caveolins (Cav-1, Cav-2, and Cav-3), the protein structure of caveolae has been noticed. Cav-1 and Cav-2 act as biomarkers for the "basal-like" phenotype in the breast carcinoma subfamily (Mercier *et al.,* 2009).

The current study showed that caveolin-1 protein was expressed in the cell membrane and the cytoplasm of 5 (10%) patients and downregulated in 45 (90%) patients. Previous studies showed that Cav-1 could be found scattered in cells and not localized in the cell membrane invaginations of breast tumors and thus considered suppressed (Lee *et al.,* 2002).

Cav-1 high levels in the stromal tissue around a breast tumor have previously been found to be highly related to decreased metastasis and increased survival (Shan-Wei *et al.,* 2012). According to earlier research, Cav-1 is downregulated in BC and acts as a tumor suppressor to impede BC's growth (Ren *et al.,* 2021). Additionally, the previous findings demonstrated that reduced expression of Cav-1 is a potent indicator of tumor relapse. Clinically, people with prostate cancer or BC are more likely to have a bad effect if they have stromal Cav-1 depletion (Ayala *et al.,* 2013; Kibria *et al.,* 2014; Witkiewicz *et al.,* 2009c). One proposed explanation for this up-regulation is the Cav-1-deficient fibroblasts showed increased expression of transforming growth factor-beta (TGFB), which was documented to induce epithelial Cav-1 expression and induce the epithelial-to-mesenchymal transition (Panic *et al.,* 2017; Gottlieb-Abraham *et al.,* 2013). Loss of Cav-1 expression has been correlated with larger tumor growth, a higher rate of nodal involvement, and more afflicted lymph nodes (El-Gendi *et al.,* 2012; Witkiewicz *et al.,* 2009c; Mercier *et al.,* 2008; Eliyatkin *et al.,* 2018; Finak *et al.,* 2008). Recent research demonstrated that CAV-1 deficiency in fibroblasts increases TGF-1 secretion, which then activates the TGF-1/Smad signalling

pathway of BCCs, promoting their metastasis and stemness (Huang *et al.,* 2022).

Cav-1 is expected to limit anchorage-independent growth while significantly reducing matrix-dependent MMP-2 production and impairing invasive potential (Bernatchez *et al.,* 2020; Fiucci *et al.,* 2002). The previous study demonstrated that Cav-1 expression prevented integrin-mediated activation of ERK1/2 (Fiucci *et al.,* 2002). Additionally, Cav-1 confers resistance to anoikis in the MCF-7 cells despite inhibiting anchorage-independent growth (Bernatchez *et al.,* 2020). Recent research shows that Cav-1 inactivates anchorage-independent growth in fibroblasts, which may indicate that it has an inhibitory effect on a proliferation signal dependent on growth factors or oncogenes. Cav-1 adversely affects cell proliferation in fibroblasts (Bernatchez *et al.,* 2020; Chen *et al.,* 2019; Galbiati *et al.,* 2001; Razani *et al.,* 2001a). Likely, Cav-1 and P-glycoprotein overexpression is the cause of the loss of anchorage-independent growth and diminished metastatic potential detected in many MDR cells (Toula *et al.,* 2004).

According to earlier research, expression levels of cyclin D1 are significantly higher in Cav-1(-/-) null mammary lesions, which is consistent with the dysplastic foci's quick formation (Razani *et al.,* 2001b). Primary embryonic fibroblasts carrying the Cav-1 (-/-) null allozyme showed enhanced rates of DNA synthesis and larger S-phase fractions, and they proliferated significantly quicker than their wild-type counterparts. Additionally, there is strong evidence that in transformed cells, expression of Cav-1 inhibited anchorage-independent growth and cellular proliferation (Bernatchez *et al.,* 2020; Chen *et al.,* 2019; Razani *et al.,* 2001b). Cav-1 overexpression suppresses cyclin D1 transcription, whereas its antisense expression raises cyclin D1 levels, proving that Cav-1 regulates cell growth (Hulit *et al.,* 2000). Therefore, it would be predicted that the rapid formation of dysplastic mammary foci in PyMT/Cav-1 (-/-) mice can be explained by the transcriptional up-regulation of cyclin D1 levels resulting from the loss of Cav-1 expression (Williams *et al.,* 2003).

From *in vitro* research on cultivated cells, Cav-1's function as a detrimental regulator of cellular proliferation is now well known (Chen *et al.,* 2019; Fridolfsson *et al.,* 2014; Wu *et al.,* 2007). In MCF-7 cells, caveolin-1 has anticancer action both *in vitro* and *in vivo* due to decreased cell proliferation and increased apoptosis (Chen *et al.,* 2019; Fridolfsson *et al.,* 2014; Wu *et al.,* 2007). Cav-1 may serve as a coupling or sensitizing factor in the signalling of apoptotic cell death in epithelial and fibroblastic cells, increasing the sensitivity of fibroblasts to apoptotic stimuli (Zundel *et al.,* 2000; Liu *et al.,* 2001). Similarly, Gargalovic and Dory observed that higher Cav-1 expression in macrophages is related to cell apoptosis (Gargalovic and Dry 2003). The previous findings showed that Cav-1 upregulation in MCF-7 cells elevated cancer cell death and decreased cancer growth *in vitro* and *in vivo* (Wu *et al.,* 2008).

In the recent investigation, in comparison to normal breast epithelial cells, the Cav-1 expression was downregulated in breast ductal carcinoma cells, there was a reciprocal link observed between Cav-1 and both EGFR and HER2 expression status (Ren *et al.,* 2021; Park *et al.,* 2005). This is consistent with the *in vitro* studies, which showed the roles of Cav-1's purposes as a negative regulator of cell signalling and a tumor suppressor in the growth of breast cancer (Ren *et al.,* 2021; Park *et al.,* 2005). Similar findings in other research revealed lower expression of caveolin-1 in human breast cancer cells and cells converted by oncogenes (Razani et al, 2001c; Sagara *et al.,* 2004; Koleske *et al.,* 1995). Further evidence supporting Cav-1's role as a tumor suppressor protein in breast cancer arises from the discovery that recombinant production of Cav-1 significantly reduced anchorage-independent growth and suppressed tumor cell proliferation (Ren *et al.,* 2021; Bernatchez *et al.,* 2020; Chen *et al.,* 2019; Lee *et al.,* 1998; Engleman *et al.,* 1997). Additionally, Cav-1 down-regulation is seen in different malignancies, including ovarian, lung, colon, and sarcomas (Racine *et al.,*

1999; Bender *et al.,* 2000; Weichen *et al.,* 2001a; Weichen *et al.,* 2001b). A previous study illustrated that the Cav-1 downregulation facilitates the phenotypical effects of EGF, internalization and transcriptional down-regulation of E-cadherin, and enhanced transactivation by β-catenin. It represents a unique mechanism explaining the effects of EGF during tumor growth (Lu *et al.,* 2003).

The previous investigation showed that the expression and discharge of the growth factors SDF-1, EGF, and FSP-1 significantly increased when Cav-1 was downregulated in fibroblasts. Additionally, it increased TIGAR expression, which could promote cancer cell growth and inhibit cancer cell death (Shi *et al.,* 2016). The prognosis of tumors is correlated with the low expression or deletion of Cav-1 expression in stromal fibroblasts (Witkiewicz *et al.,* 2010). Cav-1 depletion in breast cancer patients' stromal fibroblasts has been suggested to predict the disease's relapse, metastasis of lymph nodes, and tamoxifen opposition (Witkiewicz *et al.,* 2009; El-Gendi *et al.,* 2012). Cav-1 loss in stromal fibroblasts in patients with ER/PR/HER2 ductal carcinoma or breast cancer has been used to predict a poor clinical outcome (Yeong *et al.,* 2018; Witkiewicz *et al.,* 2009b). Breast cancer prognosis is not linked with Cav-1 expression in malignant cells (Shan-Wei *et al.,* 2012). As a result, stromal Cav1 depletion is a crucial indicator of a "lethal" cancer microenvironment (Shi *et al.,* 2016). Lower levels of Cav-1 generate larger amounts of extracellular matrix proteins and myofibroblast markers in co-cultured human breast cancer cells with fibroblasts, suggesting that Cav-1 downregulation initiates fibroblast activation in carcinogenesis (Martinez-Outschoorn *et al.,* 2010b). The previous outcomes showed that Cav-1 expression was downregulated in the cells transfected with Cav1 siRNA, proving that the Cav1 siRNA sequences had successfully stifled the expression level of the Cav1 gene (Shi *et al.,* 2016).

The relationship between lower levels of Cav-1 in the co-culture and fibroblasts and cancer cells was investigated by looking at the expression of cancer-related markers in fibroblasts and breast cancer cells (Shi *et al.,* 2016). Downregulating Cav-1 and co-cultivating breast cancer cells together enhanced SDF1 expression in fibroblasts. Moreover, Cav-1's ability to prevent tumor growth may be related to its ability to block the signaling pathways in which SDF1 is involved (Shi *et al.,* 2016).

It was strongly corroborated by the brand-clinical and molecular results indicating that stimulated Stat3 directly binds to the Cav-1 promoter, inhibiting transcription (Chiu *et al.,* 2011). On the other hand, Cav-1 controls Stat3 activation and the invasion of brain-metastatic cancer cells (Chiu *et al.,* 2011). In the previous animal model, suppressing Stat3 activation prevented breast cancer cells from migrating into the brain and metastasizing there (Chiu *et al.,* 2011). Cell proliferation and invasion are two ways that Cav-1 works to limit transformation, cancer growth, and metastasis (Chen *et al.,* 2019; Fridolfsson *et al.,* 2014; Chiu *et al.,* 2011). Cav-1 is a transcriptional regulator of cyclin D1 and an endogenous suppressor in the p42/44 mitogen-activated protein kinase cascade (Engelman *et al.,* 1998). The previous finding demonstrated that SOCS-1-induced elevation of Cav-1 expression inhibited cancer growth both *in vitro* and in nude mice (Chiu *et al.,* 2011).

Additionally, increased Cav-1 expression reduced the invasiveness of breast cancer while decreasing its expression aided in cell proliferation and breast cancer invasion (Chen *et al.,* 2019; Fridolfsson *et al.,* 2014; Chiu *et al.,* 2011). Moreover, a recent study found that caveolin-1 overexpression prevented Stat3 activation, a crucial mechanism in the development and breast tumor metastasis (Geletu *et al.,* 2019; Chiu *et al.,* 2011). Therefore, caveolin-1 expression may have an impact on breast cancer brain metastases in a variety of mechanisms (Chiu *et al.,* 2011).

Cav-1 inhibits COX-2 expression in HEK293T, HT29(ATCC), DLD-1, and ZR75 cells through a transcriptional mechanism that

is dependent on β-catenin, Tcf, and Lef (Rodriguez *et al.,* 2009). Cav-1's ability to inhibit tumors has been associated with its ability to negatively regulate -catenin-Tcf/Lef-dependent transcription (Quest *et al.,* 2008). A mechanism involving Cav-1-mediated inhibition of -catenin-Tcf/Lef-transcription adversely regulates the target genes survivin and cyclin D1 (Galbiati *et al.,* 2000; Torres *et al.,* 2006). These changes have been linked to decreased cell proliferation and an induced susceptibility to apoptosis (Chen *et al.,* 2019; Fridolfsson *et al.,* 2014; Torres *et al.,* 2006; Torres *et al.,* 2007). The previous findings present evidence that the mRNA and protein levels of COX-2 are decreased by ectopic expression of Cav-1 in breast (ZR75) and colon [HT29(ATCC) and DLD-1] cancer cells (Haertel-Wiesmann *et al.*, 2000; Araki *et al.,* 2003).

Furthermore, inhibition was observed in HEK293T cells. In addition, Cav-1 reduced reporter activity generally correlated with the stimulation of the β-catenin-Tcf/Lef pathway (TOP/FOP reporter), and particularly associated with the induction of the COX-2 gene in cancer and HEK293T cells. These findings support the idea that β-catenin-Tcf/Lef regulates COX-2 expression through transcription (Haertel-Wiesmann *et al.*, 2000; Araki *et al.,* 2003) and indicate that through this mechanism, Cav-1 suppresses the expression of COX-2 in a manner reminiscent of the survivin study (Torres *et al.,* 2006). The simplest explanation for the previous observations is that decreased synthesis of PGE2 is associated with Cav-1-dependent downregulation of COX-2 transcription. When Cav-1 is expressed in response to other apoptotic triggers (Torres *et al.,* 2006), the reduction in cell proliferation is prevented by ectopic survivin expression (Tapia *et al.,* 2006).

Further research has demonstrated that PGE2 stimulates transcription mediated by β-catenin-Tcf/Lef in HEK293, DLD-1, and LS-174T cells (Fujino *et al.,* 2002; Castellone *et al.,* 2005; Shao *et al.,* 2005). Consistent with these studies, PGE2 supplementation prevented the loss of survivin caused by Cav-1 in all cell lines examined. Previous research showed that in both mock and Cav-1-expressing cells, β-catenin moved from the cell membrane to the nucleus as a result of PGE2. PGE2-induced signalling events disrupt the Cav-1/β-catenin multiprotein complex at the cell surface, preventing β-catenin-Tcf/Lef-mediated transcription in the nucleus. It has been demonstrated that Cav-1-mediated down-regulation of β-catenin-Tcf/Lef-dependent transcription and survivin expression in cancer cells require E-cadherin (Torres *et al.,* 2007). The absence of E-cadherin in metastatic HT29(US) cells prevented observing these events. Similarly, the previous study found that in HT29(US) cells, ectopic Cav-1 expression had no effect on COX-2 mRNA levels, and restoring E-cadherin expression restored the regulation of COX-2 downstream of Cav-1. The previous findings on COX-2 support the idea that one of the most important steps in the formation of a cellular environment is the decrease of E-cadherin expression during tumor growth that is favorable and in which Cav-1's capacity to exhibit characteristics linked to tumor suppression or metastasis inhibition may be severely compromised (Quest *et al.,* 2008).

Stromal Cav-1 deletion is a novel biomarker of a fatal tumor microenvironment in the cancer-associated fibroblast compartment (Mercier *et al.,* 2008; Witkiewicz *et al.,* 2009a, Witkiewicz *et al.,* 2009b; Witkiewicz *et al.,* 2010; Witkiewicz *et al.,* 2009c; Pavlides *et al.,* 2010). The previous study demonstrated that stromal Cav-1 depletion results in a 4-fold increase in tumor volume and mass without any corresponding rise in tumor angiogenesis (Trimmer *et al.,* 2011). This supports the prior theory that oxidative stress and autophagy in cancer-associated fibroblasts provide high-energy nutrients that can be recycled and fed directly to cancer cells without the need for vascularization or blood vessels (Martinez-Outschoorn *et al.,* 2010d). The fact that oxidative stress in cancer-associated fibroblasts causes mitochondrial malfunction,

ROS generation, and the autophagic degradation of mitochondria in stromal fibroblasts is a significant finding of these investigations (Jiang *et al.*, 2022a; Martinez-Outschoorn *et al.*, 2010a; Martinez-Outschoorn *et al.*, 2010b; Martinez-Outschoorn *et al.*, 2010c; Chiavarina *et al.*, 2010). The previous findings showed that the tumor-stimulating behavior of Cav-1 defective fibroblasts could be drastically reversed by recombinant production of mitochondrially targeted SOD2 (Trimmer *et al.*, 2011). By raising the amount of collagen VI and other extracellular matrix components in the tumor/stromal milieu, stromal Cav-1 deficiency may encourage the growth of tumors (Trimmer *et al.*, 2011).

Further, the previous study examined breast cancer cell lines' apoptotic induction and migratory characteristics after Cav-1 gene silencing (Deb *et al.*, 2014). The MCF7 and MDA-MB-231 cell lines with Cav-1 silenced behave like untreated cell lines (Deb *et al.*, 2014). Although the amount of cell migration after Cav-1 knockdown was identical to control levels, less apoptosis was induced than in cells treated with control siRNA (Deb *et al.*, 2014). This information confirmed the hypothesis about a potential connection between changed Cav-1 expression and the activation of apoptosis and altered migratory properties of breast cancer cell lines (Chen *et al.*, 2019; Fridolfsson *et al.*, 2014; Deb *et al.*, 2014). According to the previous theory, Cav-1 is essential for triggering apoptosis and preventing cell migration (Jiang *et al.*, 2022b; Chen *et al.*, 2019; Fridolfsson *et al.*, 2014; Deb *et al.*, 2014). When Cav-1 is downregulated, it promotes cancer cell growth and may also boost its survival rate by suppressing the apoptotic pathway (Deb *et al.*, 2014). Therefore, Cav-1 inhibits cell migration and triggers apoptosis together (Chen *et al.*, 2019; Fridolfsson *et al.*, 2014; Deb *et al.*, 2014).

Loss of stromal Cav-1 expression significantly reduces development-free survival and serves as a powerful predictor of tumor recurrence (Witkiewicz *et al.*, 2009c). Further debate and research are necessary, considering the previous findings that the Cav-1 decrease in the cancer stroma promotes the aggressiveness of breast carcinomas (Witkiewicz *et al.*, 2009c). Cav-1 expression is required for RB tumor suppressor functional inhibition *in vivo*, which releases E2F from mammary stromal fibroblasts (Witkiewicz *et al.*, 2009c). The stromal cell loss of Cav-1 permits the signaling of transforming growth factor to be activated (Razani *et al.*, 2001b). It has been demonstrated that activated transforming growth factor-signaling in CAFs induces the production of growth-promoting proteins, including human growth factor, vascular endothelial growth factor, and interleukin-6 (Cat *et al.*, 2006). Therefore, Cav-1 expression in breast cancer stromal cells may be decreased or prevented by mutational suppression of p53 in these cells (Witkiewicz *et al.*, 2009c). The causes of Cav-1 being downregulated in breast tumor stroma are still a mystery (Witkiewicz *et al.*, 2009c). However, prior research with human breast CAFs revealed that Cav-1 mRNA transcript levels activated by about 2.3 to 2.4-fold or remained unchanged (Witkiewicz *et al.*, 2009c). This suggests that Cav-1 protein expression is lost at a post-transcriptional or post-translational stage (Mercier *et al.*, 2008). Genome-wide transcriptional profiling confirmed that Cav-1(-/-) mammary stromal fibroblasts up-regulate several genes linked to embryonic stem cells, suggesting that these cells may have greater cellular plasticity (Witkiewicz *et al.*, 2009c). According to CD31 staining (Sotgia *et al.*, 2008), the mammary stromal compartment in Cav-1(-/-) animals exhibited drastically enhanced vascularization and encouraged carcinogenesis *in vivo*, which is consistent with these observations (Williams *et al.*, 2006). Therefore, mutational suppression of p53 in breast cancer stromal cells may reduce or eliminate Cav-1 expression in these cells (Witkiewicz *et al.*, 2009c). As a result, based on these mechanistic findings, the previous data propose that, in addition to the more conventional treatment regimens, individuals with breast cancer who do not have stromal

Cav-1 may benefit from anti-angiogenic treatment (bevacizumab [Avastin]) (Witkiewicz *et al.,* 2009c).

The previous study showed that Cav-1 could be moved from the cytoplasm to the plasma membrane because of BRCA1 (Wang *et al.,* 2008). The altered invasive and metastatic abilities of BRCA1(+/+) MEFs cells could be explained by Cav-1 distribution because these traits are considerably different from those of BRCA1(-/-) MEFs cells (Wang *et al.,* 2008). The buildup of Cav-1 in plasma membranes may significantly aid the modulation of mammalian cells' capacity for invasion and metastasis (Wang *et al.,* 2008). Cav-1 has been documented to be essential for the endocytosis of E-cadherin (Chang *et al.,* 2018; Lu *et al.,* 2003). Caveolin-1 can build up in plasma membranes, increasing E-cadherin expression, decreasing β-catenin transcriptional stimulation, and lessening cancerous cells' invasiveness (Wang *et al.,* 2008). The previous findings have significant implications for metastases because invasiveness is one of the traits shared by metastatic cells (Wang *et al.,* 2008).

A previous study showed the presence of some mutations in the Cav-1 gene in invasive human breast carcinoma and oral squamous cell carcinomas (Hayashi et al. 2001). Among the recorded mutations, the P132L mutation of Cav-1 has been shown to cause inactivation of Cav-1 by producing misfolded Cav-1 oligomers that remained inside the Golgi complex and perinuclear space not directed to the cell membrane Lee, 2002). The current study revealed the absence of P132L target point mutation in all Cav-1 downregulated breast cancer patients (90%). Consequently, the alteration in the expression of Cav-1 was not due to the P132L target point mutation, but other factors may contribute to this down-expression. In a previous study, it was demonstrated that the disorder of expression of Cav-1 was due to conjugated linoleic acid (CLA) that may influence cell signaling in the breast cancer cell line (MCF-7) (Huot et al. 2010). The previous findings showed that the caveolin-1 gene was inactivated by aberrant promoter methylation in 7.3% of normal breast tissues and 25.5% of breast cancer tissues, indicating that the Cav-1 gene may be inactivated in precancerous lesions during the progression of breast cancer (Koike *et al.,* 2010).

The present results agreed with previous studies documenting the absence of sporadic P132L point mutation of Cav-1 in breast cancer patient tissues (Koike *et al.,* 2010; Patani *et al.,* 2012). On the contrary, the present results contradicted Hayashi, who found a P132L mutation of Cav-1 in six of fifty-five breast cancer patients (Hayashi *et al.,* 2001).

Conclusion: The present study found that Cav-1 protein was expressed in the plasma membrane and the cytoplasm of 5 (10%) patients and downregulated in 45 (90%) patients. To the best of our knowledge, this is the first study on Cav-1 expression in Egyptian breast cancer patients that documented the absence of P132L target point mutation in all Cav-1 downregulated Egyptian breast cancer patients (90%). Furthermore, the alteration in expression of Cav-1 in Egyptian breast cancer patient tissues is not due to the P132L target point mutation, but other factors may contribute to this down-expression.

Declarations:

Ethical Approval: This study forms part of a larger study that received ethical clearance from Institutional Review Board of Ain Shams University Hospitals Ethics Committee.

Conflict of interests: The authors declare no conflicts of interest.

Authors Contributions: Mona M. Mohamed, Salwa Sabet, Mohamed El-Shinawi and Mohamed Hosney developed the concept and directed the research. Mona M. Mohamed, Salwa Sabet, Mohamed El-Shinawi and Mohamed Hosney carried out sample collection, laboratory and data analysis as well as manuscript draft preparation. All authors have read, reviewed, and approved the content of the last version of this manuscript.

Funding: This study received fund by Avon Foundation, USA and STDF, Egypt grant #

408 .

Availability of Data and Materials: The data presented in this study are available on request from the corresponding author.

Acknowledgements: Not Applicable.

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