Original Article

Evaluation of IP3R3 Gene Silencing Effect on Pyruvate Dehydrogenase (PDH) Enzyme Activity in Breast Cancer Cells with and Without Estrogen Receptor

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Abstract

Background: Inositol 1,4,5-trisphosphate receptor (IP3R), a critical calcium ion (Ca2+) regulator, plays a vital role in breast cancer (BC) metabolism. Dysregulated IP3R in BC cells can drive abnormal growth or cell death. Estradiol increases IP3R type 3 (IP3R3) levels in BC, promoting cell proliferation and metabolic changes, including enhanced pyruvate dehydrogenase (PDH) activity, which, when reduced, leads to cell apoptosis. The study silenced IP3R3 to assess its impact on PDH.

Materials and Methods: The study used IP3R3 small interfering RNA (siRNA) to target Michigan Cancer Foundation-7 (MCF-7) and MDA-MB-231 cell lines. Transfection success was confirmed by flow cytometry. Cell viability and gene silencing were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and real-time quantitative polymerase chain reaction (PCR) assays. Protein expression and cellular activity were analyzed through western blotting and PDH activity measurement.

Results: Transfecting MCF-7 and MDA-MB-231 cells with IP3R3 siRNA achieved a 65% transfection rate without significant toxicity. IP3R3 gene silencing effectively reduced IP3R3 messenger RNA (mRNA) and protein levels in both cell lines, leading to decreased PDH enzyme activity, especially in MDA-MB-231 cells.

Conclusion: The study highlights a link between high IP3R3 gene silencing and reduced PDH activity, with higher IP3R3 expression in estrogen-independent (MDA-MB-231) compared to estrogen-dependent (MCF-7) cell lines. This suggests a potential impact on BC metabolism and tumor growth via regulation of PDH activity.

Keywords: Breast cancer, IP3R, PDH, siRNA

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Introduction

Breast cancer (BC) globally is the biggest cause of cancer-related deaths in women.[1] Each BC subtype has a different prognosis and course of treatment, and many prognostic factors determine the outcome of the disease. Metabolism alterations are the base of many cancer hallmarks, including migration, invasion, excessive proliferation, apoptosis evasion, and angiogenesis.[2] Therefore, it seems smart to focus on cellular metabolism to fight various cancers. Calcium ion signaling is one of the crucial biochemical pathways known as a notable characteristic of cancer in cancer metabolic pathways.[3]

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Ion flux deregulation, particularly calcium ion (Ca2+) flux, plays a role in several pathophysiological processes, including carcinogenesis. The intracellular control of Ca2+ release from the endoplasmic reticulum into the cytoplasm is notably managed by the inositol-trisphosphate receptor (IP3R). The proper functioning of IP3R, or its aberrations, can exert a considerable impact on various aspects of cancer cell behavior, including growth, migration, proliferation, and survival.[4] According to Singh *et al*.[5] (2017), breast tumors exhibit increases in IP3R isoform levels similar to many other malignancies.

Furthermore, prognostic elements like tumor size, regional node invasion, histologic grade, proliferation index, and hormone receptor status were positively linked with IP3R type 3 (IP3R3) expression in BC (but not IP3R type 1 [IP3R1] or IP3R type 2 [IP3R2]). IP3R3 is the subtype in BC tissue most significantly expressed.[6] More specifically, estradiol-induced IP3R3 overexpression in BC encourages Michigan Cancer Foundation-7 (MCF-7) BC cell proliferation *in vitro*.[7] Through its interactions with the voltage- and Ca2+-dependent potassium (K+) channels BKCa, IP3R3 also controls the proliferation of BC cell lines.[8] Changes in IP3R expression and activation under different pathophysiological circumstances lead to uncontrolled cell proliferation or cell death.[9,10] Malignant cells may exhibit decreased mitochondrial bioenergetics, increased adenosine monophosphate (AMP) kinases, and autophagy when IP3R-dependent calcium signals are inhibited.[11]

Metabolic reprogramming occurs along with BC, like other malignancies. The Warburg effect, or metabolic switch from glycolysis to oxidative phosphorylation (OXPHOS), is another feature of many malignancies. In the hypoxic tumor microenvironment, it increases the likelihood that cancer cells will survive and shields them from the lethal effects of oxidative damage and apoptosis.[12] The pyruvate dehydrogenase complex (PDC) and pyruvate dehydrogenase kinase (PDK) are the primary regulators of this metabolic shift. Pyruvate dehydrogenase (PDH) catalyzes the transformation of pyruvate into acetyl-CoA (coenzyme A), a component of the PDC complex.[13,14] PDH controls the glycolytic metabolism in cancer cells, acting as an oncogenic agent.[15]

One of the tumors where PDH activity is elevated is BC. Studies have been conducted on the function of estrogen receptors (ERs) in controlling metabolic processes in BC cells. 17-beta-estradiol (E2) increases glycolysis and inhibits the Krebs cycle in MCF-7 cells in the presence of high glucose concentrations by boosting protein kinase (AKT) activity. The mitochondrial pathways are triggered due to the drop in extracellular glucose. E2 then suppresses glycolysis, and raising PDH activity promotes the Krebs cycle and results in cell survival. In low glucose environments, PDH knockdown causes apoptosis and negates the effects of E2 on cell survival. The proposed investigations suggest that suppressing PDH and limiting the amount of glucose substrate will improve the treatment efficacy in ER-positive breast tumors.[16]

In malignancies, especially breast tumors, the normal physiological Ca2+ signaling activity is frequently hijacked and altered, resulting in a strong oncogenic drive within the implicated cell population.[17] In cancer cells, intracellular Ca2+ homeostasis is altered, and this disruption contributes to carcinogenesis, angiogenesis, progression, and metastasis, according to a growing body of research.[18,19] The Ca2+ generated through ER-IP3R channels are transported into mitochondria, where they govern the functioning of proteins, enzymes, and transporters crucial for organelle metabolism, impacting both the matrix and intermembrane space. Ca2+ specifically regulates PDP's (PDH phosphatase) activity. High mitochondrial [Ca2+] promotes PDP activity, which in turn promotes PDH dephosphorylation and, ultimately, its activity.[20] The cessation of the constitutive IP3R-mediated Ca2+ transport to mitochondria results in a bioenergetic crisis in transformed primary human fibroblasts, breast and prostate cancer cells, and non-tumorigenic cells. Cancer cells selectively die in response to this bioenergetic crisis.[11]

According to the studies, it was observed that the expression of IP3R3 channels is increased in BC and their role in cell metabolism and proliferation has been proven. On the other hand, the effect of estradiol on cell proliferation has been observed through increasing PDH activity. IP3R3 may mediate the effect of estradiol on increasing PDH activity. To investigate this issue, in this context we turned off IP3R3 to evaluate the level of PDH activity. By clarifying this molecular pathway, it may be possible to provide a treatment solution for BC. Although these paths are very complicated and their detailed investigation takes a lot of time.

Materials and Methods

We acquired two human cell lines, MCF-7 and MDA-MB-231, from the Pasteur Institute of Iran. IP3R3 siRNA (Cat No sc-42477.) and scramble small interfering RNA (siRNA) were procured from ABM and Mirus Bio companies, respectively. The siRNA Transfection Reagent (Dharmacon, Thermo Scientific, USA) was sourced from Santa Cruz, USA. We obtained the Pyruvate Dehydrogenase (PDH) Combo (Activity + Profiling) Microplate Assay Kit (Cat No. ab110671) from Abcam, USA. For the anti-IP3R3 antibody, we purchased Cat No. sc-377518 from Santa Cruz, USA. Trizol reagent was obtained from Invitrogen, USA. The complementary deoxyribonucleic acid (cDNA) synthesis kit was supplied by BIOFACT, Korea. In addition, we acquired the ExiLENT SYBRGreen master mix kit from Exiqon, Denmark, and the primers were ordered from Pishgam Company in Iran.

Cell Culture

The two cell lines were grown in RPMI 1640 medium (Hyclone SH30027.01), which was enriched with 10% fetal bovine serum (FBS) (Hyclone SH30070.03) and 1% penicillin-streptomycin (Hyclone SV30030). They were maintained in an incubator set at 37°C with a gas mixture of 95% air and 5% carbon dioxide (CO_2).

Transfection of IP3R3 siRNA and Scramble

About 5 × 10^5 cells were cultured in each well of the 6-well plate. After 24 h and reaching the confluency were transfected with IP3R3 siRNA and scrambled using the siRNA Transfection Reagent according to the manufacturer's instructions. Scramble is conjugated by FITC to measure the transfection rate. After 6 h, the cells were checked for the presence of fluorescein by flow cytometry to confirm the transfection.

Cell Viability

We used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the impact of siRNA on cell viability. Initially, 10^4 cells were plated in 96-well plates with RPMI 1640 + 10% FBS. After 24 h, cells were washed with phosphate-buffered saline (PBS), treated with 10 µL MTT, and incubated. Finally, we added 100 µL dimethyl sulfoxide (DMSO) to dissolve the crystals and measured the optical density at 570 nm using a microplate reader.

Real-time quantitative polymerase chain reaction (PCR)

To investigate the effect of transfected siRNA on IP3R3 silencing at the messenger RNA (mRNA) level, after 48 h, total RNA was extracted by RNA extraction kit. The cDNA was made by cDNA synthesis kit and finally, quantitative real-time PCR was performed using StepOnePlus real-time polymerase chain reaction (PCR) (AppliedBiosystems, USA). *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* as a housekeeping gene was used to normalize the results. Livak method was used to evaluate the foldchange [Table 1].

Western Blot

We assessed the expression of the IP3R3 protein through western blot analysis following a standard protocol. After 48 h of transfection, cell lysis was carried out using radioimmunoprecipitation assay buffer (RIPA Lysis Buffer System: sc-24948), as per the manufacturer's instructions. Subsequently, the extracted protein was subjected to western blot analysis using a mouse monoclonal anti-IP3R3 antibody and an anti-beta actin antibody. Beta-actin served as the ideal loading control in western blotting due to its consistent expression across all eukaryotic cell types, unaffected by cellular treatments.

Pyruvate Dehydrogenase (PDH) Activity Measurement

Forty-eight hours after transfecting with siRNA and scramble, the cells were scraped, collected, and washed using PBS. PDH activity was evaluated using the PDH Combo (Activity + Profiling) Microplate Assay Kit *according* to the *manufacturer's instructions*.

Table 1: Primers used for quantitative real-time PCR

IP3R3-F	AAGAACCAGGAGCACATTG
IP3R3-R	ACAAGGCTGACGAAGGTC
GAPDH-H-F	AGTCCACTGGCGTCTTCA
GAPDH-H-R	GAGGCATTGCTGATGATCT

IP3R=Inositol-trisphosphate receptor type 3, GAPDH=Glyceraldehyde 3-phosphate dehydrogenase , IP3R3-F=IP3R3 forward primer, IP3R3-R=IP3R3 reverse primer, GAPDH-H-F=GAPDH forward-hybridization primer, GAPDH-H-R=GAPDH reverse-hybridization primer

Results

Transfection of IP3R3 siRNA and Scramble

To determine the role of IP3R3 gene silencing in MCF-7 and MDA-MB-231 cell lines, both cell lines were transfected with target siRNA and scrambled separately. The transfection rate was around 65% [Figure 1a].

Evaluation of siRNA Effect on the Cell Viability

MTT assay was performed to study the specific cell toxicity of the siRNA. For this purpose, MCF-7 and MDA-MB-231 cell lines were transfected with siRNA. Considering Figure 1b, both the treated cell lines indicated no significant toxicity after being transfected with siRNA.

mRNA Expression Level of IP3R3

After transfection, the IP3R3 mRNA levels were measured in transfected and non-transfected MCF-7 and MDA-MB-231 cell lines by quantitative real-time PCR. The IP3R3 mRNA expression level in MDA-MB-231 was more than MCF-7 cells ($P = 0.0015$) [Figure 2a]. After 48 h of transfection, the IP3R3 level was decreased in the transfected MDA-MB-231 cells compared to un-transfected MDA-MB-231 ($P = 0.0011$) and in transfected MCF-7 cells in comparison with un-transfected MCF-7 cells ($P = 0.0001$) [Figure 2b]. These results confirmed the efficiency of siRNA on IP3R3 silencing. The IP3R3 level in cells transfected with scramble did not change significantly compared to the control group.

Protein Expression Level of IP3R3

The IP3R3 protein levels in transfected and non-transfected MCF-7 and MDA-MB-231 cell lines were measured by western blot 48 h after transfection. The IP3R3 protein expression in MDA-MB-231 cells was higher compared to MCF-7. After silencing of the IP3R3 gene, IP3R3 protein expression decreased significantly in both cell lines ($P = 0.0001$). The IP3R3 level in cells transfected with scramble did not change significantly compared to the control group ($P = 0.501$) [Figure 2c].

Pyruvate dehydrogenase (PDH) activity measurement

The results of the PDH enzyme activity test show that the activity of the enzyme is generally significantly higher in MDA-MB-231 cells than in MCF-7 cells. After confirming the silencing of the *IP3R3* gene, the activity level of this enzyme was investigated in treated and untreated cells. Figure 3 shows that the enzyme activity level has decreased significantly in the treated cells [Figure 3].

Discussion

Our present results showed that high IP3R3 gene silencing was significantly associated (linked) with decreased PDH activity. Moreover, we report increased expression of IP3R3

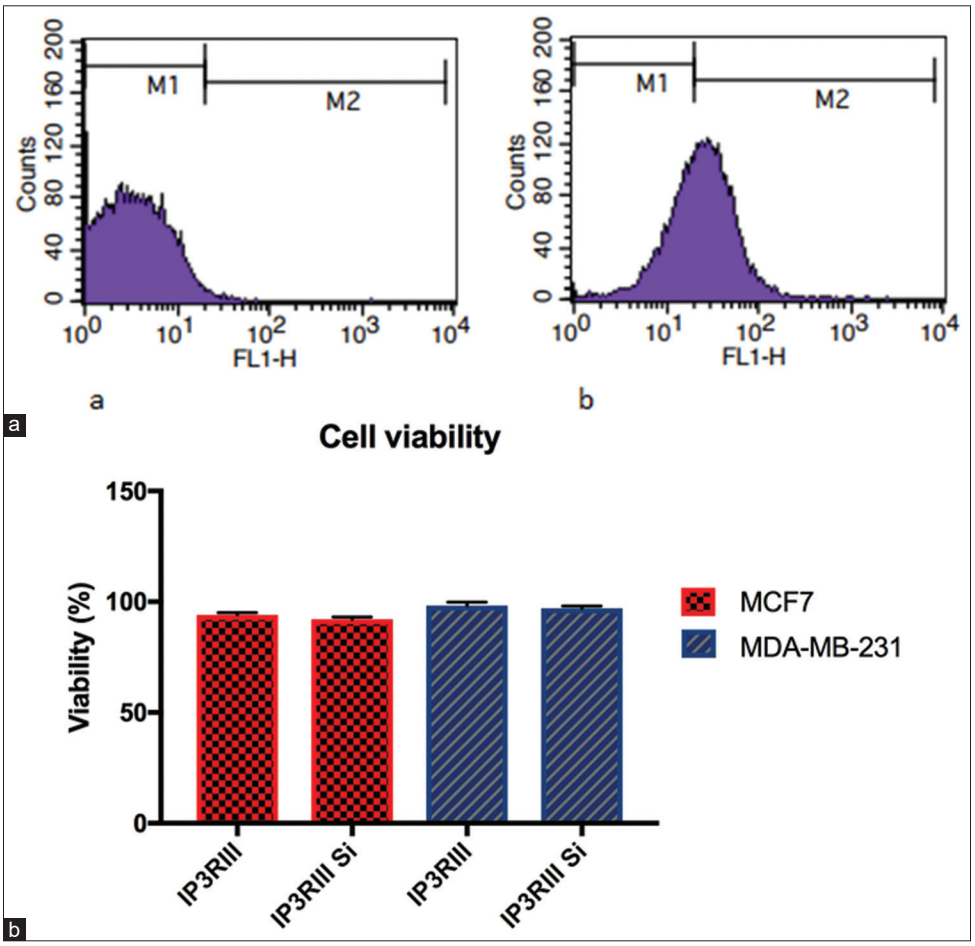

Figure 1: (a) Flow cytometry analysis for the transfection efficiency of the cells with siRNA.(a) un-transfected cells.(b) transfected cells. About 65% of the cells were transfected (b) Viability assay of MCF-7 MDA-MB-231 cell lines, transfected with siRNA. Each test was performed three times, and results are presented as the means ± SD. *Abbreviations*: MCF-7 = Michigan Cancer Foundation-7. siRNA = small interfering RNA, SD = standard deviation

in estrogen-independent (MDA-MB-231) compared to estrogen-dependent (MCF-7) cell lines. Our results conclude that in BC metabolic pathways, there is a link between the expression of IP3R3 and PDH activity. As PDH controls the transition between glycolysis and oxidative phosphorylation and thereby fosters the development of tumors,[21] IP3R3 gene silencing affects tumor growth.

Our current findings reveal distinct IP3R3 subtype expression patterns in two human BC cell lines: the estrogen-sensitive MCF-7 and estrogen-insensitive MDA-MB-231. Notably, MDA-MB-231 exhibits significantly elevated IP3R3 expression levels. Similar to our findings, it was shown that the MDA-MB-231 cell line expressed more IP3R3 RNA and protein than the MCF-7 cell line. The highly migratory MDA-MB-231 cell line was more affected by IP3R3 gene silencing than the MCF-7 cell line in terms of reduced cell migration. They have concluded that higher IP3R3 expression levels boost the ability of human BC cells to migrate.[22] Previous research contradicted our findings, stating that IP3R3 is the isoform capable of positively regulating the estrogen-dependent MCF-7 cell line's 17-beta estradiol-induced proliferation. It has been shown that E2 increases the expression of IP3R3. When E2 is added, IP3R3 inhibition through medication or the use of particular small inhibitory RNAs reduces cell proliferation.[7]

In addition, a recent study came to the general conclusion that IP3R3 expression is increased and a predictive factor in BC. Prognostic variables like tumor size, regional node invasion, histologic grade, proliferation index, and hormone receptor status were all positively linked with IP3R3 expression.[6] It was shown that IP3R3 is overexpressed in multiple cancer tissues. IP3R3 is a key player in the development of cancer as its expression level is associated with the aggressiveness of colorectal cancer,[23] while its inhibition decreases the proliferation of BC cells,[7] glioblastoma cell migration, invasion, and survival.[24] Kang *et al*.'s excellent demonstration shows that caffeine can prevent glioblastoma cells from migrating and invading *in vitro* at concentrations that inhibit IP3R3 preferentially to the two other IP3R subtypes.[24]

Although we report that PDH activity was more in the estrogen-independent MDA-MB-231 cell line, It was previously demonstrated that In MCF-7 cells, 17-estradiol (E2)

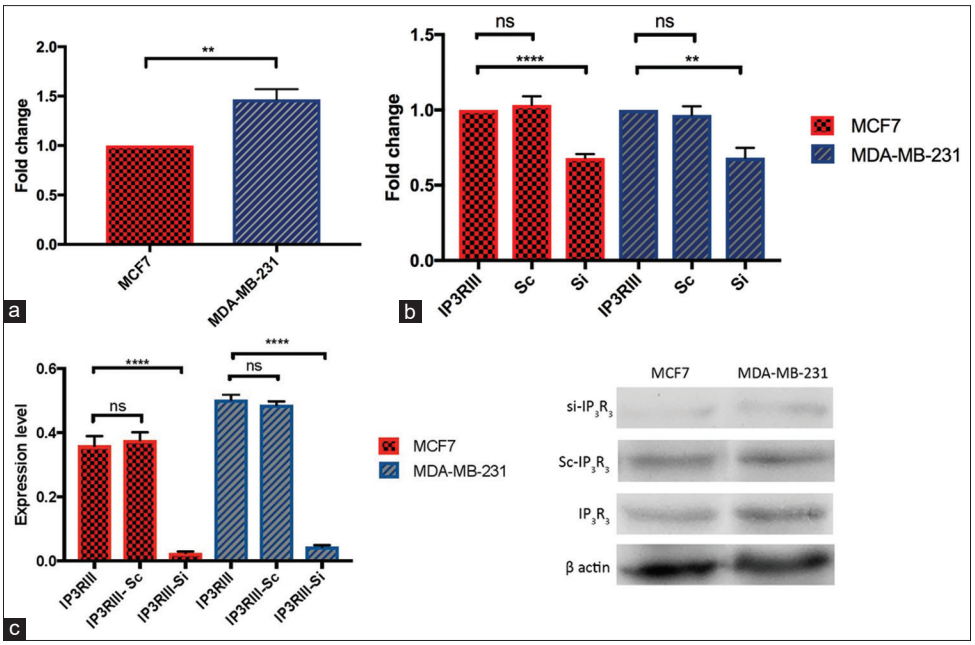

Figure 2: (a) IP3R3 mRNA relative expression in MCF-7 and MDA-MB-231 was quantified using RT-qPCR and results are expressed as means ± SD (b) The IP3R3 mRNA level was measured in the transfected MDA-MB-231 and MCF-7 cells compared to the un-transfected (c) We examined IP3R3 protein expression through Western blotting in transfected and un-transfected MCF-7 and MDA-MB-231 cells. Results are reported as the mean ± SEM of the IP3R3/β-actin protein ratio, with β-actin as the loading control. These findings represent the average of three independent experiments. *Abbreviations*: IP3R = inositol-trisphosphate receptor type 3, MCF-7 = Michigan Cancer Foundation-7, mRNA = messenger RNA. siRNA = small interfering RNA, RT-qPCR = real-time quantitative polymerase chain reaction, SEM = standard error of the mean, SD = standard deviation. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. NS = non-significant

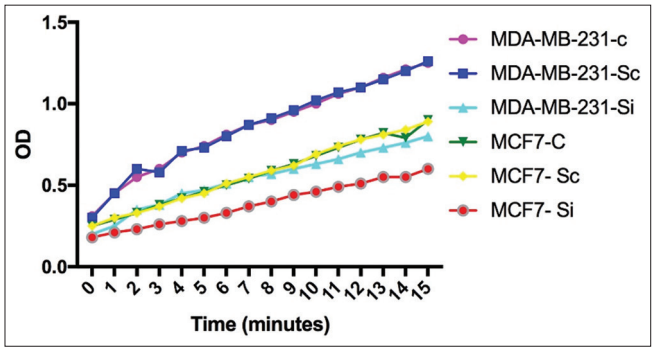

Figure 3: The pyruvate dehydrogenase enzyme activity of MDA-MB-231 and MCF-7 cells in the transfected and un-transfected conditions. *Abbreviations*: MCF-7 = Michigan Cancer Foundation-7

inhibits glycolysis and restores cell viability by promoting the tricarboxylic acid cycle through the upregulation of PDH activity. They have found a crosstalk between ERs and PDH activity in BC.[25] It was suggested that PDH knockdown and reducing glucose levels are effective treatments for BCs with ERs. Through the phosphorylation of serine 19 by AMP kinase (AMPK) and inhibition of PDH kinase 4 (PDK 4), which inactivates PDH, E2 increases PDH activity.[26]

Our gene silencing of IP3R3 did not negatively impact the viability of cells. Specifically, IP3Rs play a pivotal role in influencing cell fate, as they enable the release of Ca2+ from the endoplasmic reticulum (ER), subsequently influencing mitochondria-associated processes, including bioenergetics and apoptosis, that govern cell survival and death.[27] In contrast to these findings, a different study found that IP3R inhibition of BC cells significantly impacted autophagic cell death.[28] When IP3R was attenuated for an extended time, and IP3R failed to maintain a normal functional pool, it was typically shown[29-31] that the cell survival response (autophagy) could be harmful to cell death.

The intrinsic apoptotic pathway, induced by MOMP stimuli, is being created due to the IP3R's failure to restore its normal activity.[32,33] Other studies demonstrate that blocking IP3R in tumorigenic cells results in insufficient Ca2+ signal transmission, which halts cell cycle progression and reduces cell proliferation, causing the cell cycle to be paused by inducing apoptosis. Similarly, tumorigenic cells exhibit enhanced apoptosis when IP3Rs are blocked by exogenous pharmacological inhibitors. In addition, malignant cells may exhibit decreased mitochondrial bioenergetics, increased AMP kinases, and autophagy when IP3R-dependent calcium signals are inhibited.[11]

Investigating the unique metabolic pathways associated with different subtypes of BC could pave the way for tailored therapeutic strategies, targeting their specific alterations. The PDH complex, serving as a metabolic controller, holds a pivotal position in governing the cellular metabolic destiny. It determines whether the pyruvate generated during glycolysis will proceed through oxidative phosphorylation,

subsequently leading to mitochondrial respiration, or opt for lactate fermentation.[34] This assessed the activity levels of PDH in BC cell lines. Notably, MDA-MB-231 cells exhibited higher PDH activity compared to MCF-7 cells, indicating elevated PDH activity in estrogen-independent cell lines.[35] In a separate study, researchers examined the expression of PDH-E1A, a vital component of the PDC responsible for catalyzing the redox decarboxylation reaction within the tricarboxylic acid (TCA) cycle, across various BC cell lines. The findings established a significant correlation between PDH-E1A expression and ER expression in BC. It was observed that the majority of ER-positive patients lacked PDH-E1A expression, whereas ER-negative individuals tended to exhibit PDH-E1A expression. BC cell lines demonstrated an increase in the expression of PDH-E1A, MDA-MB-231 (4.7 times), and MCF-7 as compared to human mammary epithelial cells (2.9 folds).[35] Previous research showed that MCF-7 cells produced less lactate and used less glucose than MDA-MB-231 cells.[36,37] According to another study, Inositol 1, 4, and 5 Triphosphate Receptors are highly expressed in BC patients. Results obtained *in vitro* demonstrated that siRNA silencing and IP3R3 suppression lowered glucose uptake and pyruvate levels in MCF-7 and MDA MB-231 BC cells.[5] These alterations are connected to cancer cells' increased rate of glycolysis.

In conclusion, our study reveals a significant link between IP3R3 gene expression and BC metabolism. High IP3R3 gene silencing is associated with decreased PDH activity, impacting the transition between glycolysis and oxidative phosphorylation. In addition, we highlight distinct IP3R3 expression profiles in estrogen-dependent and -independent cell lines, with potential implications for cancer cell migration. These findings emphasize the relevance of IP3R3 in BC metabolism, offering insights into targeted therapeutic approaches for different subtypes.

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Conflicts of interest

There are no conflicts of interest.

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