Original Article

Evaluation of *MYC* Oncogene Expression in Human Breast Cancer and Its Relationship with ACSL4 and Lipin-1 Expression

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Abstract

Background: Disturbances in lipid metabolism are one of the hallmarks of cancer cells. Fatty acid synthesis and oxidation play a crucial role in the proliferation, growth, and survival of cancer cells. Several enzymes are involved in lipid metabolism. MYC is an oncogene and plays various regulatory roles in lipid metabolism. This study aimed to evaluate *MYC* expression and its association with the expressions of Lipin-1, ACSL4 (enzymes involved in lipid metabolism), in pairs of breast cancer (BC) and adjacent normal tissues to further understand the *MYC* influence on metabolic regulation.

Materials and Methods: Fifty-five pairs of samples of BC and noncancerous adjacent tissues were utilized in the present study to analyze *MYC*, *Lipin-1,* and *ACSL4* by quantitative real-time polymerase chain reaction. Further, the expression of Lipin-1 and ACSL4 proteins and a number of other clinicopathologically relevant variables were studied employing immunohistochemistry staining.

Results: *MYC* expression was substantially higher in BC tissues than in adjacent normal tissues, according to our findings. This upregulation was positively correlated with tumor size and stage. Although *MYC* expression was not correlated with that of ACSL4 and Lipin-1 expression, this may result from the complex metabolic changes that occur when cells become malignant.

Conclusions: Although further research is required to assess MYC's impact on tumor metabolic regulation, the correlations seen here between *MYC*, the pathological stage, and tumor size may indicate its prognostic significance in BC. Hence, it may be considered as a potential therapeutic target for further studies.

Keywords: ACSL4, breast cancer, Lipin-1, MYC

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Introduction

Cancer is a major cause of death worldwide. By 2030, it is estimated that a quarter (25%) of the world's population will experience at least one form of cancer.[1]

Although cancer cells differ in type and etiology,[2] they have indicated metabolic adaptation to produce the energy necessary for rapid cell division. Some evidence also suggests that all "metabolic alterations" are caused by oncogene activation in cancer cells.[1] Oncogenes have a vital role in cellular metabolism,[3] leading to a raised nutrient uptake to offer to the energetic and biosynthetic pathways.[4]

The MYC oncogene acts as a transcription factor and participates in diverse regulatory processes such as cellular

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proliferation, metabolism, apoptosis, angiogenesis, and immune evasion.[5,6] In general, MYC raises the expression of growth-related genes and suppresses that of growth-inhibiting genes.[7] Deregulated expression of MYC reprograms metabolism and contributes to tumor growth.[8] MYC induces energy generation and macromolecular synthesis via glycolysis and glutaminolysis adjustment. In addition, some studies have indicated that MYC induces sterol regulatory element-binding proteins (SREBP-1).[5] Further, SREBP-1, as a transcription factor, modulates the expression of genes involved in triacylglycerol (TG), cholesterol, fatty acid, and phospholipid (PL) synthesis.[9] Therefore, it is postulated that MYC regulates lipid metabolism.[5]

Metabolic reprogramming, such as altered lipid metabolism, is among the hallmarks of cancer cells.[1] It is indicated that changes in lipid metabolism in cancer cells are needed to increase proliferation, progression, and metastasis.[10] Studies have also shown that cancer cells can perform de novo lipogenesis at a similar rate to the liver.[11] Because fatty acids are involved in signaling and are used to synthesize many different types of lipids, including TGs as energy storage and PLs as cell membranes,[12] fatty acid oxidation (FAO), a key pathway for energy production, converts fatty acids to acetyl-CoA in the mitochondria, providing essential support for the growth and survival of cancer cells.[13]

Lipin-1 has phosphatidate phosphatase (PAP) activity. It generates diacylglycerols (DAGs) from phosphatidate and catalyzes the key regulatory step in the synthesis of TGs and PLs.[14,15] Lipin-1 overexpression has been reported in prostate cancer and breast cell lines; our previous study showed an increase in Lipin-1 in human breast cancer (BC) tumor samples.[15] We also indicated that Lipin1 inhibition represses BC cell line migration.[12]

Many metabolic processes require the activation of fatty acids to the corresponding fatty acyl-coenzyme A ester. This activation is catalyzed by the fatty acyl-CoA synthetase (ACS) enzyme family. The family comprises five isoforms (ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6) categorized based on the preferred chain length of their substrates, ranging from short to very long.[10] ACSL4 is significantly upregulated in specimens of breast, prostate, liver, and colon cancer.[8] We previously reported ACSL4 overexpression in breast tissue samples.[4]

Since there are few studies about the correlation between *MYC* and lipid metabolism,[8] understanding the role of MYC in lipid metabolism may be of interest.[5] Therefore, this work investigated Lipin-1, ACSL4 (lipid metabolism enzymes), and *MYC* expression in pairs of BC and adjacent normal tissues to better understand the MYC impact on cancer metabolic regulation.

Methods and Materials

Tissue collection

We obtained 55 pairs of human BC and adjacent normal tissues from individuals who underwent surgical excision at Ordibehesht Hospital in Isfahan, Iran, between 2016 and 2017. Following resection, the major part of the patient's specimen was fixed in formalin for pathological investigation, and a small part of the sample was frozen immediately in liquid nitrogen and stored at −80°C until ribonucleic acid (RNA) extraction. An experienced pathologist confirmed the histological details of the tumor, such as the histology, grade, size, and stage, on slides prepared from paraffin-embedded tissues. Each participant provided informed consent, and the study was approved by the Isfahan University of Medical Sciences Ethics Committee under Ethics Code 396510.

Evaluation of gene expression changes

The BioFACT™ Total RNA Prep Kit (Ver. 2.0, BioFACT, Daejeon, Korea) was used to isolate total RNA. Complementary deoxyribonucleic acid (cDNA) was produced using the BioFACT™ RTKit (BioFACT, Daejeon, Korea). The produced cDNA was then used to analyze a quantitative real-time polymerase chain reaction (RT-PCR) utilizing the BioFACT™ 2X Real-Time PCR master mix for SYBR green I (Applied Biosystems, USA). According to the planned cycle schedule, the real-time PCR reaction was carried out.[12] The sequences of the primers utilized are presented in Table 1.

The cycle threshold value is the number of cycles that pass the fluorescence intensity above the background.[16] The ΔCt method was used to compare the expression of each gene with β-actin, as an internal control gene, (ΔCt = Ct target − Ct β-actin). The $2^{-\Delta\Delta Ct}$ technique was used to calculate the fold change, representing the quantity changes between the tumor group and its control group. Finally, the results were reported as the mean ± standard error (SE).

Immunohistochemistry

Immunohistochemistry (IHC) staining was performed on paraffin-embedded tissues, as described elsewhere.[12] In brief, 5 μm-thick slices were affixed to poly-L-lysine-coated slides. The slices were dewaxed, antigen was retrieved, and endogenous peroxidase was inhibited utilizing H_2O_2. Then, the tissue slices were stained with primary antibodies: anti-Lipin-1 (ab181389, Abcam) and anti-ACSL4 (sc-271800; Santa Cruz, USA), followed by the addition of a secondary antibody conjugated to horseradish peroxidase. The visualization of these sections was then performed using the chromogenic substrate 3′-diaminobenzidine tetrahydrochloride. The background was stained with hematoxylin. Using this method, sections that had Lipin-1 and ACSL4 overexpression displayed brown staining. Using ImageJ software (version 1.52h), 40 amplification Olympus light microscope photomicrographs were quantified, and the results were shown as Pix/m^2.

Ki-67, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2) expression were further determined by the IHC technique.

Statistical data analysis

Statistical analysis was conducted by the Statistical Package for the Social Sciences (SPSS) software (version 21, IBM

Corporation). The data were expressed as the mean ± Standard Error of Mean (SEM). The results of the gene expression experiments were examined using t-tests between the tumor and control groups. The χ^2 test was used to compare associations between *MYC* expression and other parameters.

Results

MYC mRNA expression in BC and adjacent normal tissue

qRT-PCR was used to determine *MYC* expression in 55 pairs of human BC specimens and adjacent normal tissue. These findings revealed that *MYC* messenger RNA (mRNA) expression in BC tissues was considerably higher than in matched adjacent normal tissues [Figure 1]. The $2^{-\Delta\Delta Ct}$ method was used to analyze the results. Lipin-1 and ACSL4 overexpression have also been shown in our previous studies.[4,12]

ACSL4 and Lipin-1 protein expression in BC and adjacent normal tissues

To approve the gene expression outcomes, 20 pairs of BC specimens were randomly selected, and the expression of ACSL4 and Lipin-1 proteins were examined via IHC staining with their specific antibodies. Consistent with the RT-PCR data, the Lipin1 and ACSL4 expressions were considerably higher in BC tissues as compared to adjacent normal tissues [Figure 2].

The association between MYC, Lipin-1, and ACSL4 mRNA expression and clinicopathological properties of BC

Table 2 represents the relationship between *MYC*, Lipin-1, and ACSL4 mRNA expression and clinicopathological properties in BC patients. High *MYC* expression was shown to be associated with tumor size ($P = 0.033$) and tumor stage ($P = 0.047$) in these studies. No significant relationship was observed between *MYC* expression and Lipin-1 or ACSL4 expression or other clinicopathological variables (P values > 0.05).

Discussion

According to several studies, alteration in fatty acid metabolism is a hallmark of several types of cancer and is essential for adaptation to increased proliferation and tumor development.[10] Thus, inhibition of lipid metabolic pathways may be considered a therapeutic strategy.[12] In addition, dysregulated lipid metabolism involves even drug tolerance in tumor cells.

Our previous studies showed that the expression of Lipin-1 and ACSL4, important enzymes in lipid metabolism, exhibited a significant increase in tumor tissues compared to adjacent normal tissues. In these articles, we also showed that Lipin-1 and ACSL4 could be considered new independent prognostic factors through their correlation with clinicopathological variables of tumors.[4,12]

Unlike normal cells, regulators of FA synthesis in cancer cells are unknown. Some studies have demonstrated that MYC regulates almost all stages of lipogenesis by inducing SREBP-1, which is a transcription factor[5] and a key regulator in lipogenesis.[17]

A study by Chen J *et al*.[17] indicated that in hepatocellular carcinoma cells, ACSL4 upregulates SREBP-1 and its

Table 1: The sequences of the primers used in the study

Genes	Forward sequences	Reverse sequences
ACSL4	5′-AGAATACCTGGACTGGGACCGAAG-3′	5′-TGCTGGACTGGTCAGAGAGTGTAA-3′
Lipin-1	5′-CACAATCAAGGAGGAAAGTAA-3′	5′-GCTGACATTAGGCAGAAGA-3′
MYC	5′-GCTCGCCCAAATCCTGTA-3′	5′-TCCACAGACACCACATCAA-3′
β-actin	5′-GTTGTCGACGACGAGCG-3′	5′-GCACAGAGCCTCGCCTT-3′

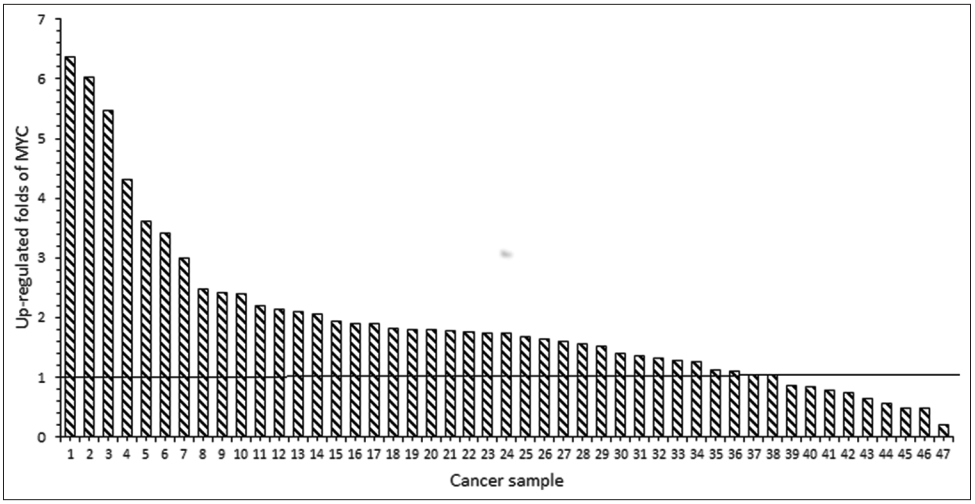

Figure 1: *MYC* mRNA expression in BC and adjacent normal tissues; real-time polymerase chain reaction was carried out to assess the *MYC* expression level. The mRNA expression data were normalized to the beta-actin (ACTB) signal. In each sample pair, the fold change of ACSL4 expression was obtained utilizing $2^{-\Delta\Delta CT}$ as columns, mean ± SEM. *$P < 0.01$ showed a significant difference from the normal group

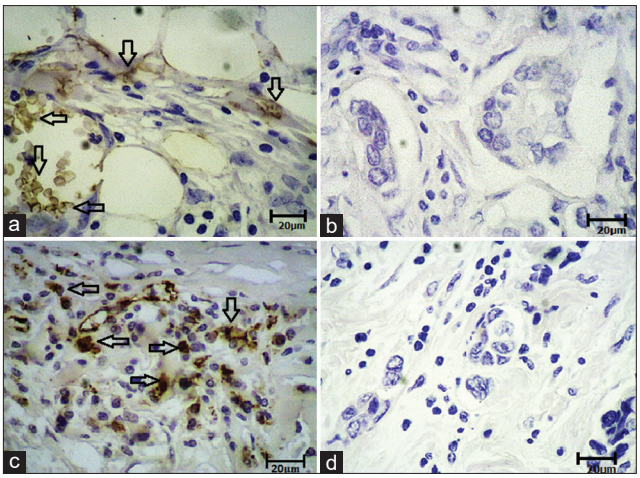

Figure 2: Photomicrograph images of IHC staining of ACSL4 and Lipin-1 in sections of (a and c) breast tumor tissue and (b and d) the adjacent normal breast tissues; brown staining displays the high expression of the proteins in breast tumor tissues. The protein expression quantification was assessed usinging ImageJ software by an Olympus light microscope with ×40 amplification (Pix/μm^2)

downstream lipogenic enzymes by mediating the stability of MYC. In addition, SERBP-1 is crucial for ACSL4-mediated regulation of lipogenesis, thus indirectly activating fatty synthesis genes.

In addition, some studies have also demonstrated that Lipin-1 is a target of SREBP-1. Lipin-1 acts as a transcriptional coactivator in the nucleus and is also a cytosolic PAP, which by activating DAG production, is involved in triglyceride synthesis.[18] Therefore, it can be expected that the expression of ACSL4, lipin-1, and MYC are related to BC tissue.

Nevertheless, in this study, we found no correlation between *MYC* expression and ACSL4 and/or Lipin-1 expression. Considering the different roles reported for ACSL4 and Lipin-1 in cancer tissues, for example, ACSL4's role in the activation of long-chain fatty acid and thus in the fatty acids β-oxidation, as well as in processes such as immune signaling transduction and increasing ferroptosis in cancer cells (ferroptosis is a recently discovered form of iron-dependent cell death, distinguished by the occurrence of lipid peroxidation induced by reactive oxygen species (ROS)),[19] and the significance of high Lipin-1 expression as a good prognostic factor in patients with BC, as reported in our previous study.[12] Therefore, the absence of correlation observed in our study likely reflects the complex regulatory processes in the lipid metabolism of cancer cells and suggests that other factors (e.g., other oncogenes and tumor suppressors) also influence the regulation of ACSL4 and Lipin-1 expression in BC. On the other hand, this may be a result of differences in the half-life of measured mRNAs or the complex metabolic changes that occur when cells become malignant.

According to the present research data, *MYC* expression was related to pathological stage and tumor size. There was no association found between *MYC* expression and that of ER, PR, HER2, and Ki-67 in BC tissues. The pathological stage is the main factor in diagnosing survival and choice of treatment for BC. Thus, the advanced stages (III and IV) of the disease have a worse prognosis than the early stages (I and II).[18] In agreement with our study, Schulze M *et al*.[20] reported the MYC target scores to be related to tumor aggressiveness and worse prognosis in ER-positive primary tumors and metastatic BC.

Ki-67 serves as a cellular proliferation marker associated with a poor prognosis. We also confirmed its relationship with histological tumor grade, ER, PR, and a poor prognosis in BC.[12]

The ER, PR, and HER2/neu expression are predictive and prognostic biomarkers in breast carcinoma.[21] The ER and PR

Table 2: The relationship between *MYC* mRNA expression and other variables

Parameter	*MYC* mRNA expression†		
	<1.73	≥1.73	*P*§
Age (years)			
≥50	15 (60%)	12 (54.5%)	0.706
<50	10 (40%)	10 (45.5%)	
Tumor size			
≥2 cm	7 (29.2%)	1 (4.8%)	0.033*
<2 cm	17 (70.8%)	20 (95.2%)	
Grade			
1	3 (12.0%)	0 (0.0%)	0.299
2	15 (60.0%)	13 (59.1%)	
3	7 (28.0%)	9 (40.9%)	
Stage			
1	3 (12.5%)	1 (4.8%)	0.047*
2	19 (79.2%)	12 (57.1%)	
3	2 (8.3%)	8 (38.1%)	
ER‡			
+	21 (87.5%)	15 (75.0%)	0.284
−	3 (12.5%)	5 (25.0%)	
PR‡			
+	18 (78.3%))	13 (65.0%)	0.334
−	5 (21.7%)	7 (35.0%)	
HER‡			
+	6 (25.0%)	7 (35.0%)	0.469
−	18 (75.0%)	13 (65.0%)	
Ki67‡			
≥20%	9 (39.1%)	10 (47.6%)	0.570
<20%	14 (60.9%)	11 (52.4%)	
Lipin-1			
<2.28	10 (43.5%)	8 (47.1%)	0.822
≥2.28	13 (56.5%)	9 (52.9%)	
ACSL4			
<1.02	9 (37.5%)	12 (54.5%)	0.246
≥1.02	15 (62.5%)	10 (45.5%)	

†The *MYC*, *Lipin-1*, and *ACSL4* mRNA expressions were measured based on ACTB in tumor and adjacent normal tissues with $2^{-\Delta\Delta ct}$ from at least two experiments. ‡Ki-67, ER: estrogen receptor; PR: progesterone receptor; and HER expression were measured using the IHC method, and the results are reported as percent of expression. §All *P* values are for the Chi-square test, and significant *P* values are shown by*

expressions are associated with lower mortality risk,[22] whereas HER2/neu overexpression indicates a poor prognosis.[23]

While an inverse correlation between ER and PR with Ki-67 was confirmed in our previous study,[4] indicating the association of Ki-67 with a poor BC prognosis, in this study, the correlation in the expression of *MYC* with other factors, that is, ER, PR, HER2, and Ki-67 is found to be insignificant. One reason for this lack of relationship could be attributed to investigating the correlation between mRNA expression and protein expression. Another reason might be that the metabolic regulations investigated herein are far more complicated. It is suggested to consider this aspect in future studies.

Conclusion

The present study demonstrated the upregulation of MYC expression in BC samples compared to the adjacent normal tissues. Although no association seems to exist between *MYC* mRNA expression and those of Lipin 1 and ACSL4, the relationship between *MYC*, pathological stage, and tumor size suggests its prognostic significance in BC. Hence, it might be considered a potential therapeutic target for further studies.

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Conflicts of interest

There are no conflicts of interest.

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