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LEP and FOXO1 genes, as a proposed tumor suppressor autophagic cell death related genes, can be targeted by antidiabetic therapy in nondiabetic breast cancer patients

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Abstract

Introduction Breast cancer can be treated effectively with personalized, gene-targeted therapies due to its molecular and genetic differences. Our study aims to identify breast cancer-specific tumor suppressor genes related to autophagic cell death and discover new drugs that target these mechanisms, even if they are not breast cancer-specific.

Materials and methods Gene intensity values of 457 tumor and 19 healthy breast tissues were used to determine downregulated and upregulated genes related to autophagy and apoptosis using Bioconductor R program via LIMMA package. Then, genes affecting survival were identified by survival analysis via Kaplan-Meier Plotter tool. Furthermore, the signalling pathways associated with these genes and targeting candidate drug components were determined by gene enrichment analysis using "KEGG pathway option" and Drug MATADOR in "ShinyGo 0.82" web-tool, respectively.

Results Breast cancer tumor tissues showed downregulation of genes related to autophagy and apoptosis (c19orf12, CRYAB, LEP, SRPX, SNCA, FOXO1) and upregulation of others (SLC7A5, ATP2A2, INHBA, ATP5IF1). Among these, SLC7A5, c19orf12, LEP, SPRX, SNCA, and FOXO1 affected patient survival and prognosis. The AMPK signaling pathway, targeting FOXO1 and LEP, was identified as key. Only the LEP gene was targeted by Metformin, Pioglitazone, Rosiglitazone, and Troglitazone.

Conclusion In our study, survival associated LEP and FOXO1 genes were identified as candidate tumor suppressor genes associated with autophagic cell death in non-obese and non-diabetic breast cancer patients. Anti-diabetic drugs such as Metformin, Pioglitazone, Rosiglitazone, Troglitazone are proposed as candidate components in the treatment processes by targeting the LEP gene in nondiabetic breast cancer patients.

Keywords Metformin, LEP gene, FOXO1 gene, Non-diabetic breast cancer patients, Autophagic cell death

1 Introduction

Breast cancer is a molecularly heterogeneous malignancy characterized by a high level of genomic instability and various genetic alterations [1, 2]. Molecular subtypes such as luminal A and B, HER2 positive, basal and normal-like are classified based on hormone receptors (estrogen and progesterone), HER2 expression levels, and BRCA gene mutations [2, 3]. These subtypes have unique genetic and epigenetic profiles. For example, high-level gene amplification is more common in HER2-positive tumors, while loss of tumor suppressor genes and copy number variations are more common in triple-negative breast cancer (TNBC) cases [1, 4]. Genome-scale analyses have enabled the identification of several subtype-specific oncogenic pathways and disease-associated genes, enabling the development of targeted treatment strategies [1, 2, 5].

Diabetes is associated with increased breast cancer risk, incidence, and mortality and women with diabetes and diabetes-related metabolic problems, especially those in the postmenopausal period, are more likely to develop breast cancer compared to those without metabolic diseases [6–10]. In addition, when women with diabetes are diagnosed with breast cancer, it is usually observed that the cancer is in advanced stages and stage [11, 12]. Recent research highlights the potential of antidiabetic agents in breast cancer treatment. For instance, metformin (1, 1-dimethylbiguanide, *Galega officinalis*), a common antidiabetic and plant-based drug, has shown promise as an anti-cancer and cancer preventive agent in breast cancer therapy in addition to its cardio- and vasculoprotective effects [7, 13–16]. Studies indicate that metformin may reduce cancer incidence and mortality in diabetic patients [13, 17]. In cancer, there are two main activated pathways such as the adenosine mono-phosphate-activated protein kinase (AMPK) pathway which are activated under carbohydrate starvation conditions and the insulin/insulin-like growth factor-1 (IGF1) signaling pathway in the presence of nutrients [18]. The drug's anti-cancer effects are linked to its impact on glucose metabolism and insulin signaling via reducing circulating glucose and insulin levels, and through decreasing insulin resistance-associated hyperinsulinemia, which promotes cancer cell growth through IGF1 and AMPK pathways [7, 13, 19].

In addition to drug agents that act via the insulin-dependent pathway, such as metformin, peroxisome proliferator-activated receptor gamma (PPARγ) ligands, including thiazolidinediones (TZDs) like pioglitazone, rosiglitazone, and troglitazone, have also shown potential in breast cancer treatment. These compounds can induce autophagy in breast cancer cells through PPARγ activation, which upregulates HIF1α and BNIP3 [20]. TZDs inhibit breast cancer cell proliferation, induce apoptosis, and suppress tumor angiogenesis and invasion both in vitro and in vivo. However, the mechanisms of action are complex, involving PPARγ-dependent and independent pathways. While preclinical studies have demonstrated antitumor effects, clinical trials have shown limited success in metastatic breast cancer patients [21]. TZDs have also shown promise as chemopreventive agents in breast cancer [22].

In summary, our study addresses a clinically relevant and timely questions.

- How autophagy-related tumor suppressor genes influence survival in breast cancer?
- How these can be pharmacologically targeted even in non-obese and non-diabetic patients.

Breast cancer is a heterogeneous disease, and despite the advancement in molecular subtyping, the role of autophagy in tumor suppression remains under-investigated, particularly in relation to survival and drug targeting. The studies mentioned above mention the effects of obesity on breast cancer in obese and postmenopausal women. However, our study focused on whether antidiabetic drugs used in the treatment of obesity are effective in the treatment of breast cancer independently of obesity through the genes associated with autophagic cell death that we identified. Therefore, the aim of our study is to first identify both autophagic and apoptosis-related genes in nonobese breast cancer patient samples. Then, we aim to determine which antidiabetic agents can target these autophagic cell death-related genes, regardless of obesity status.

Using robust computational analysis pipelines (LIMMA for differential expression, Kaplan-Meier Plotter for survival, KEGG for pathway analysis, and MATADOR for drug mapping), we provide a comprehensive approach to identifying genes that are not only differentially regulated in breast cancer tissue, but also significantly affect patient prognosis and link to actionable metabolic pathways.

Our identification of FOXO1 and LEP as survival-related, downregulated autophagy genes and their potential targeting by existing anti-diabetic drugs offers a novel therapeutic insight. This repurposing angle is of particular importance in personalized medicine, as it suggests potential non-cytotoxic adjuvants that could modulate tumor behavior in specific patient subgroups (e.g., non-obese, non-diabetic individuals).

Thus, our computational pipeline fills a specific gap: connecting transcriptomic autophagy signatures to prognostic relevance and druggability, which has not been adequately addressed in the context of breast cancer."

2 Materials and methods

2.1 Data processing

Gene intensity values of 457 tumor and 19 healthy breast tissues from breast cancer (BC) patients from GSE42568 and GSE31448 datasets from the GPL570 platform with 54,675 probes/individual were obtained from NCBI GEO Database [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array [23, 24]. Datasets and related demographic characteristics are given in Table 1.

2.1.1 Identification of autophagy and apoptosis pathway genes

In our study, the Molecular Signatures Database (MSigDB) has been used to obtain human autophagy and apoptosis pathways related genes and their gene names from "C5: ontology gene sets" subcollection. MSigDB is a well-known and widely used source of annotated genes for different biological pathways using GSEA software (https://www.gsea-msigdb.org/gsea/msigdb) [25–27]. Furthermore, autophagy and apoptosis related gene

Table 1 List of breast cancer GEO datasets

Breast Cancer Dataset	Dataset Description	Number of Patient Samples	Number of Healthy Samples
GSE42568	Breast Cancer Gene Expression Analysis	104 breast cancer tissues	15 healthy breast tissues
GSE31448	Down-regulation of ECRG4, a candidate tumor suppressor gene in human breast cancer	353 breast cancer tissues	4 healthy breast tissues

names were converted to Affymetrix IDs through "g: Profiler" tool and its "g: GOST" functional profiling section using "AFFY_HG_U133_PLUS_2" as qa target namescape (https://biit.cs.ut.ee/gprofiler/convert). In our study, 4404 probes for "Autophagy Pathways" (such as Autophagic Cell Death, Mitophagy, Pexophagy, Chaperon Mediated Autophagy, Macroautophagy, Microautophagy, Protein Targeting To Vacuole Involved in Autophagy, Autophagic Mechanism, Autophagosome Formation (Lysosome Fusion, Maturation, Membrane Docking, Organization, Autophagosome Assembly, Autophagic Vacuoles), Negative and Positive Regulation of Autophagosome Formation) and 6146 probes for "Apoptotic Signaling Pathway" were obtained from MSigDB.

2.1.2 Differentially expressed gene (DEG) analysis by using breast cancer microarray datasets

Raw data of GSE42568 and GSE31448 (19 healthy tissue, 457 BC tissues) datasets as CEL files were obtained from NCBI Gene Expression Omnibus (GEO) database. "Robust Multi-Array Average" (RMA) normalization was achieved using Bioconductor R program. On the other hand, the linear modeling method included in the R software (The R Foundation of Statistical Computing) (version 4.2.2) LIMMA package was used to determine differentially expressed genes between healthy individuals and cancer patients [28]. Upon DEG analyses, both upregulated and downregulated genes were determined in AML patients. Based on the literature, the threshold of logFC value was determined as ≥ 1.0 and ≤-1.0 for upregulated and downregulated differentially expressed genes in AML patients, respectively [29], and in our study, the same logFC value was used. The p-value of < 0.05 was accepted as the threshold value for statistical significance. In our study, after determining the up- and downregulated genes related to autophagy and apoptosis in both independent datasets (GSE42568 and GSE31448), genes commonly expressed in both autophagy and apoptosis were determined as autophagic cell death-related genes.

2.2 Survival analysis of autophagic cell death related genes in breast cancer patients

In our study, we focused on both upregulated and downregulated DEGs in breast cancer patients, and we evaluated the effects of both upregulated and downregulated genes with logFC ≥ 1 and logFC≤-1 on BC patients' prognosis via survival analysis. To achieve the survival analysis, Kaplan-Meier Plotter tool has been used. RNA-Seq data of 2976 BC patients have been included in survival analysis (Overall Survival-OS) of genes that were determined as a result of DEG analysis [30]. Gene Chip Genes with a hazard ratio value of equal or greater than 1 were selected as significantly hazardous genes; it is generally recognized that these genes caused poor prognosis of AML patients. On the other hand, genes with a hazard ratio value of equal or lower than − 1 were selected as significantly survival triggering genes.

2.2.1 Gene ontology enrichment analysis of autophagic cell death related genes

"ShinyGo 0.82" web-based tool was utilized to identify the disease specific functional biological pathways addressed by survival related DEGs, by selecting the "Kyoto Encyclopedia of Genes and Genomes-KEGG" pathway option. Furthermore, this tool was used to list gene annotations [31].

2.2.2 Novel drug targeting survival associated autophagic cell death related genetic biomarkers

In our study, Drug MATADOR from "ShinyGo 0.82" web-based tool has been used to determine the novel drug targeting autophagic cell death related genetic biomarkers in breast cancer patients. Manually Annotated Targets and Drugs Online Resource (MATADOR) is a database used for predicting protein–chemical interactions. It is a manually annotated list of relationships between protein products of such genes and chemicals (http://matador.embl.de) [32].

2.2.3 Ethics committee approval

This study made use of publicly available datasets of breast cancer patients from NCBI GEO Database. Ethics committee approval was not required because these were publicly available datasets.

3 Results

In our study, tumor suppressor genes associated with autophagic cell death specific to breast cancer were identified and new drug candidates that are not associated with breast cancer and are effective through cell death mechanisms were investigated. Gene expression data of 457 tumors and 19 healthy breast tissues were analyzed and differentially expressed genes related to autophagy and apoptosis were identified. SLC7A5, c19orf12, LEP, SPRX, SNCA and FOXO1 genes were found to affect patient survival. It was shown that LEP and FOXO1 genes are related to the AMPK signaling pathway and that the LEP gene can be targeted by some antidiabetic drugs. In conclusion, LEP and FOXO1 genes were suggested as potential tumor suppressor genes in obese and non-diabetic breast cancer patients, and it was stated that antidiabetic drugs could be treatment candidates especially for the LEP gene. The outline of the study can be seen in Figure 1.

4 Determination of both autophagic and apoptotic differentially expressed genes in breast cancer patients

As a result of differentially expressed gene analysis using R language with LIMMA package, the expression levels of genes represented by probes related to apoptosis (6146 probes) and autophagy (4404 probes) were compared in tumoral and normal breast tissues (19 healthy tissues, 457 BC tissues). As a result of the analyses, the genes that were commonly upregulated and downregulated in both independent datasets (GSE42568 and GSE31448) were determined and visualized with volcano plots (Figure 2). DEG analysis has shown that 6 autophagic cell death related genes including c19orf12 (MPAN), CRYAB, LEP, SRPX, SNCA, FOXO1 were significantly downregulated in both datasets (Table 2). Furthermore, autophagic cell death associated upregulated genes were SLC7A5, ATP2A2, INHBA, ATP5IF1 (Table 2).

4.1 Survival analysis of autophagic cell death related genes in breast cancer genes

Autophagic cell death related upregulated and downregulated DEGs were evaluated on behalf of their effects on survival rate of 2976 breast cancer patients by using RNA-Seq data in Kaplan-Meier Plotter tool. According to our results, the upregulated SLC7A5 gene was determined as hazardous gene in breast cancer patients (OS; HR: 1.31, logrank

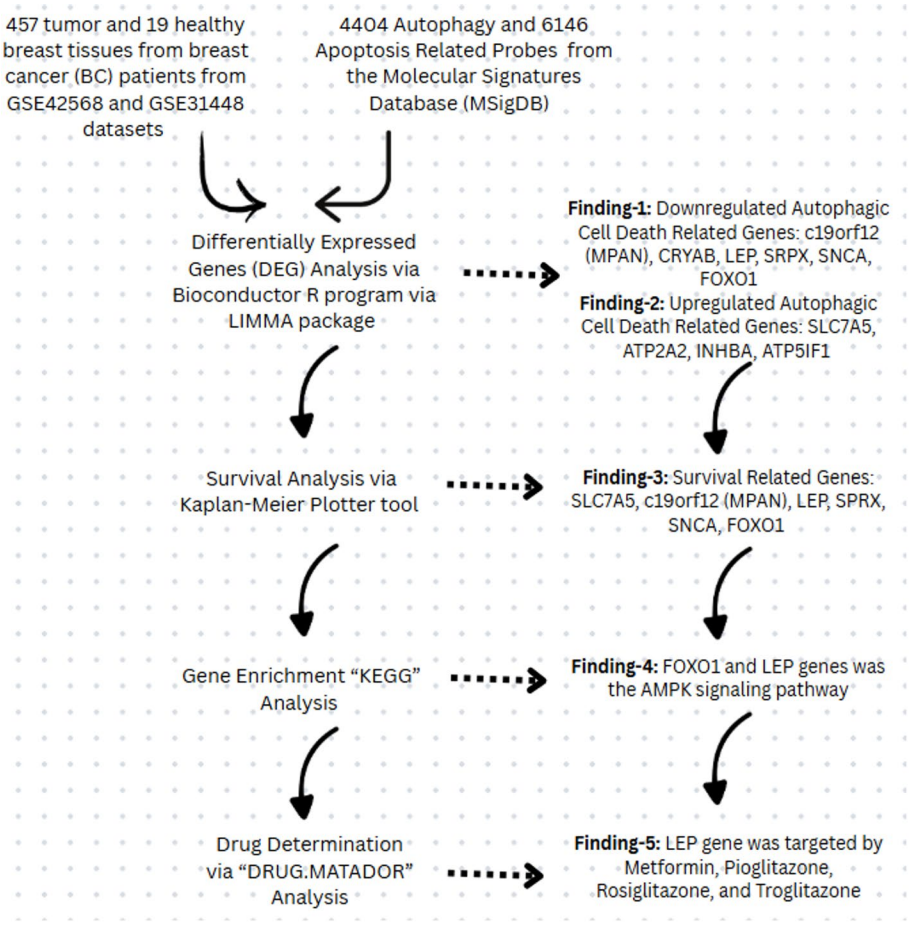

Fig. 1 Outline of the Study

P: 0.021). Besides, commonly downregulated c19orf12 (MPAN) (OS, HR: 0.69, logrank P: 0.001), LEP (OS, HR: 0.57, logrank P: 2.2e-6), SRPX (OS, HR: 0.76, logrank P: 0.025), SNCA (OS, HR: 0.48, logrank P: 2.2e-10), and FOXO1 (OS, HR: 0.56, logrank P: 0.00013) genes were determined as survival triggering genes in breast cancer patients. Besides, INHBA, ATP2A2, and CRYAB genes do not affect survival in breast cancer patients (Figure 3). Unfortunately, the ATP5IF1 gene could not be evaluated in terms of how it affects survival rate since it was not found in the microarray or RNA-Seq datasets in the survival analysis section of the KM-Plotter tool.

4.2 Gene functional enrichment and kyoto encyclopedia of genes and genomes (KEGG) analysis of survival related autophagic cell death genes

In our study, gene enrichment analysis was performed using Shinygo 0.82 tool for genes that were found to be hazardous or survival triggering in the prognosis of patients. According to the KEGG pathway analysis results, downregulated FOXO1 and LEP genes and the AMPK signaling pathway were indicated (Table 3).

4.3 Prediction of novel drug targeting survival related autophagic cell death genes in breast cancer patients

In our study, Drug.MATADOR database was used in Shinygo 0.82 tool to determine which novel drug components target the genes found to affect the survival rate of breast

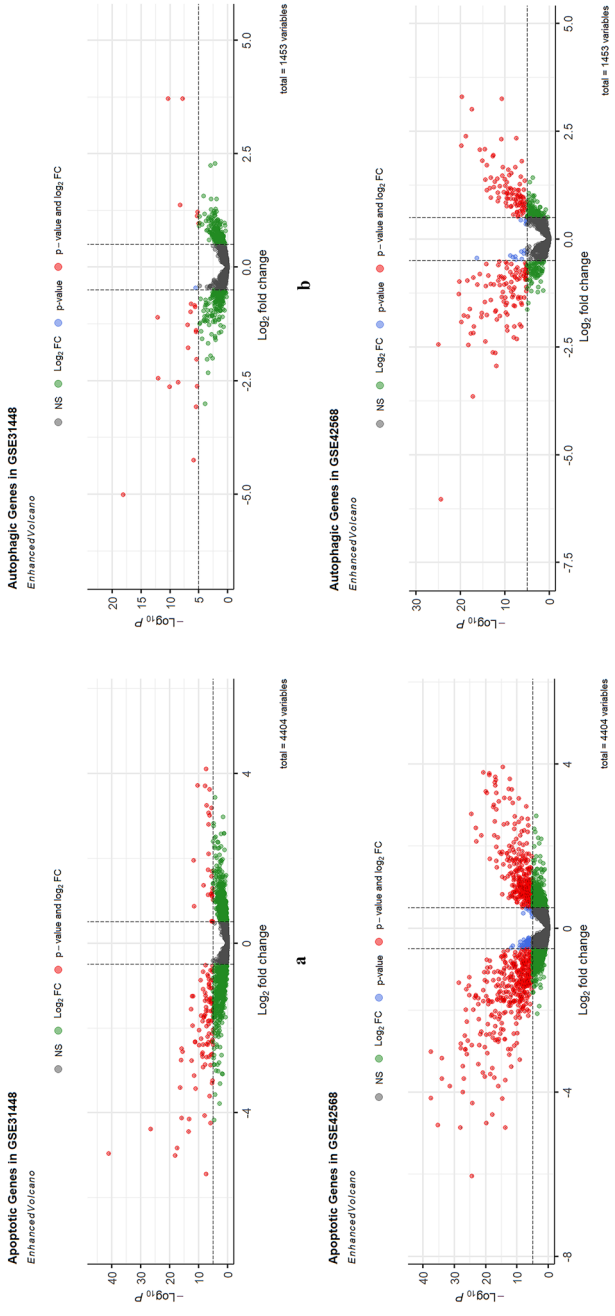

Fig. 2 Identification of Autophagic Cell Death Related DEGs in Breast Cancer. Volcano plots show upregulated and downregulated (A) apoptotic and (B) autophagic genes in GSE31448 dataset, (C) apoptotic and (D) autophagic genes in GSE42568 dataset in breast cancer patients. Upregulated and downregulated genes are shown with red dots which indicate those that are below the p-value of 0.05 and Log2FC ≤ -1 and ≥ 1 thresholds. Gray dots indicate insignificant genes

cancer patients. According to our results, the LEP gene, which was found to be downregulated in BC tissues in two different datasets, was shown as the main target and it was suggested that Metformin, Pioglitazone, Rosiglitazone and Troglitazone drugs targeting this gene could be used as novel drug components in breast cancer patients (Table 4).

5 Discussion

Targeted therapy has become increasingly important in breast cancer treatment, focusing on specific molecular subtypes and genetic alterations [33]. For example, in our study, it was shown that the leptin (LEP) gene was downregulated in breast cancer patients. Leptin gene is known as an obesity gene and its product leptin peptide hormone produced by adipose tissue, and it has a crucial role in in obesity related energy balance and body weight [34]. The literature mostly focuses on studies on the predisposition of obesity and diabetes mellitus to breast cancer in postmenopausal women, and it is emphasized that obesity and diabetes play an important role in the formation of breast cancer [35–37]. Most of these studies show that the LEP gene is upregulated in BC tissues or cell lines. For example, one study suggests that leptin and the leptin receptor are overexpressed in breast cancer cell lines MCF-7 and MDA-MB-231 cells, and that this may be due to hypoxic conditions and insulin, IGF-I or estradiol [38–40]. LEP gene expression levels were observed to be much higher in breast cancer patients with lymph node metastasis compared to those without. In addition, it was determined that overexpression of the LEP gene did not affect survival rates in these patients [41].

In another approach, in vitro and in vivo studies have indicated that co-expression of HER2 and leptin/leptin receptor may cause resistance to HER2-targeted therapies in postmenopausal obese women [42]. Similarly, it has been shown that adipose tissue-associated leptin contributes to the breast cancer progression through mitogenic, antiapoptotic and metastatic pathways, again associating it with obesity [43–47]. In addition, there are studies showing that leptin serum levels are called hyperleptinemia in breast cancer patients and that this condition causes metabolic and systemic problems (inflammation, central nervous system (CNS) impairment, etc.) and has a proinflammatory effect on breast cancer proliferation, migration and metastasis formation and exerts a role in the tumor microenvironment [47, 49].

In addition, there are studies showing that LEP gene polymorphisms (such as LEP G-2548 A, LEPrs7799039 AA) are effective in the formation of breast cancer depending on age. For example, it has been shown that the Lep-2548G/A polymorphism increases leptin expression in breast cancer cells [50, 51]. In addition, the LEPrs7799039 AA polymorphism has been associated with the risk of breast cancer in premenopausal women [52].

Contrary to the studies mentioned above, as shown in our study, there are a limited number of studies showing that the LEP gene is downregulated in breast cancer tissues. According to the results of meta-analysis and multi-omic analysis, low LEP gene expressions were associated with breast cancer patient's age and breast cancer tissue's higher state, HER2 and lymph node status and estrogen and progesterone positivity in one study [53]. Another transcriptomic study showed that the LEP gene was downregulated in direct breast cancer patients through wet laboratory experiments (54). In another study, low expression levels of LEP gene, leptin receptor and adiponectin genes were

Table 2 Commonly upregulated and downregulated autophagic and apoptotic DEGs

	IDs	Gene	Gene Description	Autophagic pathway	Apoptotic Pathway
Commonly Downregulated Autophagy and Apoptosis Related Genes	223983_s_at	**c19orf12 (MPAN)**	Chromosome 19 open reading frame 12	Autophagic Mechanism	Apoptotic Signalling Pathway
	209283_at	CRYAB	Crystallin alpha B	Autophagic Vacuoles	Apoptotic Signalling Pathway
	207092_at	**LEP**	Leptin	Negative Regulation of Autophagic Mechanism	Apoptotic Signalling Pathway
	204955_at	**SRPX**	Sushi repeat containing protein X-linked	Autophagy Mechanism and Autophagosome	Apoptotic Signalling Pathway
	204466_s_at	**SNCA**	Synuclein alpha	Chaperone Mediated Autophagy Negative Regulation of Autophagic Mechanism	Apoptotic Signalling Pathway
	202724_s_at and 202723_s_at	**FOXO1**	Forkhead box O1	Positive Regulation of Autophagic Mechanism	Apoptotic Signalling Pathway
Commonly Upregulated Autophagy and Apoptosis Related Genes	201195_s_at	**SLC7A5**	Solute carrier family 7 member 5	Negative Regulation of Autophagic Mechanism	Apoptotic Signalling Pathway
	209186_at	ATP2A2	ATPase sarcoplasmic/endoplasmic reticulum Ca2 + transporting 2	Macroautophagy Autophagosome Maturation, Organization and Membrane Docking	Apoptotic Signalling Pathway
	210511_s_at	INHBA	Inhibin subunit beta A	Autophagic Mechanism	Apoptotic Signalling Pathway
	223339_at	ATP5IF1	ATP Synthase Inhibitory Factor Subunit 1	Mitophagy Mitophagy in Response to Mitochondrial Depolarization	Apoptotic Signalling Pathway

detected specific to more aggressive breast cancer tissues using NanoString multiplexed assays and bioinformatics analyses [55].

In the current studies mentioned, downregulation of the LEP gene is associated with obesity in breast cancer patients. In addition, in these studies, the protein product of the LEP gene or downregulated LEP gene was not associated with cell death or autophagic pathway. In our study, supporting these results, breast cancer cells have downregulated leptin gene (LogFC – 6.04 in GSE42568 dataset and LogFC – 5.0 in GSE31448 dataset). In addition, the LEP gene has been associated with survival and has been identified as both an apoptotic and autophagic gene. In our study, it was determined that downregulated expressions of the LEP gene contributed to the negative regulation of the autophagic pathway. In conclusion, in our study, the LEP gene can be considered as tumor suppressor autophagic cell death gene in nondiabetic BC patients. In addition, the fact that the LEP gene contributes to the negative regulation in the autophagic pathway can be interpreted as contributing to the autophagic flux mechanism and triggering autophagic cell death by supporting the apoptotic pathway.

In addition to the LEP gene, the FOXO1 gene was also found to be downregulated in breast cancer tissues in our study (LogFC – 2.17 for the "202724_s_at" probe and – 2.03 for the "202723_s_at" probe in the GSE42568 dataset; LogFC – 1.58 for the "202724_s_at" probe and – 1.0 for the "202723_s_at" probe in the GSE31448 dataset) and was associated with survival. Mammalian forkhead members of the class O-1 (FOXO1) (also known as forkhead in rhabdomyosar coma, or FKHR) is a transcription factor involved in the regulation of cell cycle and apoptosis which shows tumor suppressive properties

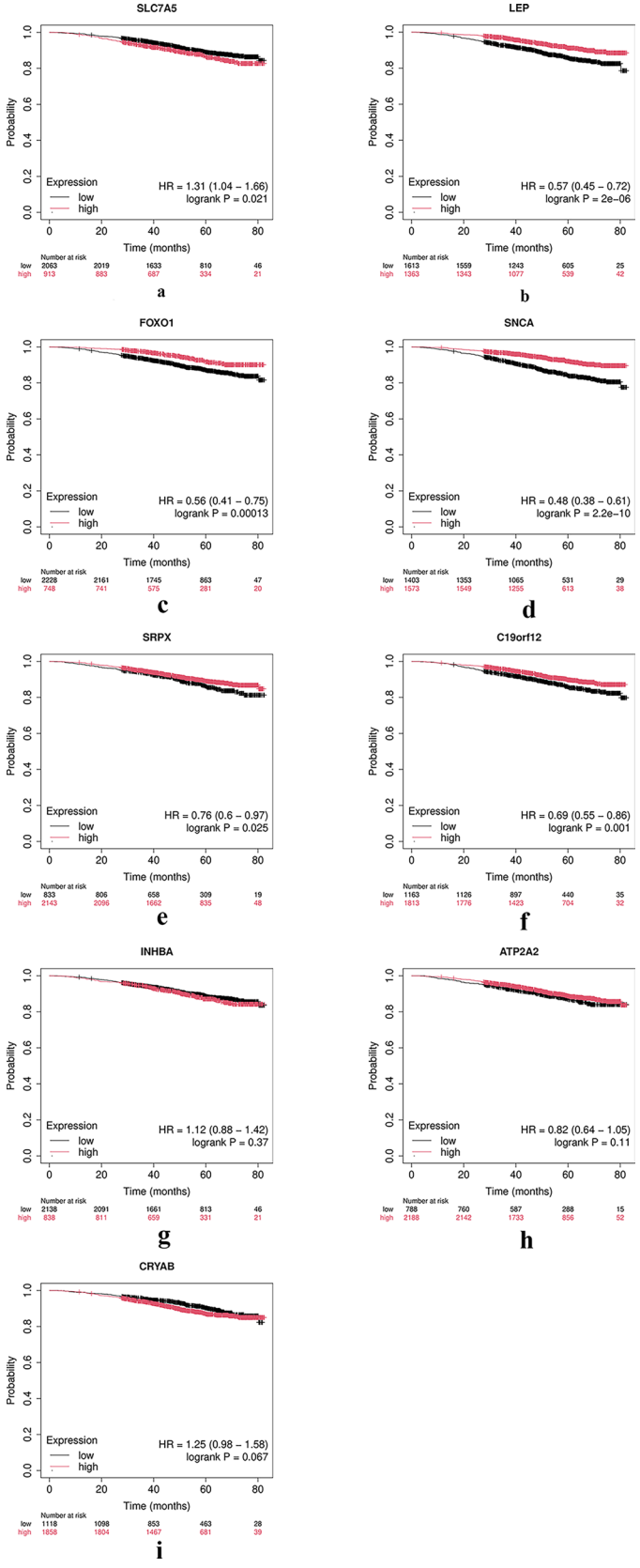

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 Kaplan Meier Plots of Survival Related Autophagic Cell Death Genes in Breast Cancer Patients The upregulated (A) SLC7A5, and downregulated (B) LEP, (C) FOXO1, (D) SNCA, (E) SRPX, and (F) c19orf12 (MPAN) genes significantly affected prognosis of breast cancer patients unlike (G) INHBA, (H) ATP2A2, (I) CRYAB genes. Red represents high gene expression levels, and black represents low gene expression levels. Logrank p values and Hazard Ratio (HR) of each gene has been included in the graphs

[56, 57]. In our study, it was shown that the FOXO1 gene, unlike the LEP gene, contributes to positive regulation in the autophagic pathway.

It has been shown that FOXO1 and LEP genes and their products play crucial roles in autophagy regulation and autophagic cell death in cancer cells. In MDA-MB-231 cells, FOXO1 enhances paclitaxel-induced autophagy through transcriptional activation of autophagy-related genes, promoting cell survival. Inhibition of FOXO1-mediated autophagy increases paclitaxel-induced apoptosis, suggesting a potential strategy to improve chemotherapy efficacy in triple-negative breast cancer [58]. Similarly, benzyl isothiocyanate (BITC) induces FoxO1-mediated autophagic death in various breast cancer cell lines, but not in normal mammary epithelial cells [59]. Furthermore, LEP promotes breast cancer cell growth by inducing autophagy through estrogen receptor signaling and AMPK/FoxO3A axis activation in breast cancer MCF-7 tumor xenograft model [60]. Autophagy inhibition decreases leptin-triggered cell proliferation, ERK activation and migration in breast cancer cells [61]. It is not yet clear how the LEP and FOXO1 genes, which were shown to be downregulated in our study, contribute to the negative and positive regulation of the autophagic pathway and apoptotic cell death mechanisms through antidiabetic agents (Metformin, Pioglitazone, Rosiglitazone, Troglitazone), respectively, and the findings of our study are novel in this respect.

Studies have indicated the FOXO1 downregulation due to promoter methylation in breast cancer tissues in Indian breast cancer patients [58, 62]. This downregulation is associated with promoter hypermethylation and correlates with clinical features such as age, tumor size, and lymph node status [58]. FOXO1 expression is nagetively correlated with lymph node-positive and HER2-overexpressing tumors and independently predicts disease-free survival. The expression of FOXO1 is regulated by the PI3K/Akt pathway and is associated with GATA3 and Annexin-1 expression [62]. Furthermore, FOXO1 lower expression levels have been correlated positively with immune checkpoint molecules and infiltration of macrophages and neutrophils in breast cancer patients in a bioinformatic analysis-based study [63]. In another study, FOXO1 mRNA levels has been determined as downregulated in breast tumors, and overexpression of FOXO1 decreases cell viability by inhibiting cell cycle progression and inducing cell death [64]. However, the role of FOXO in cancer progression is complex, as both activation and loss of FOXO function can suppress both metastasis and tumor growth [65].

FOXO proteins are negatively regulated by the PI3K-Akt signaling pathway and are activated by oxidative stress, leading to the expression of genes involved in cell-cycle arrest [66]. Additionally, leptin has been shown to inhibit the effects of AMPK activation by targeting the AMPK and PI3K/Akt pathways in breast cancer cells [67]. In our study, LEP and FOXO1 genes, which were found to be downregulated in breast cancer patients, were associated with the AMPK signaling pathway. In addition, our study showed that the LEP gene is targeted by other antidiabetic drugs (Pioglitazone, Rosiglitazone, Troglitazone), especially Metformin.

Table 3 KEGG pathway analysis

Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	Pathway	Genes
0.006588	2	121	75.63967	Path: hsa04152 AMPK signalling pathway	FOXO1, LEP

Table 4 Drug-Target prediction by using Drug. MATADOR analysis

Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	Pathway	Genes
0.010479	1	12	381.35	Metformin	LEP
0.016815	1	44	104.0045	Pioglitazone	LEP
0.016815	1	58	78.9	Rosiglitazone	LEP
0.02059	1	95	48.17053	Troglitazone	LEP

In breast cancers, the mammalian Target of Rapamycin (mTOR) pathway is dysregulated, resulting in increased proliferation, growth factor independence, and endocrine resistance [68]. In addition, studies suggest that mTOR pathway can be targeted and contribute to treatment through activation of the AMP-activated protein kinase (AMPK) signaling pathway [69]. For instance, AMPK activation with metformin, has shown promising anti-proliferative and anti-neoplastic effects in vitro and is associated with lower cancer incidence in diabetic patients in HER2 overexpressed and triple negative breast cancer models [70–76]. Our study, which shows that the AMPK pathway can be treated with metformin and other antidiabetic agents by targeting the LEP and FOXO1 genes in diabetic breast cancer patients, is novel in this respect.

Metformin, commonly used as a diabetes medication, indicates potential as a breast cancer treatment through multiple mechanisms. For instance, a presurgical trial in overweight/obese breast cancer patients found no significant reduction in tumor proliferation after metformin treatment, despite observing reductions in BMI, cholesterol, and leptin [77]. Furthermore, it induces apoptosis and autophagy in breast cancer cells, with autophagy playing a cytoprotective role [78]. Metformin's anti-cancer effects involve both caspase-dependent and poly(ADP-ribose) polymerase (PARP)-dependent cell death pathways, associated with mitochondrial enlargement in sensitive breast cancer cell lines [79]. Autophagy-related cell death may be a key mechanism for eliminating potentially tumorigenic cells, as metformin induces massive cell death in preneoplastic cells under stress conditions [80]. In non-diabetic women with early breast cancer, metformin significantly lowers fasting insulin levels, improves insulin sensitivity, and reduces weight without significant effects on quality of life. However, this study did not mention the effect of metformin against breast cancer cells and the cancer process in nondiabetic patients [81]. A meta-analysis of randomized control trials in non-diabetic breast cancer patients revealed that metformin was associated with an anti-proliferative role and improved progression-free survival [82]. The drug's mechanism of action may involve both direct effects on cancer cells and indirect effects mediated by circulating insulin [83]. It can further trigger autophagy-related cell death in preneoplastic cells, potentially eliminating tumorigenic cells [80]. In breast cancer cells, metformin induces both apoptosis and a caspase-independent, PARP-dependent cell death pathway associated with mitochondrial enlargement [79]. Metformin also increases autophagic flux in MCF-7 cells, which plays a cytoprotective role. This autophagy is mediated by ROS-induced TFE3 activation [78]. Interestingly, ferroptosis may be induced via metformin

through the inhibition of autophagy with lncRNA H19 in breast cancer cells [84]. These diverse mechanisms highlight metformin's complex effects on cancer cells, suggesting its potential as a multi-faceted anti-cancer agent. However, the response to metformin varies among cell lines, with some showing resistance to its cytotoxic effects [79]. In our study, LEP and FOXO1 genes have been determined as autophagic cell death related gene involving in AMPK pathway and can be targeted by antidiabetic drugs such as metformin, Pioglitazone, Rosiglitazone, Troglitazone. While these studies suggest potential benefits of metformin in breast cancer treatment, further research is needed to fully understand its role and effectiveness in non-obese breast cancer patients targeting LEP gene and AMPK pathway through both LEP and FOXO1 gene with autophagic cell death affect.

In our study, there are some limitations as discussed below.

- In our study, two separate patient gene intensity datasets (GSE42568 and GSE31448) were used for validation purposes and to obtain more robust results. Despite the total number of cancerous tissues being 457, the total number of healthy tissues in our analyses is only 19. This is one of the limitations of this study.
- In our study, microarray data was primarily preferred for use in DEG analyses. The reason for this is that the gene intensity data per patient tissue or healthy tissue is quite high (54,675 gene intensity data/patient-healthy tissue). In addition, to ensure the results are more robust, they need to be validated with transcriptomic data obtained through high-throughput RNA sequencing (RNA-seq), and the absence of these analyses can be mentioned as a limitation of our study. The relevant analyses will be conducted in future studies.
- Our study was conducted entirely using bioinformatics tools, and our results have not yet been validated through experimental methods (cell culture, animal models, or clinical samples from "non-obese and non-diabetic patients"). As a future perspective, it is included in our plans for upcoming studies and projects.

6 Conclusion

In our study, LEP and FOXO1 genes, which are associated with survival in breast cancer patients, were identified as candidate tumor suppressor genes associated with autophagic cell death in breast cancer patients. It is also suggested that drugs used in the treatment of diabetic diseases (Metformin, Pioglitazone, Rosiglitazone, Troglitazone) can be used in the treatment processes by targeting the LEP gene in nondiabetic breast cancer patients.

Author contributions

Ayna Duran G and Kiraz Y. designed the model and the bioinformatic analysis-based framework, analysed the data and carried out the implementation with the help of Baykara D. Ayna Duran G and Kiraz Y. wrote the manuscript and prepared figures and tables.

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Data availability

Gene intensity values of 457 tumor and 19 healthy breast tissues from breast cancer (BC) patients from GSE42568 and GSE31448 datasets from the GPL570 platform with 54,675 probes/individual were obtained from NCBI GEO Database [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array.

Declarations

Ethics approval and consent to participate
This study made use of publicly available datasets of breast cancer patients from NCBI GEO Database. Ethics committee approval was not required because these were publicly available datasets.

Competing interests
The authors declare no competing interests.

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