

Inverse *GAS5-SFXN1* Correlation in Triple-Negative Breast Cancer: Insights from Bioinformatics and Tissue Expression Analysis

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ABSTRACT

Background: Triple-negative breast cancer (TNBC) is an aggressive subtype with limited therapeutic options. Given the involvement of long non-coding RNAs, like *GAS5*, in cancer progression, this study aimed to identify *GAS5*-associated target genes through breast cancer databases and evaluate the expression levels of *GAS5* and *SFXN1* (as a potential target) in TNBC tissues.

Methods: After predicting putative *GAS5* targets via StarBase, they were validated through TCGA and GEPIA2. RT-qPCR was then performed to examine *GAS5* and *SFXN1* expression in 18 tumor and 15 adjacent normal samples. Gene expression levels were quantified using the fold-change method, and statistical analyses were performed using linear mixed models, one-sample t-tests, and Spearman's correlation.

Results: Bioinformatics analyses revealed significant differential expression of *SFXN1* in the TCGA and GEPIA2 databases. In the experimental validation phase, the mean Log₂FC values for *GAS5* and *SFXN1* in tumor samples were 0.235 and -0.450, respectively. Neither gene showed a statistically significant difference between tumor and normal tissues using the t-test (*GAS5*: $p = 0.650$; *SFXN1*: $p = 0.458$). The linear mixed model also revealed no significant difference for either gene in the same comparison. Spearman's correlation, however, demonstrated a significant negative correlation between *GAS5* and *SFXN1* expression ($r = -0.479$; $p = 0.049$).

Conclusion: While *GAS5* and *SFXN1* were not differentially expressed in TNBC tissues, their significant inverse correlation suggests a potential association that may contribute to the molecular networks underlying TNBC pathogenesis. Further functional studies are warranted to clarify this relationship. Any diagnostic or therapeutic implications remain speculative at this stage. **DOI: 10.22034/IBJ.30.4.241**

Keywords: *GAS5* long non-coding RNA, Long non-coding RNA, Triple negative breast neoplasms, *SFXN1* protein

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1. INTRODUCTION

Breast cancer (BC) remains a leading cause of cancer-related mortality among women worldwide, with triple-negative breast cancer (TNBC) contributing disproportionately to this burden^[1]. TNBC is defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), which limits targeted therapeutic options and results in a poor prognosis, high recurrence rates, and frequent metastasis^[2]. There is therefore an urgent need to identify the molecular mechanisms of TNBC that could yield novel diagnostic biomarkers and therapeutic targets.

Hereditary factors account for 5–10% of BC cases, and high-penetrance mutations in genes such as *BRCA1*, *BRCA2*, *CHEK2*, and *PALB2* markedly increase the lifetime risk^[3,4]. Epigenetic alterations, including DNA methylation, further contribute to the molecular diversity of BC^[5]. Despite advances in targeted therapies, such as CDK4/6 inhibitors, PI3K inhibitors, PARP inhibitors, and immunotherapy, TNBC remains particularly challenging due to a lack of specific molecular targets^[6]. This gap has fueled interest in novel regulatory molecules, among which long non-coding RNAs (lncRNAs) have emerged as central players in gene regulation during tumorigenesis.

lncRNAs are a large and heterogeneous class of transcripts longer than 200 nucleotides that lack protein-coding capacity but orchestrate gene expression at multiple levels^[7]. They act through chromatin remodeling, transcriptional and post-transcriptional regulation, and serve as molecular decoys, scaffolds, or guides^[8,9]. Genome-wide projects such as GENCODE and FANTOM5 have catalogued tens of thousands of human lncRNAs, many of which are dysregulated in cancer and other diseases^[10,11]. Among these lncRNAs, growth arrest-specific 5 (*GAS5*) has been identified as a potent tumor suppressor in BC^[12]. *GAS5* expression is downregulated across all major BC subtypes, and its silencing, often via promoter hypermethylation, correlates with enhanced invasion, metastasis, and drug resistance^[12]. Clinical studies have confirmed reduced *GAS5* levels in breast tumor tissues compared to adjacent normal tissues, with lower expression linked to tumor progression^[13,14]. Specifically in TNBC, low *GAS5* expression is significantly associated with poor overall and disease-free survival, and its methylation status has been linked to survival outcomes^[2]. Notably, one study has reported contradictory findings of elevated *GAS5* expression associated with advanced disease stage and lymph node metastasis, potentially reflecting differences in molecular subtypes or patient populations^[15].

Mechanistically, *GAS5* functions as a competing

endogenous RNA (ceRNA) that sponges oncogenic miRNAs, thereby derepressing tumor-suppressor genes. For instance, *GAS5* sequesters miR-21 to upregulate *PTEN* and suppress proliferation^[16]. In TNBC, *GAS5* competitively binds miR-196a-5p, restoring the FOXO1/PI3K/AKT pathway and reducing proliferation and invasion^[17]. The expression of *GAS5* itself is controlled by upstream pathways, including Notch-1^[18], *c-MYC*^[19] and the mTOR pathway; the latter is particularly relevant because *GAS5* belongs to the 5'TOP gene family and its translation is mTOR-dependent^[20]. Dual PI3K/mTOR inhibition has been shown to stably increase *GAS5* levels and enhance chemotherapy-induced apoptosis in TNBC cells^[14]. Collectively, these properties position *GAS5* as a promising diagnostic and prognostic biomarker in BC.

Circulating *GAS5* levels in plasma have been correlated with tumor burden, lymph node metastasis, and disease recurrence^[21,22], and its potential as a non-invasive biomarker is supported by studies in other malignancies^[23,24]. *GAS5* also modulates sensitivity to chemotherapy, radiotherapy, and endocrine therapy by regulating apoptosis and DNA damage repair pathways^[25,26], making it an attractive therapeutic target. Despite this progress, the downstream mRNA targets through which *GAS5* exerts its tumor-suppressive effects in TNBC remain poorly defined. Most studies have focused on general regulatory mechanisms or individual miRNA interactions in cell lines, with few integrated bioinformatics and clinical studies aimed at identifying specific mRNA targets and concurrently measuring their expression in clinical TNBC samples. In particular, no previous study has profiled both *GAS5* and a candidate target gene such as *SFXN1* in a TNBC patient cohort to evaluate their expression relationship and its potential diagnostic value. This knowledge gap limits our understanding of the *GAS5* regulatory network in this aggressive subtype and hinders the development of more precise biomarkers and treatment strategies. Therefore, the primary aim of this study was to determine the regulatory role of lncRNA *GAS5* on the genes involved in TNBC pathogenesis through integrated bioinformatics analyses and clinical sample evaluation. Our specific objectives were: (1) to comprehensively identify potential *GAS5* target genes associated with BC pathogenesis using bioinformatics databases; (2) to measure *GAS5* expression in clinical TNBC samples by real-time quantitative PCR (RT-qPCR); (3) to measure the expression of a selected *GAS5* target gene, *SFXN1*, in the same clinical samples; and (4) to evaluate the correlation between *GAS5* and *SFXN1* expression in TNBC. The exploratory goal was to evaluate the *GAS5*-*SFXN1* expression relationship and generate hypotheses

for future studies on molecular markers in TNBC. We do not claim diagnostic or therapeutic value at this stage; instead, we suggest that if confirmed functionally, this relationship might eventually be explored for such purposes.

2. MATERIALS AND METHODS

2.1. Study design

This research was a quantitative, cross-sectional, observational-analytical study that combined bioinformatics target identification with gene expression measurement in clinical samples. The study did not involve any therapeutic or pharmacological intervention. All clinical specimens were obtained from the Tumor Bank of the University of Tehran Cancer Institute (Tehran, Iran) prior to any treatment. To maintain patient confidentiality, we de-identified and coded all the samples. A total of 18 TNBC tumor samples and 15 adjacent normal tissue samples were obtained from the Tumor Bank of the University of Tehran Cancer Institute. The sample size was determined based on the availability of eligible specimens in the tumor bank. A formal sample size calculation was not performed, as no prior estimates of effect size for *GAS5* and *SFXN1* expression differences in TNBC were available to inform power calculations. The sample size was comparable to that of similar exploratory molecular studies in TNBC^[13]. Some tumor samples had matched pairs of normal tissue from the same patient. All samples were collected prior to any chemotherapy or therapeutic intervention and were stored at -80°C until RNA extraction. Inclusion criteria were histologically confirmed TNBC, availability of tumor tissue (and normal tissue where possible), adequate tissue quality and quantity for RNA extraction, and availability of baseline clinical information. Samples with prior treatment, incomplete data, or inadequate RNA quality were excluded. Samples were randomly selected from eligible specimens in the tumor bank, with an effort to ensure relative homogeneity in patient age and disease characteristics across groups. The MDA-MB-231 TNBC cell line was purchased from the Iranian Biological Resource Center (Tehran, Iran). Cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution (100 IU/mL and 100 µg/mL, respectively) and incubated in 5% CO₂ at 37°C. All experiments were performed in at least two independent replicates.

2.2. Bioinformatics analysis

Gene expression data for the breast invasive carcinoma (BRCA) cohort were obtained from The Cancer Genome Atlas (TCGA) via the Broad GDAC

Firehose portal (<https://gdac.broadinstitute.org>). The initial discovery screening was performed using the broader TCGA-BRCA cohort, which includes multiple BC subtypes and is not specific to TNBC. This approach was used to maximize statistical power. However, the final candidate gene (*SFXN1*) was selected only after considering literature reports indicating its possible involvement in TNBC. Differential expression analysis between tumor and normal tissues was performed using the edgeR package in R^[27]. Differentially expressed genes were identified using a false discovery rate (FDR) <0.05 and an absolute log fold change ($|\log_2FC|$) >1. The results were visualized using volcano plots and hierarchical clustering heatmaps. Cross-checking of gene expression patterns was carried out using the GEPIA2 platform (<http://gepia2.cancer-pku.cn>), which integrates TCGA tumor samples and GTEx normal breast tissues. Thresholds of $|\log_2FC| > 1$ and q value < 0.05 were applied. GEPIA2 was used solely for visualization and confirmation of expression patterns, not as an independent validation cohort. Potential interactions involving *GAS5* were investigated using the StarBase database (<http://rnasysu.com/encori/index.php>), restricting the analysis to experimentally supported evidence such as CLIP-Seq data. Functional enrichment analysis of Gene Ontology (GO) terms for *GAS5*-associated genes was performed using the clusterProfiler package in R. Gene symbols were converted to Entrez IDs, and duplicate entries were removed prior to analysis. Over-representation analysis was performed across the Cellular Component (CC) and Molecular Function (MF) categories, using significance thresholds of p and q values < 0.05, with Benjamini-Hochberg correction for multiple testing.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from 25 mg of each tissue sample using RNX™-Plus reagent (SinaClon, Tehran, Iran). Samples were homogenized in RNX™-Plus, followed by chloroform extraction and ethanol precipitation. The RNA pellet was dissolved in 30 µL of RNase-DNase-free water and stored at -70°C. RNA quantity and purity were assessed using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). One microgram of each RNA sample was then reverse-transcribed with random hexamer primers (SinaClon) using the Ultra™ Thermo-Resistant Reverse Transcriptase (Parstous, Tehran, Iran) according to the manufacturer's protocol. The reaction mixture was incubated at 25°C for 10 min, 55°C for 30 min, and 80°C for 5 min.

2.4. RNA quality control

The quantity and quality of the extracted RNA were

assessed by UV spectrophotometry (NanoDrop) and agarose gel electrophoresis prior to cDNA synthesis. RNA concentration was determined by measuring absorbance at 260 nm, and purity was evaluated using the A260/A280 and A260/A230 ratios. Samples with A260/A280 ratios between 2.0 and 2.2 and A260/A230 ratios between 2.0 and 2.5 were accepted as adequately pure. RNA integrity was verified by loading an aliquot of each sample on an agarose gel under RNase-free conditions. The presence of two clear and distinct bands corresponding to 28S and 18S rRNA, with an approximate intensity ratio of 2:1 and no visible smearing below the bands, was considered indicative of intact RNA. Only samples that met both the spectrophotometric purity criteria and the acceptable band pattern were used for cDNA synthesis and subsequent RT-qPCR analyses.

2.5. RT-qPCR

Specific primers for *GAS5*, *SFXN1*, and reference genes were designed using Primer-BLAST and optimized for amplification efficiency (Table S1). Melting curve analysis confirmed a single product for each amplicon. No-template controls (NTCs) were included and showed no detectable amplification. All reactions were run in duplicate, and the mean Ct was used for calculations. RT-qPCR was performed with SYBR Green master mix (Ampliqon, Denmark) in a total volume of 13 μ L containing 6.25 μ L of master mix, 0.5 μ L of each forward and reverse primer (10 μ M), 4.75 μ L of nuclease-free water, and 1 μ L of cDNA template. Thermal cycling was carried out with an initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 58°C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, with β -actin as the internal reference gene and normal tissue as the calibrator. For samples without a matched normal tissue, the mean of all normal samples was used for calibration. This approach introduces some degree of uncertainty because the normal reference is not patient-matched; however, given the exploratory nature of the study, we considered it acceptable. FC values were log2-transformed (Log₂FC).

2.6. Statistical analysis

Normality of Log₂FC distributions was assessed prior to parametric testing. For the primary analysis, a linear mixed model (LMM) was fitted to the ΔC_t values in SPSS version 27.0. This approach considered the partially paired structure of the data (some tumor specimens had matched normal tissue, while others did not). In addition, a one-sample t-test was conducted to assess whether the mean Log₂FC for each gene significantly differed from zero (representing no

change). This statistical approach is conventional in gene expression studies. The correlation between *GAS5* and *SFXN1* Log₂FC values in tumor samples was assessed using Spearman's rank correlation coefficient. A one-tailed test was applied because we had a pre-specified directional hypothesis derived from prior literature and our bioinformatics analysis that *GAS5* and *SFXN1* would be inversely correlated. For completeness, the two-sided *p*-value is also provided in the results. A *p*-value < 0.05 was considered statistically significant in all analyses. Data management and statistical analyses were performed using Microsoft Excel, GraphPad Prism version 8.4.3, and IBM SPSS Statistics version 27.0.

3. RESULTS

3.1. Bioinformatic identification of *GAS5* target genes

An initial set of 58 *GAS5*-associated candidate genes was identified using the StarBase scRNA interaction module, which integrates CLIP-seq and other high-throughput interaction data (Table S2). These candidate genes were then intersected with the expression profiles from TCGA-BRCA and GEPIA2. Differential expression analysis in TCGA further revealed that three genes (*EPAS1*, *ACSL4*, and *SFXN1*) showed significant differential expression between tumor and normal samples. To confirm these findings, differential expression analysis was also performed using the GEPIA2 platform. In GEPIA2, seven *GAS5* target genes (*ACSL4*, *TMEM33*, *EPAS1*, *PNISR*, *PI15*, *UHMK1*, and *SFXN1*) were significantly differentially expressed in BC tissues. Notably, three genes, *EPAS1*, *ACSL4*, and *SFXN1*, were validated in both TCGA and GEPIA2 analyses (Table 1). Among these genes, *SFXN1* was selected for further experimental investigation based on three pre-defined criteria: (1) a large absolute Log₂FC in TCGA, (2) a small FDR in GEPIA2, and (3) a recent functional study linking *SFXN1* to TNBC pathogenesis^[28]. The volcano plot generated from TCGA data provided an overview of the differential expression landscape, highlighting genes with significant changes (Fig. 1A). A heatmap of the *GAS5*-regulated gene expression patterns across TCGA samples revealed distinct clustering patterns between tumor and normal tissues, suggesting co-regulation or functional relationships among subsets of *GAS5* targets during disease progression (Fig. 1B). GO enrichment analysis of the *GAS5*-regulated genes identified significantly enriched terms in both CC and MF categories. In the CC category, enriched terms included “ribonucleoprotein complex binding”, “histone modifying activity”, and “protein serine/threonine kinase activity”, indicating *GAS5* involvement in RNA-

Table 1. GAS5-regulated genes with significant differential expression identified through GEPIA2 analysis.

Gene	TCGA		GEPIA2	
	Log ₂ FC	FDR	Log ₂ FC	FDR
<i>ACSL4</i>	-1.21	6.9E-46	-1.07	4.5E-50
<i>TMEM33</i>	0.53	1.8E-21	1.25	4.2E-50
<i>EPAS1</i>	-1.9	2.6E-133	-2.28	7.5E-151
<i>PNISR</i>	-	-	-1.49	2.5E-84
<i>PI15</i>	-0.91	9.0E-4	-2.65	9.9E-39
<i>UHMK1</i>	0.39	5.0E-3	1.15	3.0E-42
<i>SFXN1</i>	1.23	9.5E-45	1.18	6.4E-53

GAS5-regulated genes with significant differential expression in TCGA and GEPIA2.

protein interactions and chromatin modification. In the MF category, “protein serine kinase activity” and “protein serine/threonine kinase activity” were predominant, suggesting a role in phosphorylation-dependent signaling and cell cycle regulation. These findings highlight the potential regulatory functions of GAS5 in RNA metabolism and epigenetic modulation (Fig. 2).

3.2. Descriptive statistics

After RNA quality control, a total of 33 tissue samples yielded acceptable data for gene expression analysis: 18 tumor samples and 15 adjacent normal samples. Among the tumor samples, 11 had a paired normal sample available, whereas the remaining 7 tumor samples lacked a corresponding normal tissue. All specimens were obtained from the tumor bank of the University of Tehran Cancer Institute prior to any anticancer treatment. All patient information was coded and de-identified to ensure confidentiality. The demographic and clinicopathological characteristics of the patients are summarized in Table 2. The samples were obtained from 19 patients, with a mean age of 51.9 years (range: 31-77 years). The tumors spanned stages I to IV, and lymph node involvement was observed in seven patients. It should be noted that data for two patients were unavailable. Descriptive statistics for Δ Ct values of GAS5 and SFXN1 in tumor and normal tissues are presented in Table 3. Δ Ct was calculated by subtracting the Ct of the reference gene β -actin from the Ct of the target gene. The mean Δ Ct of GAS5 in tumor and normal samples was 0.93 and 1.18, respectively. For SFXN1, the mean Δ Ct was 8.14 in tumor samples and 7.41 in normal samples. Medians and ranges for these quantities are also included in Table 3. The relative expression values (FC) of the studied genes in tumor samples are summarized in Table 4. The mean FC values for GAS5 and SFXN1 were 2.38 and 2.20, respectively. The table also presents median and range values. It is not surprising that the mean FC of SFXN1 exceeds 1, while the mean Log₂FC is negative. This behavior occurs

because FC values are asymmetrically distributed (ranging from 0 to ∞). When averaging on the original scale, large positive outliers can pull the average upward. In contrast, averaging the Log₂-transformed values treats upregulation and downregulation more symmetrically. Figure 3 displays the individual FC values for GAS5 and SFXN1 in tumor samples. Only one value per patient per tissue type was used after averaging technical and biological replicates, which reduced the effective sample size to 13 tumor samples. In the MDA-MB-231 TNBC cell line, both GAS5 and SFXN1 were substantially downregulated (FC = 0.0005 and 0.0633, respectively). This pattern differs markedly from the tissue expression results. The contrasting expression profiles observed in MDA-MB-231 cells relative to primary tumor tissues indicate that this single cell line does not adequately capture the regulatory complexity of TNBC, underscoring the limitation of relying solely on MDA-MB-231 to represent the molecular landscape of TNBC.

3.3. Inferential statistics

The initial collection of samples contained 18 tumor and 15 normal samples from 19 patients, with 11 matched pairs. After averaging specimens from the same patient, we obtained one Δ Ct value per patient per tissue type. This result led to 13 unique tumor samples and 14 unique normal samples, with 8 matched pairs. The one-sample t-test (degrees of freedom = 12) used these 13 tumor Log₂FC values. The LMM used the full 27 samples (13 tumor, 14 normal).

3.3.1. Relative expression analysis of GAS5 and SFXN1 using LMM

To account for the partially paired structure of the data, we analyzed Δ Ct (target gene Ct minus reference gene Ct) values using LMM in SPSS version 27. Tissue type (normal = 0 and tumor = 1) served as the fixed factor, and a random intercept for patient ID was included to capture within-subject correlations. We employed a variance components covariance structure

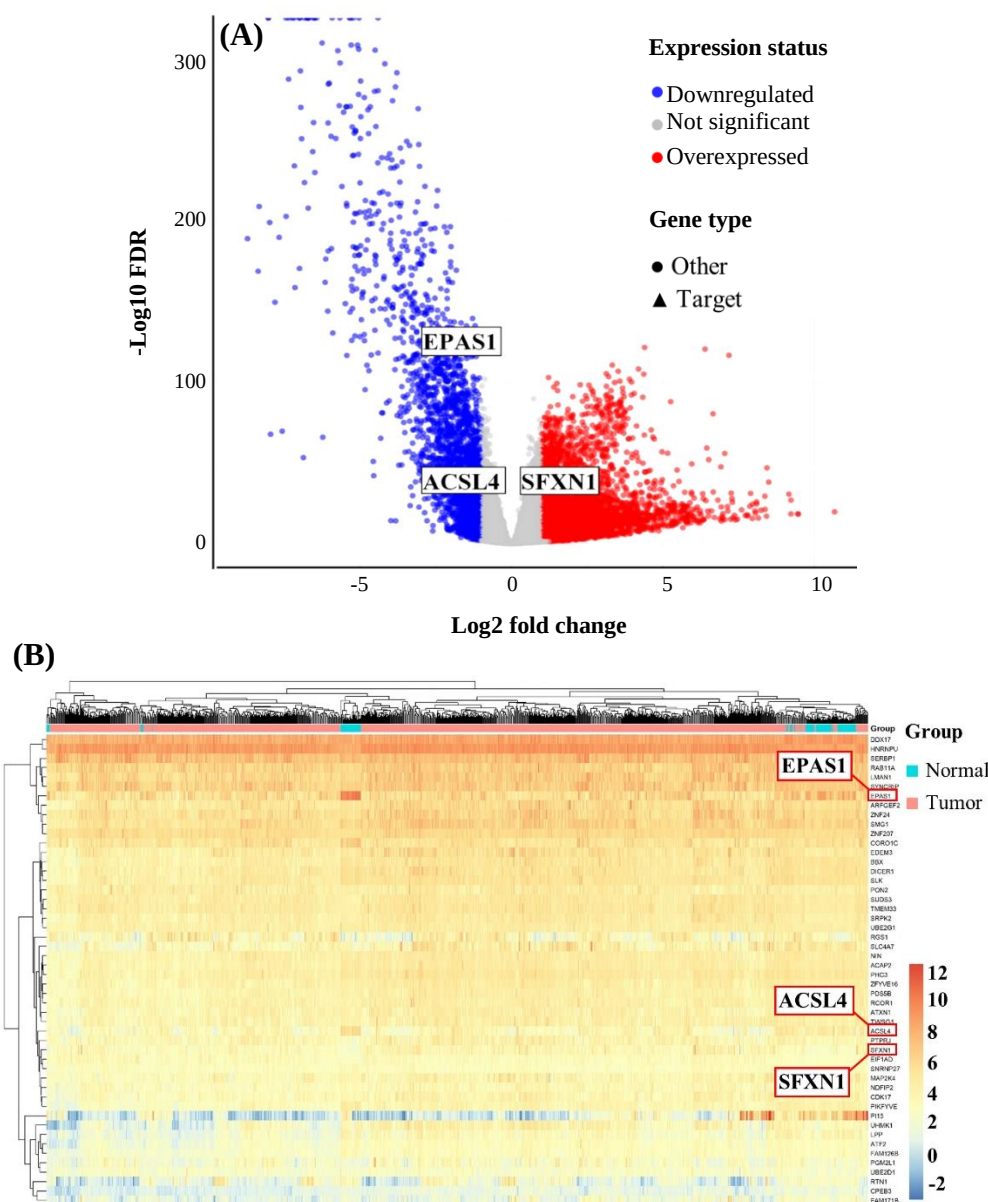


Fig. 1. (A) Volcano plot depicting differential expression of *GAS5*-associated target genes based on TCGA data. Each point represents a gene plotted according to Log_2FC and statistical significance; upregulated and downregulated candidates in tumor versus normal samples are highlighted; (B) Heatmap of expression profiles of *GAS5*-associated target genes across TCGA samples. Rows represent individual genes and columns represent tumor and normal tissue samples. Color intensity indicates normalized expression levels (Log CPM; CPM = counts per million).

and restricted maximum likelihood estimation. After averaging technical replicates, 27 samples (13 tumor and 14 normal) were included, of which 8 tumor samples were paired with normal tissue from the same patient. The lower number of samples compared with the full study group described in Table 2 reflects the averaging of technical replicates from patients with more than one tumor or normal specimen. For *GAS5*, the estimated marginal mean ΔCt was 1.059 (SD = 0.671) in normal tissue and 0.974 (SD = 0.688) in tumor tissue, with a mean difference of 0.085 (95% CI: -1.469 to

1.638). The fixed effect of tissue type was not significant ($F = 0.015$; $p = 0.905$). For *SFXN1*, the estimated marginal mean ΔCt was 7.446 (SD = 0.504) in normal tissue and 8.105 (SD = 0.523) in tumor tissue, with a mean difference of -0.659 (95% CI: -2.172 to 0.853). Again, the effect of tissue type was not significant ($F = 0.901$; $p = 0.361$). Therefore, the LMM did not reveal a significant difference in the expression of either *GAS5* or *SFXN1* between tumor and normal tissues.

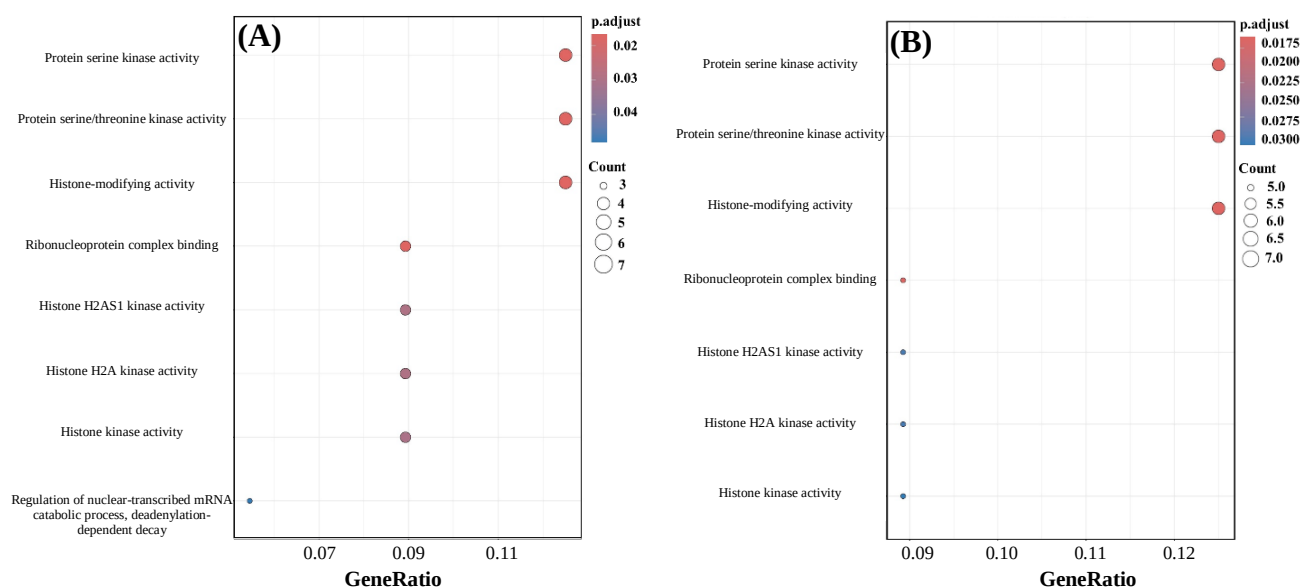


Fig. 2. GO enrichment analysis of *GAS5*-associated genes, showing the most significantly enriched terms in **(A)** CC, and **(B)** MF categories. The dot plots display significant terms ranked by adjusted *p* value.

3.3.2. Relative expression analysis of *GAS5* and *SFXN1* using one-sample t-test

Relative expression was also calculated using the $2^{-\Delta\Delta C_t}$ method, with β -actin as the internal control and normal tissue as the calibrator. Log₂FC values were first tested for normality. The D'Agostino and Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests all indicated no significant deviation from a normal distribution for either *GAS5* or *SFXN1* Log₂FC values (all $p > 0.05$; Table S3), justifying the use of parametric analyses. A one-sample t-test was used to assess whether the mean Log₂FC of each gene deviated significantly from zero (no change). Neither gene

showed a statistically significant difference. For *GAS5*, the mean Log₂FC was 0.235 ($t(12) = 0.466$; $p = 0.650$), and for *SFXN1*, the mean Log₂FC was -0.450 ($t(12) = 0.766$; $p = 0.458$). Thus, there was no evidence of differential expression for either gene in tumor tissues relative to normal tissues. It should be noted that Log₂FC is more appropriate for statistical analyses because it symmetrizes up- and downregulation, stabilizes variance, and allows parametric tests that assume normality. Conversely, raw FC values are more intuitive for descriptive statistics, as they directly communicate the magnitude of change in linear terms.

Table 2. Demographic and clinicopathological characteristics of the study patients

Variable	Value
Number of patients	19
Mean age of patients ($\pm$ SD)	51.9 ($\pm$ 14.3)
Age range	31-77
Number of samples	33
Number of tumor samples	18
Number of normal samples	15
Number of tumor samples with matched normal tissue	11
Number of tumor samples without matched normal tissue	7
Tumor stage	I (4), II (1), III (11), IV (1)
Lymph node involvement	7

Table 3. Descriptive statistics for *GAS5* and *SFXN1* expression (Δ Ct values) in tumor and normal tissues

Gene	Tissue	Number	Mean Δ Ct ($\pm$ SD)	Median Δ Ct	Min-Max Δ Ct
	Tumorous	18	0.93 ($\pm$ 1.87)	0.77	-1.77-5.18
	Normal	15	1.18 ($\pm$ 3.10)	0.65	-4.30-6.30
	Tumorous	18	8.14 ($\pm$ 1.70)	7.69	5.87-11.88
	Normal	15	7.41 ($\pm$ 2.05)	7.21	3.93-11.50

3.3.3. Correlation between *GAS5* and *SFXN1* expression

Spearman's rank correlation (one-tailed, as pre-specified) revealed a significant negative correlation ($r = -0.479$; one-tailed $p = 0.049$). For reference, the two-sided p -value was 0.099 (95% CI for r : -0.82 to 0.12). This analysis was based on 13 independent tumor samples, one per patient after averaging replicates (Fig. 4). This result indicates that in the tumor samples examined, lower *GAS5* expression was associated with higher *SFXN1* expression, and vice versa. The significant negative correlation between *GAS5* and *SFXN1* expression constitutes the principal novel finding of this study and suggests an association that could reflect regulatory mechanisms or other interactions in TNBC.

4. DISCUSSION

The present study combined a systematic bioinformatics analysis of BC datasets with gene expression profiling of clinical TNBC specimens to identify mRNA targets of the lncRNA *GAS5* and evaluate the expression relationship between *GAS5* and the selected target gene *SFXN1*. Based on the bioinformatics analysis, three candidate genes were identified: *EPAS1*, *ACSL4*, and *SFXN1*. All three candidates have established relevance in cancer biology. *EPAS1*, encoding HIF-2 α , promotes tumor progression under hypoxic conditions by enhancing angiogenesis and therapeutic resistance^[29]. *ACSL4*, a key enzyme in lipid metabolism, is implicated in the aggressiveness of TNBC and its metastatic dissemination^[30]. However, *SFXN1* was particularly suitable for this study due to its recently highlighted role in TNBC. This inner mitochondrial membrane protein is reported to be upregulated in BC and linked to poor prognosis, and has been mechanistically implicated in tumor progression and metastasis^[28,31,32].

Regarding the expression analysis, considering *GAS5*

first, neither the LMM nor the one-sample t-test revealed a statistically significant difference in *GAS5* expression between tumor and normal samples. This finding diverges from the prevailing view that *GAS5* acts as a tumor-suppressive lncRNA that is consistently downregulated in BC, including TNBC^[12,14,33,34]. Specifically, in TNBC, reduced *GAS5* expression is associated with higher clinical stage, lymph node metastasis, and poorer overall survival^[17]. Moreover, circulating *GAS5* levels correlate with metastasis, recurrence, and proliferative markers^[21,22]. However, the literature presents inconsistencies; one study reported elevated *GAS5* levels in breast tumor tissues and linked them to advanced stage and lymph node involvement^[15], while another study found no significant difference in plasma *GAS5* between patients and healthy controls^[21]. This heterogeneity underscores the need for caution when interpreting *GAS5* expression without considering the specific biological and methodological contexts. Importantly, this non-significant result should not overshadow the biological relevance of *GAS5* in the pathogenesis of TNBC. *GAS5* is well established as a multifaceted tumor suppressor that affects apoptosis, invasion, and therapy response through multiple mechanisms^[25]. As a ceRNA, it sponges oncogenic miRNAs, including miR-21, miR-222, miR-221-3p, miR-196a-5p, and miR-378a-5p. This sponging action derepresses tumor-suppressor genes such as *PTEN*, *PDCD4*, *FOXO1*, *DKK2*, and *SUFU*^[33]. In TNBC cells, *GAS5* restores FOXO1/PI3K/AKT signaling via miR-196a-5p sequestration^[17]. Moreover, *GAS5* can suppress the PI3K/AKT/mTOR, Wnt/ β -catenin, and NF- κ B pathways, enhance mitochondrial apoptosis, and inhibit DNA repair to increase radiosensitivity^[25,26,33]. Clinically, *GAS5* holds promise as a prognostic indicator and a therapeutic target. In TNBC, low *GAS5* expression or its promoter methylation has been linked to poor overall survival, disease-specific survival, and disease-free interval^[2].

Table 4. Relative expression (fold change) of *GAS5* and *SFXN1* in tumor samples

Gene	No. of samples	Mean FC ($\pm$ SD)	Median FC	Min-Max FC
<i>GAS5</i>	18	2.38 ($\pm$ 2.93)	0.80	0.24-8.94
<i>SFXN1</i>	18	2.20 ($\pm$ 4.23)	0.98	0.05-15.89

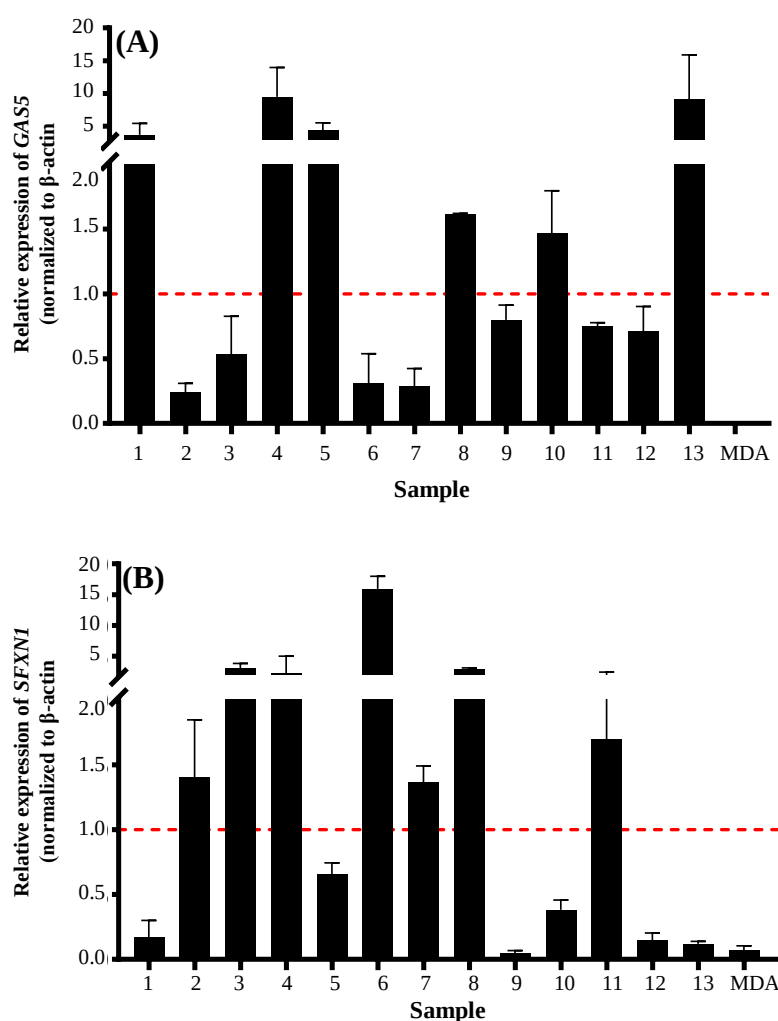


Fig. 3. Relative expression (FC) of (A) *GAS5* and (B) *SFXN1* in individual TNBC tumor samples ($n = 13$ independent tumor samples; multiple specimens from the same patient were averaged). The red dashed line at FC = 1 indicates no change. A break in the y-axis is used to accommodate a few extreme values, allowing clearer visualization of the distribution of the remaining values. MDA: MDA-MB-231 cell line (shown for reference, not included in the correlation analysis).

Restoring *GAS5* expression has been shown to enhance chemosensitivity^[14]. Nevertheless, the non-significant finding in the present study serves as a reminder that the clinical utility of *GAS5* as an independent factor requires robust validation in larger, multi-ethnic cohorts with molecularly stratified TNBC subgroups.

Parallel to the *GAS5* results, *SFXN1* expression was also not significantly altered in TNBC tissues. The mean Log_2FC was negative, and neither the one-sample t-test nor the LMM indicated a significant difference between tumor and normal samples. Biologically, *SFXN1* is an inner mitochondrial membrane protein belonging to the sideroflexin family, with a known role in serine transport and one-carbon metabolism^[31]. This pathway is fundamental for proliferating cells, as one-carbon units are essential for nucleotide and lipid biosynthesis, processes closely aligned with the anabolic demands of

cancer cells. Beyond its transport role, *SFXN1* supports mitochondrial respiratory chain function, heme metabolism, and iron homeostasis, and has been linked to redox balance and stress adaptation^[35,36]. These functions position *SFXN1* at the intersection of metabolic reprogramming, sustained proliferation, and resistance to cell death—all established hallmarks of cancer. Although *SFXN1* remains less extensively studied than classical oncogenes, a growing body of evidence suggests its potential tumor-promoting role. In pan-cancer analyses, *SFXN1* is overexpressed in multiple tumor types and has been associated with poor prognosis and immune infiltration^[37]. In non-small cell lung cancer, elevated *SFXN1* levels correlate with larger tumor size, lymph node metastasis, and advanced stage, and its knockdown reduces proliferation and invasion while inducing apoptosis^[38-40]. Specifically, in BC,

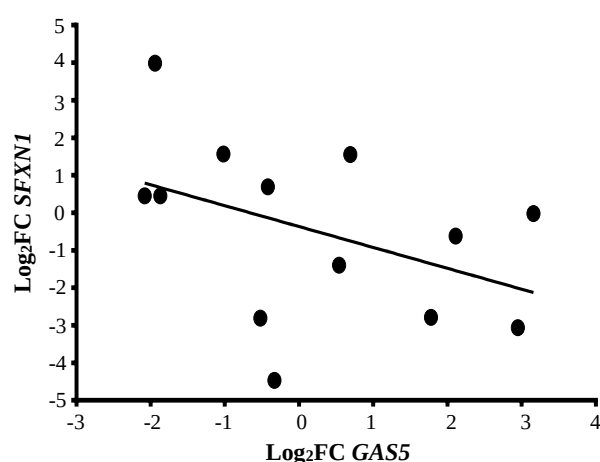


Fig. 4. Correlation between *GAS5* and *SFXN1* expression in tumor samples. Log₂FC values of *SFXN1* are plotted against Log₂FC values of *GAS5* (Spearman's $r = -0.479$; $p = 0.049$). All 13 independent tumor samples (one per patient after averaging) are shown.

higher *SFXN1* expression in tumor tissues compared to normal tissues has been associated with unfavorable outcomes^[32]. Most notably, Andriani et al.^[28] demonstrated significant upregulation of *SFXN1* in TNBC and provided functional evidence that it promotes tumor growth and metastasis by stabilizing the oncoprotein CIP2A via the inhibition of the autophagy receptor TOLLIP.

Several factors may account for the discrepancy between our non-significant findings and the majority of prior reports. First, our modest, partially paired sample size, together with the averaging of technical replicates for some patients, likely limited the statistical power needed to detect the subtle expression differences. Second, TNBC is a molecularly and micro-environmentally heterogeneous disease, and the limited size of our cohort may not have adequately represented this diversity. Third, population-specific genetic and epigenetic backgrounds, including ethnic differences inherent to our Iranian cohort, may have further influenced the observed expression patterns. Fourth, technical variables, such as tissue type, RNA quality, primer design, and normalization strategy, may be different between our approach and the public datasets, potentially introducing additional variability. Finally, within the complex regulatory network linking lncRNAs, miRNAs, and target mRNAs, a theoretical negative feedback loop could simultaneously alter the expression levels of both the target gene and the upstream lncRNA, further complicating the observed pattern^[12,33].

Despite the absence of a significant individual differential expression, Spearman's correlation analysis of Log₂FC values revealed a significant negative

correlation between the two genes. Samples with lower *GAS5* expression tended to show higher *SFXN1* expression, and vice versa. lncRNAs can correlate with the expression of their target genes, both positively and negatively. For instance, the lncRNA LINC00839 exhibits a significant positive correlation with its target gene *CCNE1*^[41], while the sense-antisense pair lncRNA NOP14-AS1 shows an inverse expression pattern with its mRNA counterpart, *NOP14*, in response to DNA damage^[42]. This regulatory relationship is influenced by several factors, including genomic context; lncRNAs acting in cis (overlapping or proximal to the target gene) tend to show more positive correlation with nearby genes compared to those acting in trans (distant or on a different chromosome)^[43]. Additionally, coordinated regulation at both transcriptional and post-transcriptional levels can create a tight coupling between lncRNAs and their targets^[44]. However, caution is warranted, as strong inverse correlations can also arise from independent regulatory mechanisms rather than direct interactions^[42]. Furthermore, data heterogeneity due to different experimental approaches may affect the reliability of predicted correlations^[45].

Similar expression relationships involving *GAS5* have been reported across several cancer types. In non-small cell lung cancer, *GAS5* expression is negatively correlated with the proliferation marker Ki67 and with EGFR^[46]. In gastric cancer, reduced *GAS5* levels are associated with increased miR-23a, with functional analyses supporting a ceRNA-mediated regulatory mechanism^[47]. Similarly, in BC, *GAS5* has been reported to correlate positively with APOBEC3C and negatively with miR-103, illustrating that *GAS5* can participate in multi-component regulatory axes, with varying correlation patterns depending on the interacting partners^[48]. Large-scale co-expression and network analyses have further demonstrated that lncRNAs are embedded in complex transcriptional and post-transcriptional networks in cancer and can exhibit both positive and negative correlations with coding genes in a tissue-specific manner^[49,50]. However, it is essential to emphasize that a statistically significant correlation does not establish causality. The observed inverse relationship between *GAS5* and *SFXN1* could result from direct regulation, indirect regulation through intermediaries such as miRNAs or transcription factors, or the influence of a common upstream pathway that coordinately alters the expression of both molecules in opposite directions. Definitive resolution of this question will require functional experiments, specifically knockdown and overexpression of *GAS5* in appropriate TNBC cell lines, followed by measurement of *SFXN1* expression at the RNA and protein levels. If directional modulation of *GAS5* leads to corresponding

changes in *SFXN1*, subsequent studies could delineate the precise regulatory mechanisms, including potential miRNA intermediaries or transcription factors. At present, this correlation does not support any diagnostic or therapeutic applications, and such possibilities remain entirely speculative and would require extensive functional and clinical validation.

Beyond the relatively small sample size from a single-center Iranian cohort and the inherent molecular heterogeneity of TNBC, this study has additional limitations that should be considered when interpreting the results. First, the discovery screening was conducted using the broader TCGA-BRCA cohort, which includes multiple BC subtypes. Complete annotations for ER/PR/HER2 were not uniformly available to reliably restrict the analysis to TNBC cases. Second, using the mean of all normal samples as a calibrator for unmatched tumors may have biased the Log₂FC estimates, potentially underestimating the true expression differences. Third, gene expression was assessed only at the mRNA level by RT-qPCR. Without corresponding validation methods at the protein level, the functional consequences of the observed expression changes remain uncertain. Fourth, no functional experiments (e.g., knockdown, overexpression, or luciferase reporter assays) were performed to directly test the regulatory relationship between *GAS5* and *SFXN1*. Fifth, pre-analytical variables, such as tissue handling, ischemia time, and cellular composition, may have contributed to variability, despite rigorous quality control measures. Finally, the cross-sectional design of the study prevented the assessment of dynamic expression changes during disease progression or in response to treatment. Further functional studies, including knockdown and overexpression of *GAS5* in TNBC cell lines followed by proteomic and phenotypic assays, are needed to establish the mechanistic basis of the *GAS5-SFXN1* relationship. Also, any potential diagnostic or therapeutic relevance would require extensive prospective validation.

5. CONCLUSION

This study combined bioinformatics analyses with gene expression profiling in clinical TNBC samples to investigate the regulatory role of lncRNA *GAS5* and its potential target gene *SFXN1*. Neither *GAS5* nor *SFXN1* showed significant differential expression between tumor and normal tissues. However, a significant negative correlation was identified between the expression levels of *GAS5* and *SFXN1*. This inverse expression pattern provides preliminary, hypothesis-generating evidence for a potential association between these two molecules in TNBC. While this correlation does not demonstrate regulation, causality, or clinical relevance at this stage, it suggests that the *GAS5-SFXN1*

axis may play a role in the molecular networks involved in the pathogenesis of this aggressive BC subtype. These findings warrant further functional and mechanistic studies to determine whether *GAS5* directly modulates *SFXN1* expression and to further explore the clinical significance of this association.

DECLARATION

Acknowledgments

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Generative AI and AI-assisted technologies

Grammarly and Typeset were used to refine the English Language in this manuscript. The authors declared that the manuscript was not generated by AI.

Ethical approval

The study protocol was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (ethical code: IR.SBMU.MSP.REC.1403.043).

Consent to participate

Informed consent was obtained from all participants by the tissue bank.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

PSD: conception, design of the study, data acquisition and analysis, writing original draft, review, and editing; MVO, MP, and AAF: acquisition of data, software, visualization, review, and editing; SMY: supervision, conception, design of the study, funding acquisition, review, and editing.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare no competing interests.

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Supplementary information

The online version contains supplementary material.

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