Gazi Med J 2026;37(1):83-91

DOI: http://dx.doi.org/10.12996/gmj.2025.4604

miR-99b-5p as a Modulator of KLF4 Expression and Drug Response in Luminal B Breast Cancer via a Ubiquitin-Mediated Mechanism

miR-99b-5p'nin Ubikütin-Aracılı Mekanizma ile Luminal B Meme Kanserinde KLF4 Ekspresyonunu Düzenlemesi

Senem Noyan, Bala Gür Dedeoğlu

Biotechnology Institute, Ankara University, Ankara, Türkiye

ABSTRACT

Objective: Luminal B breast cancer (BC) exhibits aggressive behavior and distinct molecular features, often leading to resistance to therapies such as trastuzumab and tamoxifen. MicroRNAs have emerged as key regulators of cancer progression and drug response. This study explores the role of miR-99b-5p in modulating treatment resistance in Luminal B BC.

Methods: Both trastuzumab-sensitive BT-474 cells and trastuzumab-resistant BT-474 cells were employed to evaluate the functional role of miR-99b-5p through mimic and inhibitor transfections. Gene and protein expression levels were measured using qRT-PCR and Western blotting, respectively. Bioinformatic analyses were performed using the ENCORI, UALCAN, GeneMiner, ROCplot, and UbiBrowser databases.

Results: Our results demonstrated that miR-99b-5p expression was initially downregulated following tamoxifen or trastuzumab treatment, but was subsequently elevated in trastuzumab-resistant cells, suggesting a dynamic regulatory role in therapeutic adaptation. Bioinformatic analyses identified an association between miR-99b-5p and Kruppel-like factor 4 (KLF4), a zinc-finger transcription factor implicated in BC progression and therapy sensitivity. Biological assays provided evidence that miR-99b-5p positively regulates KLF4 and BCL2 protein levels, potentially promoting cell survival and contributing to drug resistance. Mechanistically, we identified TRAF7 as a downstream target of miR-99b-5p, and TRAF7 modulates KLF4 protein stability through ubiquitin-mediated degradation. Suppression of TRAF7 by miR-99b-5p reduces KLF4 ubiquitination, enhancing its stability and downstream signaling.

ÖZ

Amaç: Luminal B tipi meme kanseri, artmış tümör agresifliği ve özgün moleküler özellikleriyle karakterize olup, trastuzumab ve tamoksifen gibi hedefe yönelik tedavilere karşı direnç gelişimiyle sıklıkla ilişkilidir. MikroRNA'lar (miRNA), kanser progresyonu ve tedavi yanıtının temel düzenleyicileri olarak öne çıkmaktadır. Bu çalışmada, miR-99b-5p'nin Luminal B meme kanserinde tedavi direncini düzenleyici rolü araştırılmıştır.

Yöntemler: Trastuzumab'a duyarlı ve trastuzumab dirençli BT-474 meme kanseri hücrelerinde, miR-99b-5p'nin fonksiyonu, özgül mimik ve inhibitörler kullanılarak değerlendirilmiştir. Gen ve protein ekspresyon düzeyleri sırasıyla qRT-PCR ve Western blot yöntemleriyle analiz edilmiştir. Ayrıca, ENCORI, UALCAN, GeneMiner, ROCplot ve UbiBrowser gibi biyoinformatik veri tabanları aracılığıyla hedef genlerin ekspresyon profilleri ve tedavi cevabı ile ilişkisi incelenmiştir.

Bulgular: miR-99b-5p ekspresyonu, tamoksifen veya trastuzumab uygulaması sonrasında ilaca duyarlı BT-474 hücrelerinde azalırken, trastuzumab dirençli hücrelerde artış göstermiştir; bu durum, terapötik adaptasyonda dinamik bir düzenleyici rolüne işaret etmektedir. Biyoinformatik ve deneysel analizler, miR-99b-5p'nin çinko parmak transkripsiyon faktörü (KLF4) ile ilişkili olduğunu ortaya koymuştur. Hücre temelli analizlerde miR-99b-5p'nin KLF4 ve BCL2 protein düzeylerini artırarak hücre sağkalımını desteklediği ve ilaç direncine katkıda bulunduğu gözlemlenmiştir. Mekanistik olarak, miR-99b-5p'nin hedeflediği TRAF7 olarak bilinen E3 ubikütin ligazın KLF4'ün ubikütinasyonu aracılı yıkımını düzenlediği; TRAF7'nin baskılanmasıyla KLF4 ubikütinasyonunun azaldığı ve protein stabilitesinin arttığı belirlenmiştir.

Cite this article as: Noyan S, Gür Dedeoğlu B. miR-99b-5p as a modulator of KLF4 expression and drug response in Luminal B breast cancer via a ubiquitin-mediated mechanism. Gazi Med J. 2026;37(1):83-91

Address for Correspondence/Yazışma Adresi: Senem Noyan, Biotechnology Institute, Ankara University, Ankara, Türkiye
E-mail / E-posta: senemnoyan@gmail.com/snoyan@ankara.edu.tr
ORCID ID: orcid.org/0000-0001-6455-3702

Received/Geliş Tarihi: 10.12.2025
Accepted/Kabul Tarihi: 30.12.2025
Publication Date/Yayınlanma Tarihi: 19.01.2026

ABSTRACT

Conclusion: These findings reveal a novel miR-99b-5p/TRAF7/KLF4 axis that promotes drug resistance in Luminal B BC through post-transcriptional and ubiquitin-mediated mechanisms. Targeting this regulatory pathway may represent a promising strategy to overcome therapy resistance and may warrant further investigation in biomarker development and targeted treatment design.

Keywords: Luminal breast cancer, miR-99b-5p, post-transcriptional regulation and drug response

ÖZ

Sonuç: miR-99b-5p/TRAF7/KLF4 ekseni, Luminal B meme kanserinde post-transkripsiyonel ve ubikütin aracılı mekanizmalarla tedavi direncinin gelişiminde rol oynamaktadır. Bu düzenleyici ağın hedeflenmesi, tedaviye direnç gelişimini engellemeye yönelik yeni terapötik stratejilerin geliştirilmesine katkı sağlayabilir. Bulgular, bu eksenin daha kapsamlı fonksiyonel ve çoklu omik yaklaşımlarla incelenmesini gerekli kılmaktadır.

Anahtar Sözcükler: Luminal meme kanseri, miR-99b-5p, transkripsiyon sonrası regülasyon ve ilaç yanıtı

INTRODUCTION

Given the heterogeneity of breast cancer (BC), targeted therapeutic approaches have become essential in enhancing treatment efficacy (1). The routine assessment of biomarkers, including progesterone receptor, estrogen receptor (ER), HER2, and Ki-67, is essential for accurate diagnosis and to inform adjuvant treatment strategies in BC management (2). Molecular analytical techniques have significantly refined the identification of prognostic and predictive biomarkers, enabling more accurate patient stratification (3). The molecular classification system introduced by Sorlie et al. (4), based on gene expression profiling, established a framework for distinguishing biologically distinct subtypes of BC. Among these, the Luminal subtypes, typically distinguished by the expression of ER/PR and the absence of HER2 amplification, are routinely classified by immunohistochemistry and further subclassified into Luminal A and Luminal B based on molecular profiling, with each subtype carrying distinct prognostic significance. Additionally, multigene expression assays such as Oncotype DX, PAM50, MammaPrint, and EndoPredict offer improved risk stratification and inform decisions regarding the potential benefit of adjuvant chemotherapy when used alongside endocrine therapy, thereby advancing the paradigm of personalized BC management (5,6).

For hormone-receptor-positive Luminal A and Luminal B BC, adjuvant endocrine therapy is the cornerstone of treatment. These tumors are generally responsive to anti-estrogen therapies, including tamoxifen and aromatase inhibitors, and, when applicable, to HER2-directed therapies (7). Tamoxifen inhibits ER signaling, thereby reducing tumor proliferation, and is typically given long-term to lower recurrence risk (7). Trastuzumab, a monoclonal antibody targeting HER2, is primarily used to treat HER2-positive Luminal B tumors, where it inhibits HER2 signaling (8). It also helps overcome resistance to endocrine therapy by modulating the interplay between HER2 and ER signaling pathways (9). Nevertheless, prolonged anti-estrogen and HER2-targeted therapies often lead to intrinsic or acquired drug resistance, limiting treatment effectiveness and promoting disease progression.

MicroRNAs (miRNAs) are small, non-coding RNAs that control gene expression post-transcriptionally (10) and, in BC, influence major oncogenic and tumor-suppressive pathways by regulating processes such as proliferation, apoptosis, and metastasis (11). Epigenetic regulation mediated by miRNAs has emerged as a central focus of recent research aimed at developing prognostic and predictive models (12). Numerous miRNAs are significantly upregulated or downregulated depending on the BC stage, the risk of tumor recurrence, and the overall survival, underscoring their potential as valuable biomarkers and therapeutic targets (13). Dysregulated miRNA expression is closely linked to various malignancies, including BC (14,15); altered expression profiles not only distinguish cancerous from healthy tissues but also enable classification of distinct molecular subtypes (16). This underscores the significance of miRNA profiling as a promising tool in personalized medicine, particularly for the early detection and accurate classification of BC subtypes (17). Specifically, in Luminal BC, miRNA profiling provides significant prognostic and predictive value beyond that provided by traditional endocrine therapies (18). Distinct miRNA expression patterns have been shown to differentiate between Luminal A and Luminal B subtypes, with certain profiles correlating with tumor grade, proliferation rate, and clinical outcomes (19). These findings indicate that miRNAs acting both as molecular biomarkers for disease detection and prognostic assessment, and as valuable surrogates for guiding and personalizing therapeutic interventions.

Kruppel-like factor 4 (KLF4) plays a fundamental role in BC, integrating multiple signaling pathways that regulate cell proliferation, differentiation, apoptosis, and maintenance of cancer stem cells (20). Due to its dual and context-dependent roles, acting either as a tumor suppressor or as an oncogene, tight regulation of KLF4 expression and activity is essential (21). Recent studies have highlighted the importance of post-transcriptional regulatory mechanisms in modulating KLF4 protein levels, particularly in BC (22). These mechanisms include miRNAs, non-coding RNAs, RNA-binding proteins, mRNA modifications, and alternative splicing events. This study presents a comprehensive overview of the post-transcriptional regulation of miR-99b-5p, which is downregulated in response to drug treatment in Luminal BC. It explores the underlying molecular mechanisms driving this regulation and examines the impact of miR-99b-5p on tumor behavior and therapeutic response. We hypothesize that therapeutic pressure from tamoxifen or trastuzumab in Luminal B BC dynamically modulates miR-99b-5p levels, which, in turn, regulate KLF4 expression through an ubiquitin-mediated mechanism, thereby influencing the drug response. Recent evidence suggests that post-transcriptional regulation of key transcription factors, including KLF4, plays a pivotal role in BC progression and therapy response (23). However, the mechanisms controlling KLF4 protein stability remain incompletely understood. Emerging data implicate E3 ubiquitin ligases such as TRAF7 in modulating protein degradation pathways in cancer. Yet, the interplay between miRNAs and ubiquitin ligases in regulating KLF4 stability in Luminal B BC has not been elucidated. Here, we investigate the role of miR-99b-5p in modulating KLF4 expression through targeting TRAF7, unveiling a novel miR-99b-5p/TRAF7/KLF4 regulatory axis that may contribute to therapy resistance and BC progression.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

BT-474 BC cells, either trastuzumab-sensitive or -resistant, were grown in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin under standard culture conditions at 37 °C with 5% CO_2 in a humidified incubator. In addition to the BT-474 BC cell line, the SK-BR-3 and MCF7 cell lines were used. SKBR3 cells were cultured in McCoy's medium, and MCF7 cells were cultured in DMEM high glucose medium. Tamoxifen (Tocris) and trastuzumab (Roche) were used in this study; their IC_{50} values were determined in our previous research (24). BT-474 (trastuzumab-resistant) cells were continuously treated with 6 µg/mL trastuzumab to maintain the resistant phenotype (25,26).

As this study involves *in vitro* cell culture experiments, ethics committee approval is not required according to the guidelines of the Scientific and Technological Research Council of Turkey (TUBITAK) for such studies.

miRNA Transfection

Overexpression of miR-99b-5p was induced using a miR-99b-5p mimic (Qiagen, Cat. No. MSY0000689), with a scrambled negative control (Qiagen, Cat. No. SI03650318); downregulation was achieved using a miR-99b-5p inhibitor (Dharmacon, Cat. No. IH-300658-05) and its corresponding scrambled control (Dharmacon, Cat. No. IN-001005-01). Cells were plated in 6-well plates (3×10^5 cells/well) and cultured overnight under standard conditions (37 °C, 5% CO_2, humidified atmosphere). Transfection was carried out the following day using 25 nM of the mimic or 100 nM of the inhibitor. Cell pellets were collected 48 and 72 hours after transfection for RNA isolation and protein extraction, respectively.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from the harvested cell pellets using TRIzol reagent (Invitrogen), and complementary DNA (cDNA) was synthesized from 1 µg of RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad). qRT-PCR analysis was then performed on a LightCycler® 480 system (Roche) using a SYBR Green-based master mix. Primer sequences and corresponding reference IDs are listed in Table 1. The expression of miR-99b-5p in BC cells was evaluated by reverse-transcribing total RNA using the miScript II RT Kit (Qiagen, Cat. No. 218160) according to the manufacturer's instructions. Quantification of miR-99b-5p was performed by qRT-PCR using a specific miScript Primer Assay (Qiagen, Cat. No. MS00032165) and the miScript SYBR® Green PCR Kit (Qiagen, Cat. No. 218075) on the LightCycler® 480 system (Roche). Each reaction was conducted in duplicate using 5 ng of cDNA. The endogenous control RNU6 (Qiagen, Cat. No. MS00033740) was used for normalization, and relative expression was quantified via the 2^−ΔCT approach.

Table 1. Primer sequences.

Gene name	Sequences 5'---3'	RefSeq ID
KLF4	F: ACCCACACAGGTGAGAAACC	NM_004235.6
	R: ATGTGTAAGGCGAGGTGGTC	
BCL2	F: CTTTGAGTTCGGTGGGGTCA	NM_000633.3
	R: GCCGGTTCAGGTACTCAGTC	
TRAF7	F: ACCACAGGGACCAGAATGGA	NM_032271.3
	R: GTCCTGCAGTGCTGCTTGTA	
GAPDH	F: TTCGACAGTCAGCCGCAT	NM_002046.7
	R: TGAAGGGGTCATTGATGGCA	

Western Blot

Total protein was extracted from cell pellets using RIPA buffer (Cell Signaling Technology, Cat. No. 9806) and quantified by the Bradford assay (Thermo Scientific). Equal amounts (15 µg per sample) were separated by SDS-PAGE at 100 V for 2 hours. Proteins were transferred to membranes by wet transfer, and subjected to immunoblotting with specific primary antibodies, and then incubated with corresponding secondary antibodies. The following primary antibodies were used: KLF4 (Invitrogen, Cat. No. MA5-41214, 1:1000), BCL2 (Cell Signaling Technology, D17C4, 1:1000), and β-actin (ACTB, BioLegend, Cat. No. 643801, 1:1000). S The secondary antibodies used were HRP-conjugated anti-mouse IgG (Cell Signaling Technology, Cat. No. 7076) and HRP-conjugated anti-rabbit IgG (Cell Signaling Technology, Cat. No. 7074). Immunoreactive bands were visualized using the WesternBright Sirius Kit (Advansta, Cat. No. K12043-D20) and imaged with the LI-COR Odyssey Imaging System.

Bioinformatic Analysis

The expression of miR-99b-5p in BC was analyzed using the ENCORI and UALCAN databases. KLF4 expression was evaluated separately in tumor versus normal tissues, as well as across PAM50 molecular subtypes, utilizing GTEx and The Cancer Genome Atlas (TCGA) datasets through the GeneMiner platform. Additionally, the association between miR-99b-5p expression and drug response was investigated using ROCplot analysis. The substrate proteins of E3s were predicted using UbiBrowser.

Statistical Analysis

Statistical significance for pairwise comparisons was assessed using Student's t-test, with p-values below 0.05 deemed statistically significant. Statistical analyses were conducted using SPSS software (IBM Corporation, New York, USA).

RESULTS

miR-99b-5p Expression Dynamics in Drug Response and Resistance

Our previous research analyzed miRNA expression profiles that were altered in response to therapeutic regimens, including tamoxifen and trastuzumab. We observed that miR-99b-5p expression was significantly downregulated following exposure to either drug (24). Supporting these findings, an independent study reported that the overexpression of miR-99b-5p promotes proliferation of BT-474 BC cells (27). In this study, we aimed to elucidate the regulatory role of miR-99b-5p, which is consistently suppressed following drug exposure (Figure 1a). miR-99b-5p expression was markedly upregulated in trastuzumab-resistant BT-474 cells (Figure 1c), suggesting a potential role in acquired resistance mechanisms. Consequently, its predictive value for trastuzumab response in patients with luminal BC was assessed by receiver operating characteristic (ROC) curve analysis (n = 47). The analysis revealed a 1.1-fold change that was statistically

significant (p=0.005). The ROC analysis yielded an area under the curve of 0.833, demonstrating robust discriminative performance; sensitivity (true positive rate) was 0.71, specificity (true negative rate) was 1.0, and the optimal threshold was 14.74 (Figure 1b). Collectively, these results demonstrate that miR-99b-5p expression is acutely downregulated in response to tamoxifen and trastuzumab, but is significantly elevated in resistant cell populations, highlighting its potential dual role in drug sensitivity and resistance.

Bioinformatic Evidence for the Regulatory Role of miR-99b-5p on KLF4 in Breast Cancer

KLF4 expression was analyzed in BC using datasets from GTEx and TCGA. The analysis revealed that KLF4 expression was significantly downregulated in breast tumor tissue compared with both normal breast tissue and adjacent non-tumorous tissue samples. PAM50-based subtype-specific analysis further demonstrated that the Luminal B subtype exhibits the lowest KLF4 expression levels (Figure 2a). In contrast, data from the ENCORI database indicated that miR-99b-5p is significantly upregulated in BC tissues. Consistently, analysis of the BRCA dataset showed elevated miR-99b-5p expression across all PAM50 subtypes relative to normal tissue (Figure 2b). Bioinformatic analyses identified a potential correlation between miR-99b-5p and KLF4 expression, suggesting a post-transcriptional regulatory interaction. Collectively, these findings imply that miR-99b-5p may modulate KLF4 expression in BC, particularly within the Luminal B subtype.

Regulatory Effects of miR-99b-5p on KLF4 and BCL2 Expression in Luminal Breast Cancer Cells

Expanding upon our previous research that suggests a regulatory interaction between miRNA and KLF4, and considering the observed downregulation of miR-99b-5p following drug treatment, we investigated the effects of miR-99b-5p modulation on KLF4 expression in BT-474 cells, a representative model of the Luminal B BC subtype. qRT-PCR (Figure 3a) and protein expression analyses (Figure 3b) revealed that miR-99b-5p inhibition led to a significant reduction in KLF4 levels, while its overexpression did not elicit a notable change. In parallel, suppression of miR-99b-5p resulted in decreased BCL2 protein levels, supporting a positive correlation between KLF4 and BCL2 expression. Notably, tamoxifen-treated BT-474 cells exhibited increased miR-99b-5p expression, and this increase was accompanied by significant upregulation of both KLF4 and BCL2 relative to the scrambled control. A similar pattern of miR-99b-5p induction and associated protein upregulation was observed following trastuzumab treatment (Figure 3c). Collectively, these findings suggest a context-dependent positive regulatory relationship between miR-99b-5p and expression of KLF4, potentially contributing to tumor cell proliferation.

KLF4 Expression is Downregulated by miRNA Inhibition Through an Indirect Ubiquitination Mechanism

The observed decrease in KLF4 expression following miR-99b-5p inhibition was hypothesized to result from enhanced KLF4 degradation mediated by a ubiquitination-related gene targeted by miR-99b-5p. We propose that miR-99b-5p indirectly regulates KLF4 protein levels by suppressing a gene involved in KLF4 ubiquitination. Inhibition

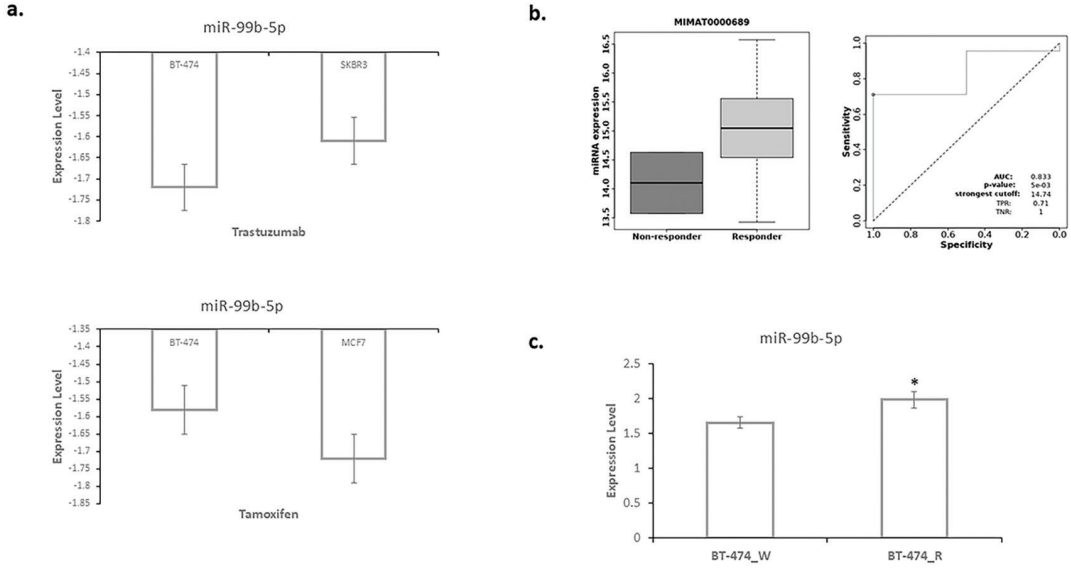

Figure 1. Expression levels of miR-99b-5p in Luminal breast cancer cells. (a) Luminal breast cancer cell lines were treated with tamoxifen (10 µM) or trastuzumab (6 µg/mL) for 48 hours, and miR-99b-5p expression levels were assessed by qPCR-array. (b) Predictive performance of miR-99b-5p expression in response to trastuzumab was evaluated using receiver operating characteristic curve analysis in Luminal breast cancer patients (n =47), stratified by median follow-up time. The area under the curve was 0.833, with a statistically significant p-value (p=0.005). The optimal cut-off value for miR-99b-5p expression was 14.74, yielding a true positive rate of 0.71 and a true negative rate of 1.0. (c) miR-99b-5p expression levels were measured in parental (BT-474_W) and trastuzumab-resistant BT-474 (BT-474_R) cells using qRT-PCR, normalized to U6 snRNA. Data are presented as mean ± standard deviation from two independent experiments. Statistical significance was determined using the Student's t-test (*p<0.01).

KLF4: Kruppel-like factor 4, AUC: Area under the curve, TPR: True positive rate, TNR: True negative rate, miRNA: MicroRNA.

of miR-99b-5p leads to upregulation of this target gene, thereby promoting KLF4 ubiquitination and reducing its protein stability. To investigate this post-transcriptional regulatory mechanism, predicted miR-99b-5p target genes were intersected with known E3 ubiquitin ligases implicated in KLF4 degradation. This analysis yielded *FBXO22*, *FBXO32*, *FZR1*, and *TRAF7* as common candidates (Figure 4a). Subsequent evaluation based on expression levels, presence of a miR-99b-5p binding site within the 3'-untranslated region (3'-UTR), and literature evidence led to the selection of TRAF7 for further analysis. Comprehensive bioinformatic interrogation of the TCGA BC cohort identified a statistically significant overexpression of TRAF7 in tumor specimens compared to matched normal tissues. In contrast, KLF4 expression was lower in tumors than in normal samples (Figure 4b). Based on this inverse correlation (Figure 4c), we evaluated TRAF7 expression in BT-474 cells after transfection with a miR-99b-5p mimic. Overexpression of miR-99b-5p led to a marked decrease in *TRAF7* expression (Figure 4d). Furthermore, target prediction analysis revealed a putative miR-99b-5p binding site within the 3'-UTR of *TRAF7*, supporting the notion that miR-99b-5p directly interacts with *TRAF7* mRNA. Collectively, these data

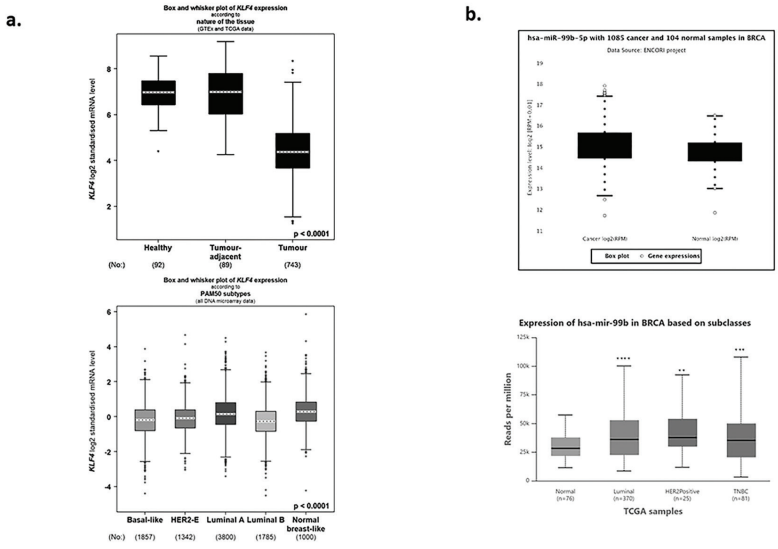

Figure 2. Bioinformatic analysis of miR-99b-5p and KLF4 expression in breast cancer. (a) KLF4 expression was analyzed using transcriptomic datasets from GTEx and TCGA, showing significant downregulation in breast tumor tissues compared to both normal breast tissue and adjacent non-tumorous samples (healthy, n =92; tumor adjacent, n =89; tumor, n =743; p<0.0001). Subtype-specific analysis based on PAM50 classification revealed that the Luminal B subtype exhibits the lowest KLF4 expression levels among subtypes (basal-like, n =1857; HER2-enriched, n =1342; Luminal A, n =3800; Luminal B, n =1785; normal-like, n =1000; p<0.0001). (b) Conversely, miR-99b-5p expression was significantly upregulated in breast cancer tissues compared to normal samples, as assessed via the ENCORI database (tumor, n =1085; normal, n =104; p<0.001). Furthermore, analysis of PAM50 subtypes in the UALCAN BRCA dataset showed that all breast cancer subtypes exhibit elevated miR-99b-5p levels compared to normal tissue (normal, n =76; Luminal A, n =370; HER2-enriched, n =25; TNBC, n =81) Differences between normal and breast cancer subtypes were evaluated using the two-sided Wilcoxon rank-sum test. Bars represent median expression values. Statistical significance is indicated as follows: ****p<0.0001, ***p<0.001, **p<0.01.

KLF4: Kruppel-like factor 4.

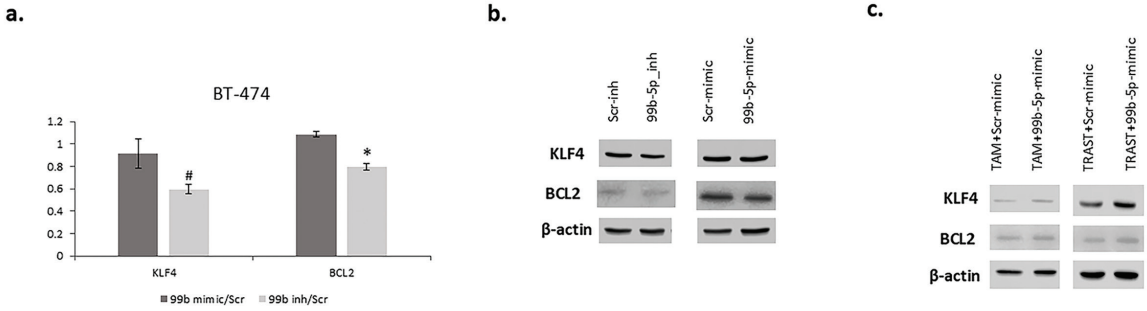

Figure 3. miR-99b-5p regulates KLF4 and BCL2 expression in BT-474 Luminal breast cancer. Modulation of miR-99b-5p in BT-474 cells, a model of Luminal B breast cancer, was performed to investigate its impact on KLF4 and BCL2 expression. qRT-PCR (a) and protein analyses (b) demonstrated that inhibition of miR-99b-5p significantly reduced KLF4 expression, whereas miR-99b-5p overexpression did not cause notable changes (two biological replicates, #p<0.01, *p<0.05). Concurrently, suppression of miR-99b-5p led to decreased BCL2 protein levels, suggesting a positive correlation between KLF4 and BCL2 expression. (c) Upon treatment with tamoxifen, BT-474 cells showed increased miR-99b-5p expression accompanied by significant upregulation of both KLF4 and BCL2 proteins compared to scrambled controls. A similar induction of miR-99b-5p and associated protein upregulation was observed following trastuzumab treatment.

KLF4: Kruppel-like factor 4, Scr: Scrambled control, TAM: Tamoxifen, TRAST: Trastuzumab.

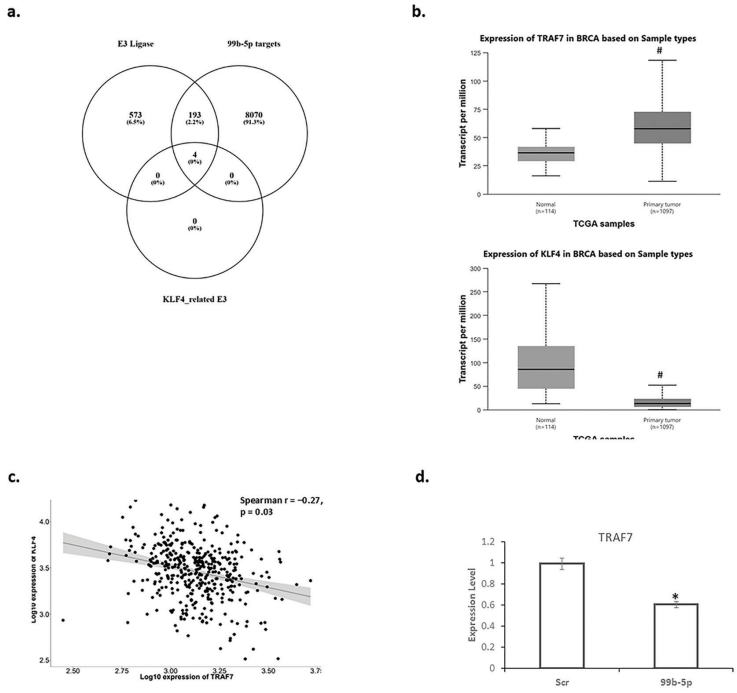

Figure 4. Indirect regulation of KLF4 by miR-99b-5p through targeting the E3 Ligase TRAF7. (a) Venn diagram showing the intersection between predicted miR-99b-5p target genes and known E3 ubiquitin ligases associated with KLF4 degradation. Four common candidates, *FBXO22, FBXO32, FZR1*, and *TRAF7*, were identified for further analysis. (b) Expression analysis of TRAF7 and KLF4 in breast cancer tissues using UALCAN database. Box plots illustrating *TRAF7* and *KLF4* mRNA expression levels in breast tumor tissues compared to normal breast tissues. TRAF7 expression is significantly elevated in tumor samples, whereas KLF4 expression is decreased, indicating an inverse expression pattern between the two genes in breast cancer (#p<0.001). (c) Negative correlation between *TRAF7* and *KLF4* expression in breast cancer. Scatter plot showing a significant inverse correlation between *TRAF7* and *KLF4* mRNA expression levels in breast cancer samples, as determined by Spearman correlation analysis (r= –0.27, p=0.03; n =403). (d) Relative *TRAF7* mRNA expression levels in BT-474 cells transfected with scrambled control or miR-99b-5p mimic. Overexpression of miR-99b-5p significantly reduced *TRAF7* expression compared to scrambled controls (two biological replicates, *p<0.05), indicating that *TRAF7* is negatively regulated by miR-99b-5p in Luminal breast cancer cells.

KLF4: Kruppel-like factor 4, Scr: Scrambled control.

imply that miR-99b-5p modulates KLF4 protein levels indirectly by targeting TRAF7, which in turn regulates KLF4 degradation via the ubiquitin–proteasome system.

DISCUSSION

Features of Luminal B BC include distinct profiles of somatic point mutations, DNA methylation patterns, and gene copy-number alterations (28) and are associated with aggressive clinical behavior. Its prognosis parallels those of HER2-enriched and basal-like subtypes, whereas Luminal A BC generally exhibits a more favorable outcome. Notably, Luminal B tumors demonstrate higher relapse rates within the first five-years post-diagnosis, which decline over time, alongside a metastatic dissemination pattern similar to that observed in basal-like and HER2-enriched cancers (29).

miRNAs serve as master regulators of BC development and drug response by integrating signals from diverse cellular pathways (30). Their capacity to target multiple genes simultaneously not only drives tumor progression and metastasis but also modulates chemotherapeutic efficacy, making them promising biomarkers and therapeutic targets for overcoming drug resistance in BC (31). Both tamoxifen and trastuzumab, key therapeutic agents for hormone receptor-expressing and HER2-overexpressing BCs, respectively, exhibit variable clinical responses that may be modulated by miRNAs (24,32,33). In line with these findings, our study revealed that miR-99b-5p expression decreased following treatment with tamoxifen and trastuzumab, regardless of cell line or drug, suggesting that miR-99b-5p may be downregulated in response to therapeutic pressure in luminal BC. Moreover, we observed increased miR-99b-5p levels in trastuzumab-resistant cells, further supporting the hypothesis that this miRNA is dynamically regulated by drug exposure and may play a role in acquired resistance mechanisms.

KLF4, a zinc-finger transcription factor, exhibits a context-dependent dual role in BC, acting either as an oncogene or as a tumor suppressor, and is regulated by a complex post-transcriptional network (34). miRNAs represent one of the principal mechanisms regulating KLF4 via post-transcriptional control (35). By binding to the 3' untranslated region of KLF4 mRNA, these small non-coding RNAs induce either degradation of the target mRNA or inhibition of its translation. A previous study revealed that KLF4 mediates estrogen signaling in BC by promoting estrogen-induced transactivation and cell proliferation (23). Mechanistically, estrogen downregulates pVHL, a key regulator

of KLF4 degradation, leading to KLF4 accumulation, which in turn drives BC progression (23). Moreover, another study demonstrated that KLF4 enhances sensitivity to tamoxifen in BC by suppressing the MAPK/ERK and p38 signaling pathways. High KLF4 expression is associated with an improved therapeutic response and a favorable prognosis, suggesting that targeting the KLF4/MAPK regulatory axis may constitute a novel therapeutic approach to overcome tamoxifen resistance in BC (36). Our study, building upon this evidence, reveals that upregulation of miR-99b-5p in the presence of tamoxifen leads to increased expression of both KLF4 and BCL2. This suggests that elevated levels of miR-99b-5p may compromise therapeutic efficacy by modulating the expression of key regulators of drug response, including KLF4. These findings indicate a potentially oncogenic role for miR-99b-5p in the context of endocrine resistance and underscore the complexity of KLF4 regulation in tamoxifen-treated luminal BC.

E3 ubiquitin ligases, which control protein turnover by targeting substrates for ubiquitin-dependent proteasomal degradation, play critical roles in signal transduction, cellular homeostasis, and cancer development (37); their dysregulation has been implicated in BC progression, metastasis, and therapy resistance (38). Among E3 ligases, members of the TRAF family, particularly TRAF7, have emerged as context-dependent regulators of tumorigenesis (39). TRAF7, which also functions as an adaptor protein in TNF signaling pathways, promotes or suppresses KLF4 expression, the depending on tumor type. In hepatocellular carcinoma, TRAF7 facilitates KLF4 degradation via ubiquitination, while in meningiomas, TRAF7 loss leads to increased KLF4 expression (40). Our findings suggest that in Luminal B BC cells, miR-99b-5p positively regulates KLF4 protein levels by targeting *TRAF7*. An inverse correlation between miR-99b-5p and *TRAF7* expression was observed by bioinformatic analysis and was further supported by the identification of a putative miR-99b-5p binding site within *TRAF7* 3′ UTR, suggesting a direct post-transcriptional regulatory interaction. Functional suppression of *TRAF7* by miR-99b-5p is postulated to attenuate KLF4 ubiquitination, thereby promoting its stabilization by preventing proteasomal degradation. Taken together, this miR-99b-5p/TRAF7/KLF4 axis may contribute to BC cell survival and drug response, highlighting the context-dependent role of ubiquitin-mediated regulation in BC.

Collectively, these post-transcriptional regulatory mechanisms constitute a highly adaptable and evolutionarily conserved network, enabling BC cells to dynamically modulate KLF4 expression in response to microenvironmental cues, therapeutic pressure, and intrinsic genomic alterations. A comprehensive understanding of this regulatory landscape not only deepens our insight into BC pathophysiology but also reveals promising therapeutic opportunities aimed at correcting dysregulated KLF4 signaling and re-establishing cellular homeostasis. In this context, our findings support the existence of a miR-99b-5p/TRAF7/KLF4 regulatory axis that governs KLF4 protein stability through a ubiquitin-dependent mechanism, potentially contributing to therapy resistance in Luminal B BC. These data demonstrate the central importance of post-transcriptional and ubiquitin-mediated modulation in BC progression and drug responsiveness. Targeting specific components of this axis, particularly miRNA–E3 ligase interactions, may represent a novel and effective strategy to restore tumor suppressor activity and overcome resistance in hormone receptor–positive BCs.

Study Limitations

Although this study provides important insights into the role of miR-99b-5p in modulating KLF4 expression and drug resistance mechanisms in Luminal B BC through *in vitro* models, further validation in in vivo systems, such as animal models, is essential to substantiate the mechanistic findings. The translation of these results into a clinical context presents additional challenges, as the complex interactions within the tumor microenvironment and the variability in patient-specific factors may influence the regulation and functional activity of the key molecules involved. Consequently, it is crucial that future studies incorporate in vivo validation and clinical cohort analysis to confirm the clinical relevance of targeting the miR-99b-5p/TRAF7/KLF4 axis and to assess its therapeutic potential in patient populations.

CONCLUSION

Our findings delineate a novel post-transcriptional regulatory axis whereby miR-99b-5p indirectly enhances KLF4 protein stability through the targeted suppression of the E3 ubiquitin ligase TRAF7. This regulatory pathway appears to modulate therapeutic response in Luminal B BC cells, particularly by influencing sensitivity to tamoxifen and trastuzumab. This evidence supports the notion that miR-99b-5p may function as a positive regulator of KLF4 and BCL2, contributing to therapy response dynamics and potentially to resistance mechanisms. Hormone-dependent BCs represent the predominant subtype, rendering acquired or intrinsic resistance to endocrine therapies a significant clinical obstacle. Consequently, extensive research efforts have focused on characterizing the molecular mechanisms underlying hormone resistance, identifying robust biomarkers indicative of the resistant phenotype, and investigating alternative molecular targets to overcome these resistance mechanisms. One promising strategy is the use of epigenetic drugs that target reversible epigenetic modifications and, when combined with chemotherapy or endocrine therapy, may help restore therapeutic responsiveness. Moving forward, integrating multi-omics approaches, including transcriptomics, epitranscriptomics, proteomics, and functional genomics, will be essential to achieving a more comprehensive understanding of how post-transcriptional regulation of key factors, such as KLF4, contributes to BC progression and therapy resistance. In particular, investigations into the temporal dynamics of miRNA expression and the interplay between lncRNAs and circRNAs under different treatment conditions will be critical for the development of next-generation therapeutic strategies capable of effectively modulating these complex regulatory networks.

Ethics

Ethics Committee Approval: As stated by The Scientific and Technological Research Council of Turkey (TUBITAK), ethics committee approval is not required for *in vitro* cell culture studies.

Informed Consent: Patient consent was not required.

Footnotes

Authorship Contributions

Concept: S.N., Design: S.N., Data Collection or Processing: S.N., B.G.D., Analysis or Interpretation: S.N., B.G.D., Literature Search: S.N., Writing: S.N. B.G.D.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

REFERENCES

1. Liang Y, Zhang H, Song X, Yang Q. Metastatic heterogeneity of breast cancer: molecular mechanism and potential therapeutic targets. Semin Cancer Biol. 2020; 60: 14–27.
2. Korde LA, Somerfield MR, Carey LA, Crews JR, Denduluri N, Hwang ES, et al. Neoadjuvant chemotherapy, endocrine therapy, and targeted therapy for breast cancer: ASCO Guideline. J Clin Oncol Off J Am Soc Clin Oncol. 2021; 39: 1485–505.
3. Bischoff H, Espié M, Petit T. Neoadjuvant therapy: current landscape and future horizons for ER-positive/HER2-negative and triple-negative early breast cancer. Curr Treat Options Oncol. 2024; 25: 1210–24.
4. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001; 98: 10869–74.
5. Jia Z, Yue F, Chen X, Narayanan N, Qiu J, Syed SA, et al. Protein arginine methyltransferase PRMT5 regulates fatty acid metabolism and lipid droplet biogenesis in white adipose tissues. Adv Sci (Weinh). 2020; 7: 2002602.
6. Mondal S, Preetam S, Deshwal RK, Thapliyal S, Rustagi S, Alghamdi S, et al. Advances in prognostic and predictive biomarkers for breast cancer: integrating multigene assays, hormone receptors, and emerging circulating biomarkers. Clin Chim Acta. 2025; 578: 120513.
7. Shien T, Iwata H. Adjuvant and neoadjuvant therapy for breast cancer. Jpn J Clin Oncol. 2020; 50: 225–9.
8. Giordano SH, Temin S, Kirshner JJ, Chandarlapaty S, Crews JR, Davidson NE, et al. Systemic therapy for patients with advanced human epidermal growth factor receptor 2-positive breast cancer: American Society of Clinical Oncology clinical practice guideline. J Clin Oncol Off J Am Soc Clin Oncol. 2014; 32: 2078–99.
9. Sharaf BM, Giddey AD, Alniss H, Al-Hroub HM, El-Awady R, Mousa M, et al. Untargeted metabolomics of breast cancer cells MCF-7 and SkBr3 treated with tamoxifen/trastuzumab. Cancer Genomics Proteomics. 2022; 19: 79–93.
10. Saliminejad K, Khorram Khorshid HR, Soleymani Fard S, Ghaffari SH. An overview of microRNAs: biology, functions, therapeutics, and analysis methods. J Cell Physiol. 2019; 234: 5451–65.
11. Weidle UH, Dickopf S, Hintermair C, Kollmorgen G, Birzele F, Brinkmann U. The role of micro RNAs in breast cancer metastasis: preclinical validation and potential therapeutic targets. Cancer Genomics Proteomics. 2018; 15: 17–39.
12. Diener C, Keller A, Meese E. Emerging concepts of miRNA therapeutics: from cells to clinic. Trends Genet. 2022; 38: 613–26.
13. Tarighati E, Keivan H, Mahani H. A review of prognostic and predictive biomarkers in breast cancer. Clin Exp Med. 2023; 23: 1–16.
14. Quiat D, Olson EN. MicroRNAs in cardiovascular disease: from pathogenesis to prevention and treatment. J Clin Invest. 2013; 123: 11–8.
15. Dehwah MAS, Xu A, Huang Q. MicroRNAs and type 2 diabetes/obesity. J Genet Genomics. 2012; 39: 11–8.
16. Cantini L, Bertoli G, Cava C, Dubois T, Zinovyev A, Caselle M, et al. Identification of microRNA clusters cooperatively acting on epithelial to mesenchymal transition in triple negative breast cancer. Nucleic Acids Res. 2019; 47: 2205–15.
17. Zubor P, Kubatka P, Dankova Z, Gondova A, Kajo K, Hatok J, et al. miRNA in a multiomic context for diagnosis, treatment monitoring and personalized management of metastatic breast cancer. Future Oncol. 2018; 14: 1847–67.
18. Kudela E, Samec M, Koklesova L, Liskova A, Kubatka P, Kozubik E, et al. miRNA Expression profiles in luminal a breast cancer-implications in biology, prognosis, and prediction of response to hormonal treatment. Int J Mol Sci. 2020; 21.
19. Lowery AJ, Miller N, Devaney A, McNeill RE, Davoren PA, Lemetre C, et al. MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/neu receptor status in breast cancer. Breast Cancer Res. 2009; 11: R27.
20. Yu F, Li J, Chen H, Fu J, Ray S, Huang S, et al. Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion. Oncogene. 2011; 30: 2161–72.
21. He Z, He J, Xie K. KLF4 transcription factor in tumorigenesis. Cell death Discov. 2023; 9: 118.
22. Zhou Z, Song X, Chi JJ, Gius DR, Huang Y, Cristofanilli M, et al. Regulation of KLF4 by posttranslational modification circuitry in endocrine resistance. Cell Signal. 2020; 70: 109574.
23. Hu D, Zhou Z, Davidson NE, Huang Y, Wan Y. Novel insight into KLF4 proteolytic regulation in estrogen receptor signaling and breast carcinogenesis. J Biol Chem. 2012; 287: 13584–97.
24. Noyan S, Gurdal H, Dedeoglu BG. Involvement of miR-770-5p in trastuzumab response in HER2 positive breast cancer cells. PLoS One. 2019; 14.
25. Noyan S, Gür Dedeoğlu B. miR-770-5p-induced cellular switch to sensitize trastuzumab resistant breast cancer cells targeting HER2/EGFR/IGF1R bidirectional crosstalk. Turkish J Biol = Turk Biyol Derg. 2024; 48: 153–62.
26. Dolapçi İB, Noyan S, Polat AY, Gürdal H, Dedeoğlu BG. miR-216b-5p promotes late apoptosis/necroptosis in trastuzumab-resistant SK-BR-3 cells. Turkish J Biol. 2023; 47: 199–207.
27. Noyan S, Gür Dedeoğlu B. Upregulation of miR-99b-5p Modulates ESR1 expression as an adaptive mechanism to circumvent drug response via facilitating ER/HER2 crosstalk. Balkan Med J. 2025; 42: 150–6.
28. Weinstein JN, Collisson EA, Mills GB, Shaw KRM, Ozenberger BA, Ellrott K, et al. The Cancer Genome Atlas Pan-Cancer analysis project. Nat Genet. 2013; 45: 1113–20.
29. Li ZH, Hu PH, Tu JH, Yu NS. Luminal B breast cancer: patterns of recurrence and clinical outcome. Oncotarget. 2016; 7: 65024–33.
30. An X, Sarmiento C, Tan T, Zhu H. Regulation of multidrug resistance by microRNAs in anti-cancer therapy. Acta Pharm Sin B. 2017; 7: 38–51.
31. Bhatia A, Upadhyay AK, Sharma S. miRNAs are now starring in "no time to die: overcoming the chemoresistance in cancer". IUBMB Life. 2023; 75: 238–56.
32. Zhang W, Xu J, Shi Y, Sun Q, Zhang Q, Guan X. The novel role of miRNAs for tamoxifen resistance in human breast cancer. Cell Mol Life Sci. 2015; 72: 2575–84.
33. Rezaei Z, Sebzari A, Kordi-Tamandani DM, Dastjerdi K. Involvement of the dysregulation of miR-23b-3p, miR-195-5p, miR-656-5p, and miR-340-5p in trastuzumab resistance of HER2-positive breast

cancer cells and system biology approach to predict their targets involved in resistance. DNA Cell Biol. 2019; 38: 184–92.

34. Frazzi R. KLF4 is an epigenetically modulated, context-dependent tumor suppressor. Front cell Dev Biol. 2024; 12: 1392391.
35. Frazzi R, Farnetti E, Nicoli D. circRNA/miRNA networks regulate KLF4 in tumor development. Non-coding RNA. 2025; 11.
36. Jia Y, Zhou J, Luo X, Chen M, Chen Y, Wang J, et al. KLF4 overcomes tamoxifen resistance by suppressing MAPK signaling pathway and predicts good prognosis in breast cancer. Cell Signal. 2018; 42: 165–75.
37. Sampson C, Wang Q, Otkur W, Zhao H, Lu Y, Liu X, et al. The roles of E3 ubiquitin ligases in cancer progression and targeted therapy. Clin Transl Med. 2023; 13: e1204.
38. Qi J, Ronai ZA. Dysregulation of ubiquitin ligases in cancer. Drug Resist Updat Rev Comment Antimicrob Anticancer Chemother. 2015; 23: 1–11.
39. Zotti T, Scudiero I, Vito P, Stilo R. The emerging role of TRAF7 in tumor development. J Cell Physiol. 2017; 232: 1233–8.
40. He H, Wu Z, Li S, Chen K, Wang D, Zou H, et al. TRAF7 enhances ubiquitin-degradation of KLF4 to promote hepatocellular carcinoma progression. Cancer Lett. 2020; 469: 380–9.