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The role of eugenol in modulating PRKAA1 expression and cellular energy homeostasis in breast cancer

Ali Serat[1,2], Nader Bagheri[3], Pouyan Zandi[1,2], Reza Yarahmadi[1,2], Zahra Yazdani Chamheydari[4], Seyed Abbas Mirzaei[1], Hadi Raeisi Shahraki[5], Hossein Teimori[1*]

[1]Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran
[2]Student Research Committee, Shahrekord University of Medical Sciences, Shahrekord, Iran
[3]Clinical Biochemistry Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran
[4]Department of Genetics, Faculty of Biological Sciences and Technology, Shahid Ashrafi Esfahani University, Isfahan, Iran
[5]Department of Epidemiology and Biostatistics, School of Health, Shahrekord University of Medical Sciences, Shahrekord, Iran

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ABSTRACT

Introduction: Eugenol, a natural phenolic compound obtained from clove and cinnamon, has been reported to show anticancer properties in breast cancer models. Metabolic reprogramming is a hallmark of breast cancer and can be regulated by AMPK. The PRKAA1 gene encodes the catalytic subunit of AMPKα1. This study aimed to investigate the effects of eugenol on cell viability and PRKAA1 expression in human breast cancer cell lines. PRKAA1 expression patterns and prognostic relevance in breast cancer patient datasets were also analyzed.

Methods: RNA-seq data from ENCORI/StarBase were employed to compare PRKAA1 expression in tumor and normal breast tissues. Kaplan–Meier analysis evaluated possible associations between PRKAA1 expression levels and overall survival. MCF-7 and MDA-MB-231 cell lines were treated with eugenol, and cell viability was assessed using the MTT assay. PRKAA1 expression levels were measured by RT-qPCR after eugenol treatment and compared with controls.

Results: Bioinformatics analysis showed lower PRKAA1 expression in breast cancer compared with normal breast samples (P = 4.3e-24, false discovery rate [FDR] = 4.4e-23). Survival analysis showed no significant association between PRKAA1 expression and overall survival in breast cancer patients (P = 0.69; hazard ratio [HR] = 1.07). Eugenol reduced the viability of both cell lines and showed greater toxicity with increasing concentrations and exposure time. Eugenol treatment downregulated PRKAA1 expression (P < 0.001).

Conclusion: Eugenol treatment altered PRKAA1 expression in both cell lines. These findings may suggest a potential link between eugenol induced cytotoxicity and changes in energy homeostasis genes; however, further studies are required to clarify the role of AMPK signaling in this context.

Implication for health policy/practice/research/medical education:
This study offers preliminary evidence linking eugenol exposure to reduced cell viability and modulated PRKAA1 mRNA expression in breast cancer, suggesting a potential role for this plant-derived compound in cancer research. These findings support further translational research into the use of natural bioactive compounds as exploratory candidates for modulating cancer cell metabolism. From a research and medical education perspective, this work highlights the importance of distinguishing between transcript-level associations and definitive mechanistic pathways. Ultimately, these results provide a conceptual foundation for future studies to validate eugenol's effects at the protein and functional levels, ensuring a more comprehensive understanding of its potential therapeutic relevance.

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***Corresponding author**: Hossein Teimori,
Email: hteimori@skums.ac.ir

Introduction

Breast cancer is among the most prevalent and deadly malignancies in women around the world (1,2). Malignant cells display specific modifications in their metabolism that support their progression and survival under stress, a process known as metabolic reprogramming (3). This reprogramming allows malignant cells to fulfill the increased energy and anabolic demands necessary for uncontrolled growth. One characteristic feature of cancer metabolism is the Warburg effect, defined by elevated glucose consumption and lactate synthesis even in aerobic conditions (4).

Cellular energy homeostasis is maintained by regulatory pathways that sense and adapt to fluctuations in nutrient and energy status. A key regulator of this homeostasis is the AMP-activated protein kinase (AMPK) complex (5), which acts as a metabolic checkpoint controlling the switch between anabolic growth and catabolic survival processes (5,6). This complex monitors cellular energy levels by sensing the concentrations of ATP, ADP, and AMP (7). AMPK activation is triggered by cellular energy deficits and acts to restore essential energy levels by inhibiting biosynthetic pathways whilst promoting energy-generating processes (6,7). In cancer cells, alterations in AMPK signaling influence metabolic adaptation and can affect proliferation, survival, and response to metabolic stress (8).

The catalytic ability of AMPK depends on its α subunit, of which the protein kinase AMP-activated catalytic subunit alpha 1 (PRKAA1) gene encodes the α1 isoform (AMPKα1) (9). Also, PRKAA2 gene encodes the α2 isoform (AMPKα2) (10). This α catalytic subunit is essential for AMPK's kinase activity and its ability to phosphorylate downstream targets governing metabolism (11,12). Through phosphorylation events, AMPK inhibits energy-demanding anabolic pathways such as protein and lipid biosynthesis while stimulating fatty acid oxidation and glucose absorption, therefore preserving ATP levels during energy stress (13). Hence, disruption of PRKAA1 expression or activity can alter the dynamic balance between growth and survival. Many studies have shown that reduced AMPK signaling may promote metabolic reprogramming that favors tumor progression, which highlights AMPK as a tumor suppressor (8,14,15). In contrast, some articles introduce AMPK as a tumor promoter because of the role it has in tumor cell's protection from cellular energy stress (16,17). It was observed that knockout of AMPK in cancer cells made them more vulnerable to cellular death by glucose starvation or detachment from the extracellular matrix. This might suggest that AMPK has a role in preventing these cell death events (16,18).

Plant-derived compounds have drawn interest as possible therapeutic compounds targeting cancer metabolism. Eugenol (4-allyl-2-methoxyphenol) is a natural phenolic compound isolated from clove and cinnamon essential oils (19). Clove (*Syzygium aromaticum*) is the primary botanical source of eugenol, where this compound constitutes approximately 70–90% of clove essential oil (20). This compound has shown antibacterial, anti-inflammatory, and antioxidant properties (21,22). In addition, it displayed cytotoxic effects against diverse cancer cell lines, including suppression of proliferation and induction of programmed cell death (21,23,24). Some studies have demonstrated that eugenol inhibits breast cancer cell growth and can trigger apoptosis through mitochondrial pathways, downregulate oncogenic factors such as E2F1/survivin, and influence autophagy pathways (25-27). Hence, eugenol may alter or disrupt key intracellular signaling pathways that influence cancer cell viability. Although the anticancer properties of eugenol have been linked to apoptotic and autophagic mechanisms (25,26), no study to date has specifically addressed eugenol's effects on the expression of particular energy sensors such as PRKAA1 in breast cancer cells. Understanding whether eugenol impacts energy homeostasis via modulation of PRKAA1 can reveal novel metabolic mechanisms underpinning its anticancer actions. Hence, this study investigated the impact of eugenol treatment on cellular viability and PRKAA1 expression in two distinct breast cancer cell lines. The main purpose was to demonstrate a potential link between eugenol treatment and metabolic regulation through PRKAA1 expression in cancer cells. In addition, this study also focused on clarifying the possible role of PRKAA1 as a biomarker and prognostic marker via bioinformatics analysis.

Materials and Methods

MCF-7 and MDA-MB-231 cell lines were received from Shahrekord University of Medical Sciences. Roswell Park Memorial Institute (RPMI) medium and Dulbecco's Modified Eagle's Medium (DMEM) were from Gibco (Grand Island, NY, USA), fetal bovine serum (FBS) was purchased from Arvand Cell (Shahrekord, Iran), and Trypsin-EDTA from Bio Idea Co (Tehran, Iran). Eugenol (CAS 97-53-0, Catalog No. 818455) was purchased from Merck (Germany) and cell proliferation kit I (MTT) from Roche (Indianapolis, IN, USA). qPCR SYBR master mix, Total RNA Extraction Mini-Kit Plus YTzol, and cDNA synthesis kit were purchased from Yekta Tajhiz Azma (Tehran, Iran). The DNase I kit was ordered from ATR-MED (Tehran, Iran).

Bioinformatics expression analysis and pathways

Breast cancer RNA-seq data for PRKAA1 were employed from the ENCORI/StarBase v2.0 database (28), which contained transcriptomic datasets downloaded from The Cancer Genome Atlas (TCGA). Gene expression values were scaled via log2 (FPKM + 0.01) method. PRKAA1 expression levels in breast cancer tissues and normal

breast tissues were examined and visualized using box plot provided by the platform to assess differential expression patterns. In addition, the potential clinical significance of PRKAA1 expression was assessed through Kaplan–Meier survival analysis and log-rank test using publicly available breast cancer datasets from the same database. Samples were divided into low and high PRKAA1 expression groups to assess the possible association between PRKAA1 expression levels and overall survival (P value < 0.05 considered statistically significant).

Functional pathway information was examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (29) to explore the metabolic pathways associated with PRKAA1. This analysis was used to identify pathways related to AMPK signaling and cellular energy metabolism in which PRKAA1 participates.

Cell culture

MDA-MB-231 cells were cultured in DMEM medium, while MCF-7 cells were cultured in RPMI. Both media contained 10% FBS and 1% penicillin-streptomycin. Incubation conditions were as follows: 37 °C, 90% humidity, with 5% CO2. When 70% confluency was reached, MCF-7 cells were passaged by 0.25% trypsin-EDTA, but 0.05% trypsin-EDTA was used to passage MDA-MB-231 cells. Trypsin neutralization was done by using medium supplemented with 10% FBS.

MTT assay

For the MTT assay, 96-well plates were utilized. Seeding density was approximately 1×10^4 cells per well. After seeding, cells were incubated for 24 hours to allow attachment. Due to eugenol's high hydrophobicity, dimethyl sulfoxide (DMSO) was used as a solvent. Then, different amounts of eugenol were added to the medium to make treatment concentrations ranging from 100 µM to 3000 µM. DMSO concentration was kept under 0.1% in all treatments. After the removal of the old medium from the wells, treatment concentrations were added in technical triplicates. Important considerations are eugenol's sensitivity to light and oxidation, so every part regarding handling eugenol was done in low light and with minimal exposure to oxygen. Also, storage was in amber vials or amber microtubes. After the end of treatment time, 10 µL of MTT solution (Roche cell proliferation kit I) was added to each well and incubated for 4 hours. Then, 100 µL SDS solution was added to each well and underwent an overnight incubation. After that, the 96-well plate was placed in ELISA reader, and absorption was measured at 570-630 nm. Experiments were repeated 3 times.

RNA extraction, RT- qPCR, and analysis

Cells were seeded in a 6-well plate; each well contained 5×10^5 cells before treatment. Total RNA was extracted and purified with the Total RNA Extraction Mini–Kit Plus YTzol (Yekta Tajhiz Azma) and DNase I Kit (ATR-MED) according to the manufacturer's protocol. RNA concentrations, 260/230 and 260/280 ratios were measured by NanoDrop™ 2000 (Thermo Scientific). Two micrograms of total RNA from each sample was used for cDNA synthesis by YTA cDNA Synthesis kit. The qPCR runs were performed by YTA Smart SYBR Green qPCR Master Mix (Yekta Tajhiz Azma) in Corbett's RotorGene 3000. ACTB and GAPDH were used as internal controls. Quantitative analysis of gene expression was done using the Pfaffl method (hybrid of the standard curve and ΔΔCt methods) and normalized according to the geometric averaging of multiple internal control genes approach (30,31). Each sample was evaluated in technical triplicate. AlleleID 7 was used for primer design, and primer specificity was verified in silico by Primer-BLAST (NCBI) and uMelt Quartz melting curve prediction tool, then compared with melt curve of RT-qPCR runs. Primers are provided in Table 1.

Statistical analysis

Data analysis, visualization, and graph designs were done using GraphPad Prism 10. Data normality was assessed using Shapiro–Wilk test. For normally distributed data, one-way ANOVA followed by Dunnett's post hoc test was applied. For non-normally distributed data, the Kruskal–Wallis test followed by Dunn's multiple comparisons test was used. Data are presented as mean ± SEM from three independent experiments for each cell line (n = 3). A P value < 0.05 was considered significant. Bar graphs were used for visualization.

Table 1. Primer sequences used for RT-qPCR

Gene	Type	Sequence (5′ – 3′)	Amplicon length (bp)
PRKAA1	Forward	AAGATATCAGGGAACATGAATGGT	176
	Reverse	ATCCTGGTGATTTCTGTTGTAAAG	
GAPDH	Forward	ACACCCACTCCTCCACCTTT	112
	Reverse	TCCACCACCCTGTTGCTGTA	
ACTB	Forward	CTCACCATGGATGATGATATCGC	163
	Reverse	AGGAATCCTTCTGACCCATGC	

Results

Expression of PRKAA1 at significantly lower levels in breast cancer tissue samples compared with normal breast tissue samples

Comparison of PRKAA1 expression between tumor tissues (n = 1104) and normal tissues (n = 113) was performed using ENCORI transcriptomic data (Figure 1). The box plots show that the median expression level is significantly lower in cancer samples than in normal samples. While cancer tissues displayed a larger overall distribution and a greater number of low-expression outliers, the overall data showed a significant reduction of PRKAA1 expression in tumor tissues (P = 4.3e-24, false discovery rate [FDR] = 4.4e-23).

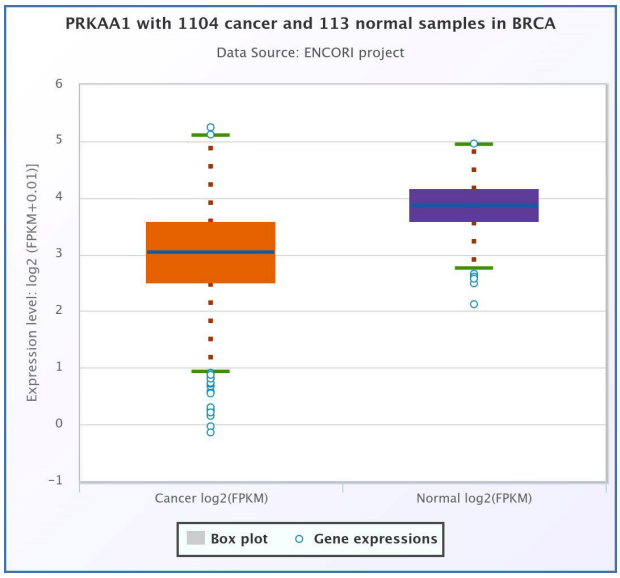

Figure 1. Differential expression of PRKAA1 between cancer (n = 1104) and normal (n = 113) breast tissues based on ENCORI data. Box plots display log2(FPKM + 0.01) values for each group. Tumor samples show lower median PRKAA1 expression compared with normal tissues, indicating a significant downregulation of PRKAA1 in breast cancer samples (P = 4.3e-24, FDR = 4.4e-23).

No significant association between PRKAA1 expression and overall survival in breast cancer patients

Overall survival analysis for PRKAA1 expression in breast cancer patients was performed using Kaplan–Meier curves (Figure 2). Data from patient samples were categorized into low- and high-expression groups (n = 541 per group). The survival curves for the two groups largely overlapped, and the log-rank p test showed no significant difference (P = 0.69). The hazard ratio (HR) was 1.07, indicating no significant survival advantage or disadvantage associated with PRKAA1 expression levels in this dataset. This suggests that PRKAA1 may not serve as a prognostic marker in breast cancer but may still reflect alterations in tumor metabolic states.

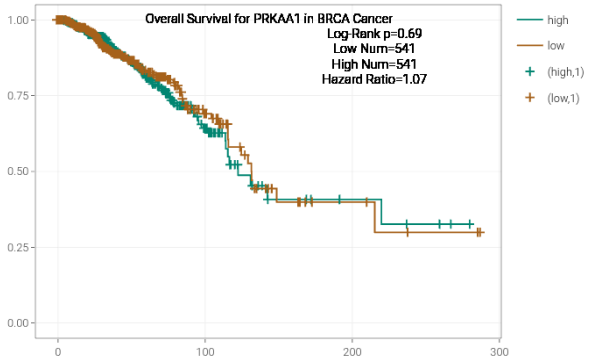

Figure 2. Kaplan–Meier overall survival curves for PRKAA1 expression in breast cancer patients (BRCA cohort). Patients were divided into high expression (n = 541) and low expression (n = 541) groups. The difference between the groups was not statistically significant (log-rank p = 0.69; HR = 1.07), indicating no prognostic association between PRKAA1 expression level and overall survival.

PRKAA1 participates in metabolic and growth-regulatory pathways

In order to define the biological role of PRKAA1, the AMPK signaling pathway diagram (Figure 3) was examined. PRKAA1 encodes the catalytic α1 subunit of AMPK, a metabolic master regulator. As shown, AMPK integrates signals from nutrient status, cytokines, upstream kinases (LKB1, CAMKKβ), and hormones such as leptin. AMPK interacts with various downstream effectors that are involved in pathways such as glucose metabolism (PFK2, PEPCK, GLUT4), lipid metabolism (ACC1/2, FAS, SCD1), mitochondrial biogenesis (PGC 1α), cell cycle modulation (Cyclin D, Cyclin A), autophagy (ULK1), mTOR signaling, and protein synthesis (eEF2K, eEF2). This illustrates that PRKAA1/AMPK is positioned at a hub controlling energy homeostasis, cell growth, and metabolic stress responses.

Eugenol inhibits viability and shows dose- and time-dependent cytotoxic effects on breast cancer cells

Treatment with increasing concentrations of eugenol was performed at 24 and 48 h time points to evaluate its cytotoxic effects on these two cell lines. Then, the MTT assay was used to assess cell viability. In MCF-7 cells, eugenol treatment led to a marked decrease in viability at both 24 hours and 48 hours (Figure 4A). At lower concentration (250 μM), viability was slightly reduced compared with the control. At 500 μM, viability decreased more substantially, with further reduction observed as concentration increased (750 μM). Higher concentrations (1000 μM and 1500 μM) resulted in a marked loss of viability, with values approaching minimal metabolic activity, indicating greater cytotoxicity. At both 24 hours and 48 hours, viability decreased more with longer incubation, consistent with a time-dependent effect of eugenol.

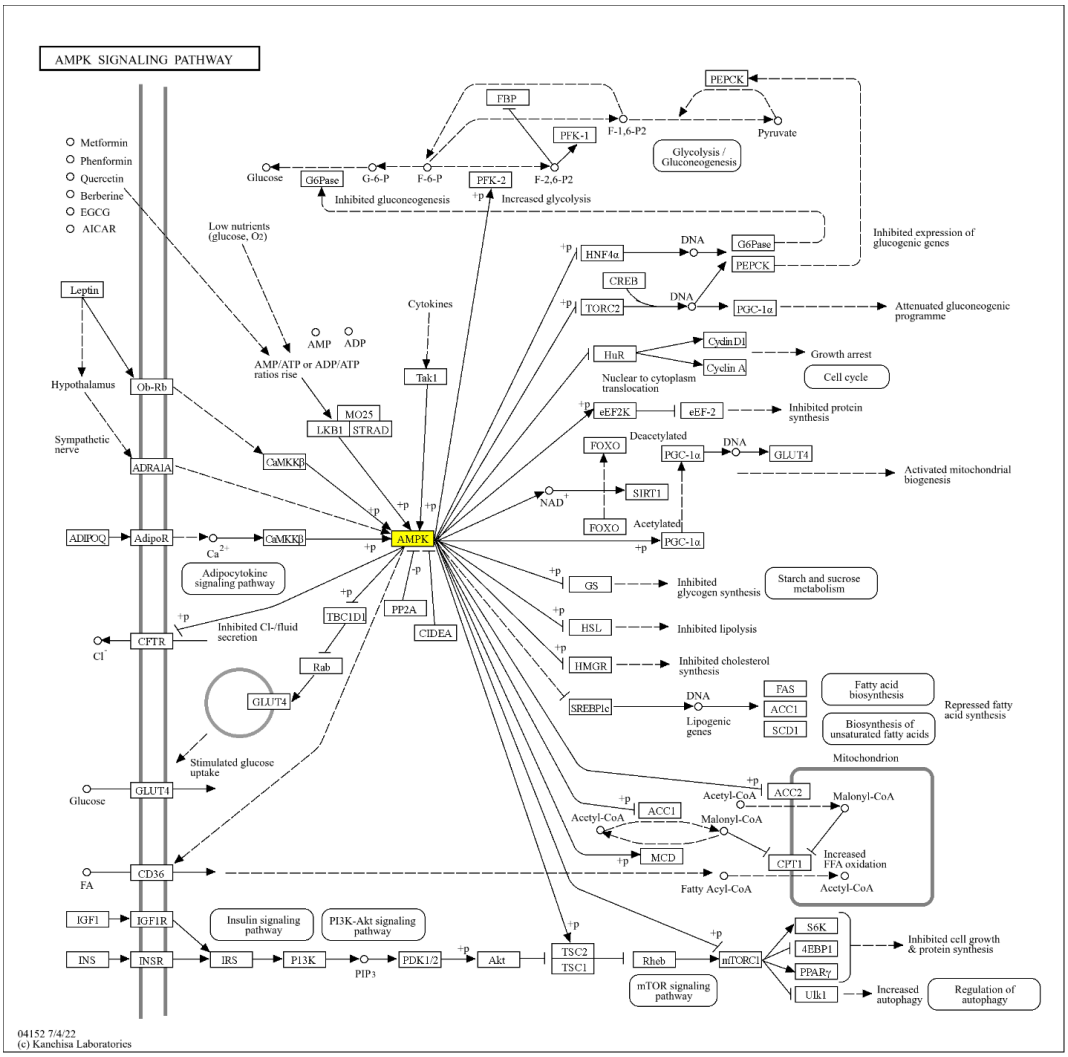

Figure 3. AMPK signaling pathway schematic highlighting the position of PRKAA1 (encoding AMPKα1). AMPK is highlighted in yellow. This protein complex integrates nutrient and hormone signals to regulate glucose metabolism, lipid metabolism, mitochondrial biogenesis, autophagy, protein synthesis, and cell cycle progression.

In MDA-MB-231 cells, a similar dose-dependent effect was observed on viability (Figure 4B). At 500 μM, a slight non-significant reduction at 24 hours and a moderate reduction in viability at 48 hours was observed. Increasing the concentration to 1000 μM resulted in further decreases, and by 1500 μM and above, MDA-MB-231 viability was significantly reduced. At the highest tested concentrations (2000–3000 μM), cell viability was at or near baseline (close to 0%), exhibiting a strong cytotoxic effect of eugenol in this breast cancer cell line. Overall, these results show that eugenol inhibits the viability of both MCF-7 and MDA-MB-231 cells and exhibits cytotoxicity in a dose- and time-dependent manner. In addition, MCF-7 showed higher sensitivity to eugenol compared with MDA-MB-231.

Eugenol suppresses PRKAA1 expression in both cell lines

Expression of the metabolic regulator PRKAA1 was measured by real-time qPCR following eugenol IC_{30} treatment (750 μM for MCF-7, 900 μM for MDA-MB-231) in order to determine whether eugenol influenced the expression of PRKAA1. In MCF-7 cells treated with 750 μM eugenol (Figure 5A), PRKAA1 expression was significantly suppressed to approximately 0.50-fold at 12 hours and 0.35-fold at 24 h relative to untreated controls ($P < 0.001$). Likewise, treatment with 900 μM eugenol in MDA-MB-231 cells (Figure 5B) significantly reduced PRKAA1 transcript levels to approximately 0. 25-fold at 12 h and 0.45-fold at 24 hours compared with untreated controls ($P < 0.001$). These data indicate that eugenol significantly downregulates PRKAA1 expression across both MCF-7 and MDA-MB-231 cell lines.

Discussion

The PRKAA1 gene encodes the catalytic α1 subunit of the AMPK complex, a key mediator of cellular energy

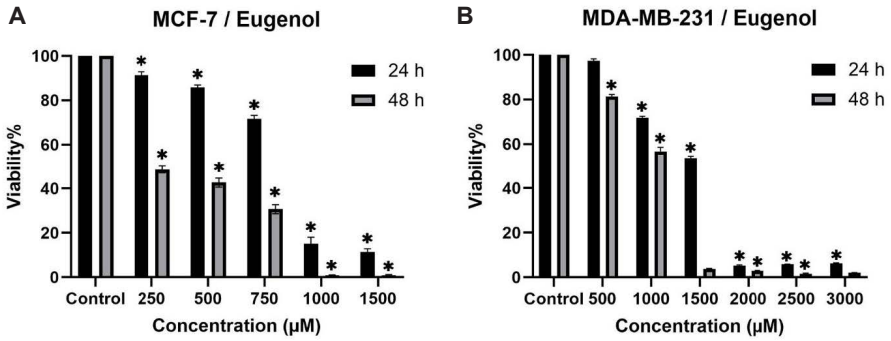

Figure 4. Effect of eugenol on breast cancer cell viability. (A) MCF-7 viability after treatment with 0–1500 μM eugenol for 24 h (black bars) and 48 h (gray bars). (B) MDA-MB-231 viability after 0–3000 μM eugenol treatment for 24 h and 48 h. Viability was expressed as a percentage of the untreated control. Each column represents the mean ± SEM of three independent experiments (*$P < 0.05$ vs. control).

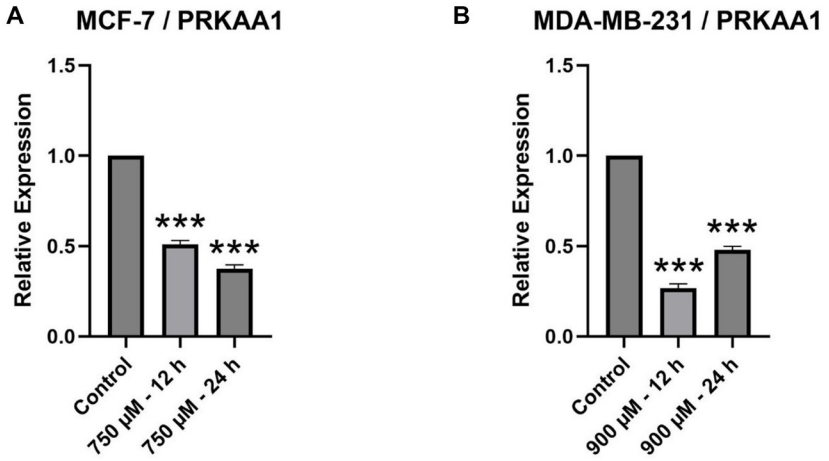

Figure 5. Downregulation of PRKAA1 expression by eugenol. Relative mRNA expression of PRKAA1 in MCF-7 (A) and MDA-MB-231 (B) cells after eugenol treatment, compared with control. Bars represent mean ± SEM of 3 independent experiments (***$P < 0.001$ vs. control). Statistical comparisons were performed within each cell line relative to its own control and time points.

homeostasis (7,9). AMPK complex acts as a central metabolic sensor, which maintains energy balance by regulating anabolic and catabolic pathways in response to cellular stress and nutrient availability (5). Unlike many oncogenes or tumor suppressor genes, PRKAA1 shows relatively low cancer specificity and is therefore not considered a marker for a particular cancer type (32). However, increasing evidence indicates that AMPK can play dual and contradictory roles in cancer (14-16). Hence, while the physiological role of AMPK in normal cells is well established, its function within the tumor environment is still complex and context-related.

AMPK is generally known as a tumor suppressor due to its roles in inhibiting cellular proliferation and growth; this effect occurs through the suppression of the mTOR signaling pathway, reduction in fatty acid synthesis, and increasing levels of cell cycle regulatory proteins such as p21 and p27, which participate in growth arrest under metabolic stress (33,34). However, some articles reported tumor-promoting roles for AMPK in certain metabolic conditions and contexts, especially in cancer cells adapting to nutrient deprivation or hypoxia (17,35,36). These findings indicate that AMPK signaling may either act in suppression or promotion of tumor progression depending on metabolic conditions and cellular context.

Previous studies investigating the biological effects of eugenol have reported various pharmacological benefits, such as anti-inflammatory, antioxidant, and anticancer properties (21,23). In the context of diabetes, eugenol has been shown to activate or upregulate AMPK signaling, resulting in better metabolic regulation (37). Another study observed AMPK upregulation after eugenol treatment in rat testicular tissue (38). Additionally, methyl eugenol treatment showed AMPK/GSK3β axis activation in the mouse kidney tissue (39). However, in our study, eugenol treatment induced the suppression of PRKAA1 expression (α1 catalytic subunit of AMPK complex) in the context of breast cancer. Although PRKAA1 encodes the catalytic α1 subunit of AMPK, changes in PRKAA1 mRNA expression do not necessarily reflect AMPK protein abundance or

kinase activity. Therefore, the observed downregulation of PRKAA1 following eugenol treatment should not be interpreted as direct inhibition of AMPK signaling but rather as a transcriptional change potentially associated with metabolic or cytotoxic stress.

Bioinformatics analysis using RNA sequencing data from the ENCORI/StarBase database displayed that PRKAA1 transcript levels were significantly lower in breast cancer samples compared to the normal breast samples, suggesting that PRKAA1 dysregulation may have a specific role associated with breast cancer initiation and progression. However, a Kaplan–Meier analysis conducted using the same database showed no significant association between PRKAA1 expression and overall survival, suggesting that PRKAA1 mRNA levels alone are unlikely to serve as a strong prognostic biomarker in breast cancer cohorts.

Our results further showed that eugenol significantly reduced cell viability, with the effect increasing at higher concentrations and longer exposure. The effective concentrations observed in this study are similar to those reported in a previous study evaluating the cytotoxic effects of eugenol in breast cancer cell lines (27). Our RT-qPCR results showed a significant downregulation of PRKAA1 expression in eugenol-treated breast cancer cells relative to untreated controls. Notably, this effect was observed in both cell line models, suggesting that the response to eugenol might occur independently of hormone receptor status. Also, these data suggest that the expression decrease in MDA-MB-231 cells peaks at 12 h and then is slightly reversed in 24 h treatment. However, MCF-7 cells show a more stable decrease with longer eugenol treatment. This might show an underlying trait in MDA-MB-231 cells that needs further study. It is also possible that the downregulation of PRKAA1 expression represents a secondary consequence of decreased cell viability rather than a primary driver of cytotoxicity, emphasizing the need for functional validation to determine this.

Considering AMPK's fundamental involvement in metabolic regulation, the observed reduction in PRKAA1 expression may contribute to alterations in cellular energy sensing and metabolic balance in cancer cells. If AMPK activity under certain conditions supports tumor cell survival by facilitating adaptation to metabolic stress, downregulation of PRKAA1 could impair this adaptive response. As a consequence, disruption of the balance between anabolic and catabolic pathways may limit tumor cells' ability to respond to fluctuations in energy availability. Such a metabolic imbalance could potentially lead to energy deprivation and reduced glucose utilization, contributing to decreased cellular viability (16). This supports the hypothesis that eugenol may act as an efficient sensitizer for chemotherapy in breast cancer. However, this claim needs further research to be confirmed.

According to the notion that AMPK signaling in breast cancer may be context dependent, different subtypes of breast cancer may not show the same role or regulation levels of this gene. These findings show the need to further explore how modulation of this gene might affect cellular metabolism in different subtypes of breast cancer.

This study has several limitations. First, the conclusions regarding AMPK signaling are based solely on PRKAA1 mRNA expression without assessment of AMPK protein levels or phosphorylation status. Second, no functional assays were performed to directly evaluate apoptosis or metabolic activity. Third, the relatively high concentrations of eugenol used in vitro may limit physiological relevance. Finally, the findings are restricted to cell culture models and should be interpreted as preliminary and hypothesis-generating.

In summary, the findings of this study suggest that eugenol reduces breast cancer cell viability and is associated with decreased PRKAA1 expression in both ER-positive and triple-negative breast cancer cell lines. These results support the hypothesis that modulation of AMPK-related metabolic pathways may contribute to the anticancer effects of eugenol. Rather than establishing a direct mechanistic pathway, these findings provide exploratory evidence linking eugenol exposure to changes in metabolic gene expression in breast cancer cells. However, further studies are required to investigate AMPK protein activity, phosphorylation status, possible α2 isoform or PRKAA2 compensation, and downstream metabolic signaling with the purpose of better understanding the mechanistic relationship between eugenol treatment and PRKAA1/AMPK-mediated metabolic regulation in breast cancer cells.

Conclusion

In this research on two breast cancer subtype cell lines and eugenol treatment, the findings suggest that eugenol treatment was associated with reduced viability of breast cancer cells and modulation of PRKAA1 expression, suggesting a potential link with cellular energy-related pathways. However, further mechanistic and protein-level studies are required to clarify the role of eugenol besides AMPK signaling in mediating these effects.

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Authors' contribution

Conceptualization: Ali Serat.
Data curation: Ali Serat.
Formal analysis: Ali Serat.
Funding acquisition: Hossein Teimori, Ali Serat.
Investigation: Zahra Yazdani Chamheydari.
Methodology: Seyed Abbas Mirzaei, Nader Bagheri.

Project administration: Hossein Teimori.
Resources: Ali Serat, Zahra Yazdani Chamheydari.
Software: Pouyan Zandi, Reza Yarahmadi.
Supervision: Hossein Teimori.
Validation: Hadi Raeisi Shahraki.
Visualization: Pouyan Zandi.
Writing–original draft: Ali Serat.
Writing–review & editing: Nader Bagheri, Seyed Abbas Mirzaei, Hossein Teimori.

Conflict of interests

The authors have no conflicts of interest.

Declaration of AI-assisted tools in the writing procedure

AI tools such as Grammarly and Quillbot were utilized for text refinement and grammar correction throughout manuscript writing and editing. All AI-assisted content was then reviewed and edited by author(s) to ensure that the originality and accuracy of the article are preserved.

Ethical considerations

All research protocols were approved by the Ethics Committee of Shahrekord University of Medical Sciences, Shahrekord, Iran (IR.SKUMS.MED.REC.1403.108).

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