Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Notch signaling mediated repressive effects of resveratrol in inducing caspase-dependent apoptosis in MCF-7 breast cancer cells

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Article Info

Article history:

Received: February 05, 2024
Accepted: April 24, 2024
Published: August 31, 2024

Use your device to scan and read the article online

Abstract

Resveratrol, a potent anticancer bioactive compound, has been shown to trigger apoptosis in numerous cancer cells. Although Notch signaling promotes breast cancer apoptosis, it is unclear whether resveratrol induces apoptosis in MCF-7 cells via influencing the Notch pathway. This study aimed to evaluate the effect of resveratrol on modulating Notch signaling targets and provide critical information for employing resveratrol in breast cancer therapy. Thus, in this study, we have deciphered the effect of resveratrol against three potent genes (Notch1, Jagged1, and DLL4) of the notch signaling pathway. For mechanistic studies, in silico, and in vitro analysis was executed to investigate the apoptotic-inducing potential of resveratrol against three selected oncogenes involved in the progression of breast cancer. Docking analysis revealed the inhibitory potential of resveratrol against all three selected targets of the Notch pathway (Notch1: -5.0; Jagged-1: -5.9; DLL4: -5.8). In vitro, findings further displayed a significant reduction in cell viability in resveratrol-treated MCF-7 cancer cells, which were concomitantly related to the downregulation of Notch-1, Jagged-1, and DLL4. Moreover, the antiproliferative efficacy of resveratrol was correlated with apoptosis and modulation in the expression of Bax, Bcl-2, cyclin D1, CDK4, p21, and caspase-3 activation. Taken together, these experimental findings suggested that apoptotic inducing potential of resveratrol was mediated through a novel mechanism involving suppression of the Notch signaling pathway.

Keywords: Breast cancer, Notch signaling, Docking, Caspase, Apoptosis.

1. Introduction

Breast cancer is the most prevalent type of carcinoma in women and one of the most deadly worldwide [1, 2]. Chemotherapy and surgery is frequently used in the postoperative care of breast cancer to stop recurrence. Unfortunately, recurrent drug resistance and severe toxicities negatively impact patients' quality of life [3, 4]. According to their cellular origins, squamous cell carcinomas and adenocarcinomas are two different types of breast cancers, which are one of the main causes of cancer-related death among women in developing nations [5]. Therefore, clinical personnel needs to investigate more dependable and non-toxic therapeutic approaches in the adjuvant treatment of breast malignancies.

Resveratrol, a non-flavonoid polyphenol that is a naturally occurring stilbene, has anti-cancer, anti-inflammatory, antioxidant, and cardioprotective characteristics. It is also a phytoestrogen [6, 7]. By altering the several signal-transduction pathways that regulate cell growth and division, inflammation, apoptosis, metastasis, and angiogenesis, resveratrol influences a number of cancer phases from initiation and promotion to progression [8, 9]. Resveratrol inhibits proliferation by causing apoptosis, according to *in vitro* research, which has shown this to be true. They include cyclins and cyclin-dependent kinases (CDKs), and resveratrol alters their relative proportions, inhibiting the cell cycle at the G0/G1 phase [10].

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.8.12

Substantial evidence indicates that the Notch signaling pathway may play a significant role in the etiology of breast cancer and, thus, may be a novel therapeutic target, even though the underlying mechanism that causes breast cancer is still unknown [11, 12]. Previous reports corroborated that the notch pathway is one of the key signaling pathways in drug-resistant cancer cells. Additionally, down-regulation of this pathway resulted in reduced cancer cell proliferation and invasion by inducing drug sensitivity. The notch pathway consists of four main notch receptors (a group of transmembrane proteins) along with their respective ligands (Dll4 and Jagged1) [13, 14]. Ligand receptor binding would consequently result in γ-secretase mediated cleavage of notch receptor and the associated release of NICD (notch intracellular domain) [15, 16]. These facts have further motivated us to target these three crucial notch components with resveratrol to elucidate a plant-based compound as a potent drug candidate for BC management. Additionally, this research is intended to investigate the mechanism associated with the inhibitory efficacy of resveratrol by targeting key notch components responsible for the progression of breast cancer. Altogether, in the present study, we demonstrated that resveratrol induced human breast cancer cell death via targeting crucial notch signaling components in breast cancer by employing both *in silico* and *in vitro* approaches. The results suggest that resveratrol can be considered a potential therapeutic agent for treating human breast cancer.

2. Materials and methods

2.1. *In silico* analysis

Resveratrol was used as a ligand to target three crucial components of the notch pathway (Notch1, Jagged1, and Dll4 proteins). The PDB file of these targets (Notch1: 1TOZ, Jagged1:4CCO, and Dll4: 5MVX) was downloaded from rcsb.org as a crystal structure. The criteria behind selecting these structures was the lowest energy. CB Dock and Patch Dock servers [17, 18] were used to identify the binding efficacy of resveratrol against these three target proteins by examining their binding sites and ligand stability. The complex structure was visualized using chimera software (UCSF) [19]. Additionally, the drug-likeness properties [20] of resveratrol were also investigated by employing the Swiss ADME tool (online). Lipinski's rule of five (Molecular Weight=<500; logP=<5; H bond donors=<5; H bond acceptor =<10) [21].

2.2. Investigation of cell viability by MTT assay

DMEM growth medium was used to cultivate MCF-7 cancer cells (NCCS, India) in an incubator (5% CO_2 + 37ºC) for 24 hours. The effectiveness of resveratrol as an inhibitor of MCF-7 cells was evaluated using the MTT assay [22]. After 24 hours (at 37°C) of incubation, resveratrol treatment (0-65 M) was given to MCF-7 cells and left incubated for further 24 hours. Following the addition of MTT dye, each well was given 4 hours (at 37°C) of incubation. The formazan (purple color precipitate) is then gently shaken into a solution with DMSO. Cell survival was evaluated using a microplate reader to measure final absorbance (at 490 nm).

2.3. Investigation of caspase-3 activity and caspase-3 inhibitors

Caspase-3 activity was investigated in resveratrol-treated MCF-7 cells by utilizing the Caspase-3 Colorimetric Assay Kit (BioVision) [23]. Both untreated and resveratrol-treated MCF-7 ($3x10^6$) cells were treated with cell lysis buffer (chilled) and incubated for 10 minutes (on ice). Lysed cells were centrifuged (10,000xg) for 1 min and the remaining supernatant was utilized for further analysis. Cell lysate (50 µl) was then mixed with reaction buffer (10 mM DTT) in 96 well plates. Thereafter, DEVD-pNA (5 µl) substrate was added (to each well) and left incubated for 1 hour (37°C). % change in caspase activity was analyzed by taking absorbance at 405 nm. Further, MCF-7 cells were treated with Z-DEVD-FMK inhibitor (50 µM) to characterize the cytotoxicity of resveratrol for 2 h and then treated with resveratrol (0-65 µM) for 24 h. Cell viability was thereafter evaluated using an MTT assay.

2.4. Determination of intracellular ROS (reactive oxygen species) level and NAC (N-acetyl-L-cysteine) efficacy

ROS generation in resveratrol-treated MCF-7 cells was assessed using DCFH-DA dye. 10 µM dye was added to resveratrol-treated MCF-7 ($1.5x10^4$ cells in a 12-well plate) and incubated for 30 minutes at 37ºC. A Fluorescence microscope was used to record the images for qualitative analysis of ROS generated in resveratrol-treated cells [24]. Quantitative estimation of ROS was done by recording the fluorescence intensity.

ROS generation was validated by using NAC (ROS inhibitor) in resveratrol-treated MCF-7 cells. MCF-7 cells were pretreated with NAC for 2 hours followed by resveratrol treatment and left for 12 hours. PBS-washed MCF-7 cells were stained with DCFH-DA dye and left incubated for 30 minutes (37°C). Fluorescence intensity (emission wavelength at 528 nm and excitation wavelength at 485 nm) was observed by a multiwall microplate reader. The correlation between ROS generation and apoptosis induction was investigated using an MTT assay in NAC-treated MCF-7 cells [25].

2.5. Real Time PCR analysis

To examine whether resveratrol-treated MCF-7 cells exhibit any modulatory effect on the transcription of apoptotic (anti-apoptotic or pro-apoptotic) genes, the mRNA expression of target genes in resveratrol-treated MCF-7 cells or DMSO control cells (Table 1). Total RNA extracted from resveratrol-treated MCF-7 cells after 24h post-treatment using TRIzol Reagent (Invitrogen manufacturer's protocol). RT-PCR was executed using SuperScript III one-step RT-PCR with Platinum Taq DNA polymerase kit (Invitrogen) [25]. Relative expression of both control and treated sample was normalized to β-actin mRNA and evaluated by $2^{(-\Delta\Delta Ct)}$ method:

Target mRNA expression = $2^{-[(Ct \text{ of the gene of interest}) - (Ct \text{ of internal control})]}$, where, Ct stands for threshold cycle for every transcript.

2.6. Statistical Analysis

GraphPad Prism software (version 7.0) [27] was used to analyze the experimental data. Statistical analyses were performed using one-way ANOVA. Error bars for SEM are shown. Where indicated in the figures, degrees of P-value significance are as follows: *p<0.01 and **p<0.001.

3. Results

Table 1. Selected primers used in this study.

Gene	Forward Primer	Reverse Primer
Bax	AAGAAGCTGAGCGAGTGT	GGAGGAAGTCCAATGTC
Bcl-2	TCCATGTCTTTGGACAACCA	CTCCACCAGTGTTCCCATCT
Cyclin D1	ATGTGTGCAGAAGGAGGTCC	CCTTCATCTTAGAGGCCACG
CDK4	CAGTGTACAAGGCCCGTGATC	ACGAACTGTGCTGATGGGAAG
Notch1	GAGGCTGCTGGACGAGTA	GAGGCTGCTGGACGAGTA
Jagged1	ACTGGCACGGTTGTAGCACTG	TGGTTAATGGTTATCGCTGTATCTG
DLL4	TGGTTAATGGTTATCGCTGTATCTG	GTGGGTCAGAACTGGTTATTG
$P21^{cip1}$	GCAGAGGAAGACCATGTGG	TGTGATGATGGTGAGGATGG
β-actin	GTCTGTGATGCCCTTAGATG	AGCTTATGACCCGCACTTAC

3.1. Docking analysis of resveratrol against the crucial targeted protein of breast cancer

The pharmacokinetic analysis of resveratrol was determined by utilizing an online available software Swiss ADME (Table 2). The molecular characteristics of a compound are shown by Lipinski's rule of five, which are crucial for lead selectivity and optimization of a prospective orally active medication in clinical applications. An orally active chemical shouldn't typically have more than one Lipinski violation if its bioavailability is being jeopardized. It's interesting to note that resveratrol has not demonstrated Lipinski's violation, blood-brain barrier (BBB) permeability or permeability-glycoprotein (P-GP) substrates. P-GP is an ATP-dependent bioavailability protein pump that removes medications from biological systems. The pharmacokinetics and survivability of pharmaceutical medications are reduced by the natural release of pharmaceuticals back into the stomach lumen via PGPp (which are supposed to be PGPp substrates). Patch Dock and CB Dock an online docking server were utilized for the docking investigation of resveratrol against three crucial targets of the notch signaling pathway in breast cancer. Table 3 and Table 4 show the comparative *in silico* analysis of resveratrol against three target proteins. These preliminary findings revealed that resveratrol showed a more binding affinity with the targeted protein in comparison to the standard drug 5-FU. However, further studies are still needed to validate these binding affinities.

Table 2. Physicochemical properties of Resveratrol (Ligand).

Physicochemical properties of Resveratrol	
Formula	$C_{14}H_{12}O_3$
Molecular weight	228.24 g/mol
Num. heavy atoms	17
Num. aromatic heavy atoms	12
Num. rotatable bonds	2
Num. H-bond acceptors	3
Num. H-bond donors	3
Molar Refractivity	67.88
TPSA	60.69 Å²
Log Po/w (iLOGP)	1.71
GI absorption	High
BBB permeant	Yes
P-gp substrate	No
CYP2C19 inhibitor	No
Lipinski	Yes; 0 violation
Ghose	Yes
Veber	Yes
Egan	Yes
Muegge	Yes

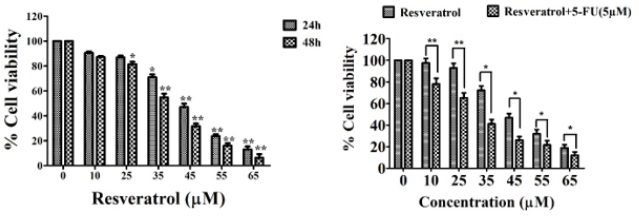


Fig. 1. At increasing dosages of resveratrol for 24 and 48 hours, the MTT assay revealed that (A) resveratrol reduced the viability of MCF-7 cancer cells and (b) combined effect of resveratrol+5FU in MCF-7 cancer cells (% cell vitality (determined by the MTT test) was normalized to untreated cells (control). SEM and means from three separate trials were shown; *p<0.01, **p<0.001 were given in contrast to corresponding control values.

3.2. Resveratrol reduced cell viability in MCF-7 breast cancer cells

The effectiveness of resveratrol in inhibiting MCF-7 cell proliferation was evaluated using the MTT test. For 24 hours, MCF-7 cells were subjected to varying dosages of 5-FU (1–5 μM) or resveratrol. When compared to the control, MCF-7 cells treated with resveratrol clearly showed a significant reduction in cell viability, which was dose-dependent (Figure 1). The IC_{50} value (IC_{50}=114.07μM) was determined using Origin software (Data Analysis and Graphing Software), which was then used to choose the appropriate doses for additional analysis.

3.3. Resveratrol-induced caspase-3 activity in MCF-7 cancer cells

Caspases are a class of cysteine proteases that cleave proteins at aspartic acid residues to cause apoptosis. In order to determine whether caspase activation was the cause of resveratrol-induced apoptosis in MCF-7 cancer cells. After 24 hours, MCF-7 cells treated with resveratrol showed a significant elevation of caspase-3 activity (Figure 2A). In comparison to untreated (control) MCF-7 cells, Figure 2(A) showed a considerable increase in caspase-3 activity. Hence, MCF-7 cells treated with resveratrol showed a dose-dependent increase in caspase-3 activity. MCF-7 cells were pretreated with 50 μM of caspase 3 inhibitor (Z-DEVD-FMK) for 2 hours and then were

Table 3. Docking results of resveratrol with selected targets using Patch Dock and CB Dock.

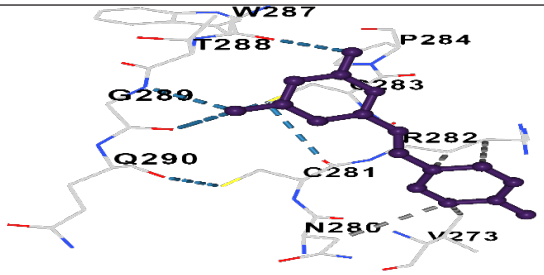
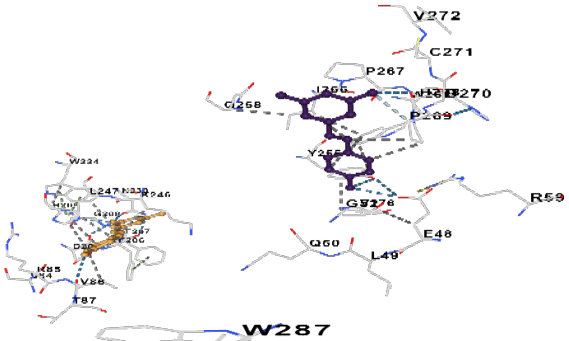
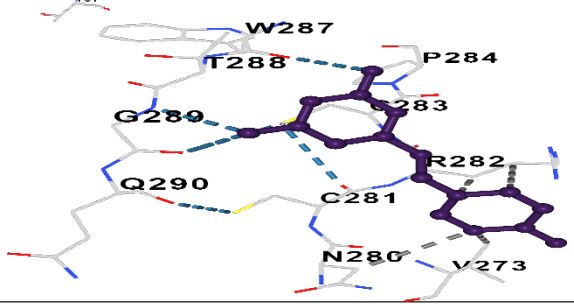
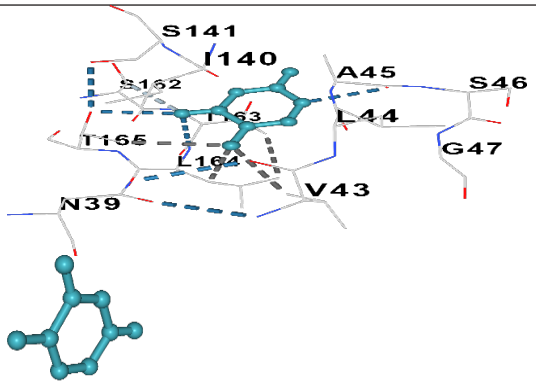
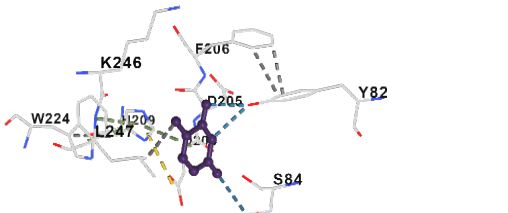
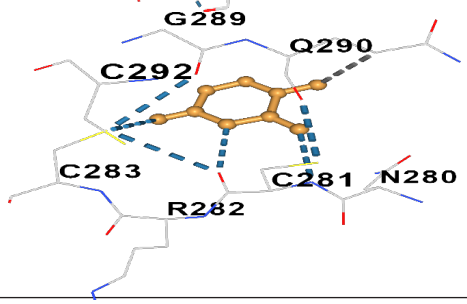
Target	Patch Dock		CB DOCK	Complex
	Score	Area	Vina score	
DLL4	3456	366.3	-5.9	
Jagged 1	3480	387.2	-5.8	
Notch1	2894	315.4	-5.0	

Table 4. Docking results of 5-Fluorouracil with selected targets using Patch Dock and CB Dock.

Target	Patch Dock		CB DOCK	Complex
	Score	Area	Vina score	
DLL4	2028	238.5	-4.5	
Jagged 1	2106	255.6	-4.3	
Notch1	1692	178.8	-3.4	

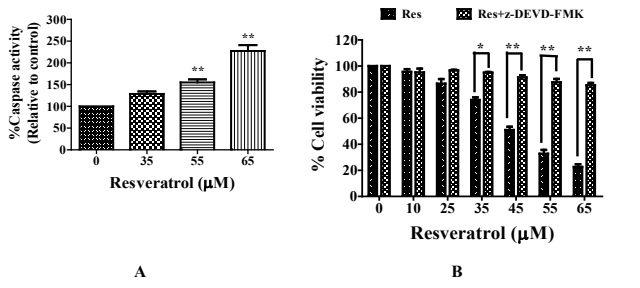


Fig. 2. Effects of resveratrol administration on (A) caspase-3 cleavage in MCF-7 cancer cells treated for 24 hours. (B) Caspase-3 inhibitor (Z-DEVD-FMK) was used as a pretreatment on MCF-7 cells to prevent caspase cleavage. SEM and means from three separate trials were shown; *p<0.01, **p<0.001 were given in contrast to corresponding control values.

exposed to specific dosages of resveratrol for 24 hours to demonstrate whether resveratrol-induced cytotoxicity in MCF-7 cancer cells was linked to the activation of caspase-3. Thereafter, an MTT assay was utilized to investigate cell viability. Caspase-3 inhibitor pretreatment may have lessened the cytotoxicity that resveratrol therapy had on MCF-7 cancer cells (Figure 2B). Together, these results provide compelling confirmation of the critical function of caspase-3 activation in resveratrol-induced apoptosis.

3.4. Resveratrol augmented the ROS (reactive oxygen species) level in MCF-7 cells

Fluorescence microscopy was used to examine ROS production in order to show how it affects the growth inhibition and apoptosis induction of MCF-7 carcinoma cells treated with resveratrol. According to Fig. 3A, cells treated with resveratrol for 12 hours had significantly higher intracellular ROS levels. Moreover, the quantitative study demonstrated increased ROS generation in a dose-dependent manner (Figure 3B). Moreover, MCF-7 cancer cells were pretreated with a ROS inhibitor to further support the notion that resveratrol is responsible for the elevation in ROS levels (NAC, N-acetyl-L-cysteine). Quantitative analysis showed that the high ROS level in NAC (10 mM) pretreatment MCF-7 cancer cells was reduced, which clearly supported our findings that resveratrol might increase ROS level in MCF-7 cancer cells (Figure 3C and D).

3.5. Effect of Resveratrol on Modulation of Notch-1, p21, Bax, Jagged-1, and DLL4 mRNA expression in MCF-7 cells

To explain the mechanism behind apoptosis in resveratrol-treated MCF-7 cancer cells, we inspected the mRNA transcript level of apoptosis-controlling genes by using RT-PCR. Resveratrol treatment also decreased the expression of Bcl-2 after 24h of treatment. However, a significant increase was observed in the gene expression of Bax in resveratrol-treated cells (Figure 4B). RT-PCR is used to explain how resveratrol might modulate Notch-1, Jagged-1, and DLL4 mRNA transcript levels (breast cancer oncogenes) that affect cell cycle progression. Notch-1, Jagged-1, and DLL4 mRNA expression were determined after 24 h of treatment of MCF-7 cells with resveratrol. Figure 4A depicted a significant reduction in Notch-1, Jagged-1, and DLL4 mRNA expression levels in resveratrol-treated MCF-7 cancer cells. Moreover, in MCF-7 cells treated with resveratrol, a substantial decrease in CDK4

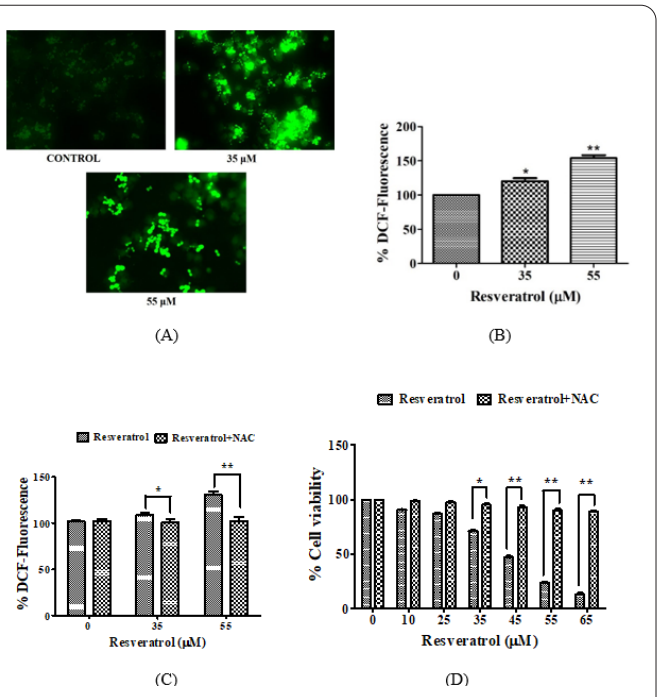


Fig. 3. Increased ROS generation in resveratrol-treated MCF-7 cells (A) Increased ROS generation in DCFH-DA stained MCF-7 cancer cells exposed to selective doses of rutin observed by fluorescence microscopy. Presented data are representative of three independent experiments. (B) ROS quantification in terms of % DCFDA fluorescence in rutin-treated MCF-7 cells. (C) Percent cell viability of MCF-7 cells pretreated with NAC and then treated with resveratrol (0–65 µM) for 24 h estimated by MTT assay. (D) ROS level in MCF-7 cells pre-exposed with NAC (ROS inhibitor), and then treated with resveratrol. SEM and mean of three independent experiments were presented: *P<0.01, **P<0.001, in comparison to related control value.

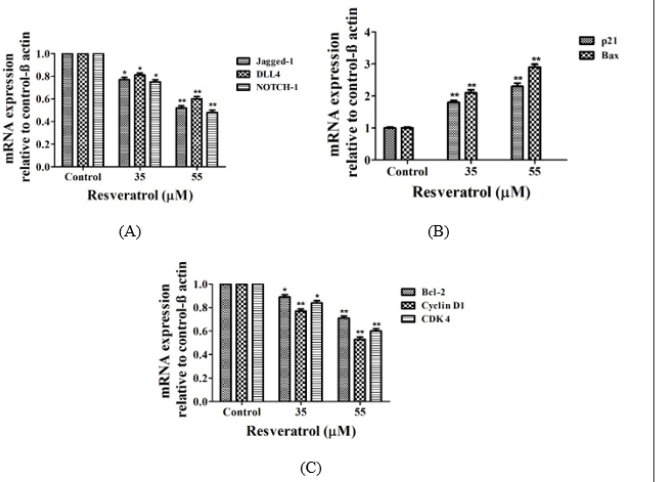


Fig. 4. Modulation of cell cycle and related gene mRNA expression by resveratrol analyzed using RT-PCR, this graph displays the level of expression of (A) selected Target genes (B) apoptotic genes (Bax and p21) (C) expression level of cell cycle regulatory gene (Cyclin D1, Bcl-2 and CDK4). Three independent experiments' SEM and means were provided; *p<0.01, **p<0.001 in contrast to the corresponding control value.

and cyclinD1 mRNA expression levels was seen (Figure 4C).

4. Discussion

Several biological processes, including angiogenesis,

are influenced by the conservative mechanism known as notch signaling [28]. Angiogenesis is a crucial physiologic component of cancer. Gap ligand Dll4 is mostly expressed in artery endothelial cells during embryonic development [29]. Severe vascular abnormalities caused by Dll4 haploid deficiency result in the demise of the embryo [30]. While Dll4/Notch signalling lowers the signal in the vascular growth factor pathway by decreasing the expression of VEGFR2 and raising the expression of VEGFR1, the vascular growth factor increases the expression of Dll4 and Notch1 [31]. In this study, the expression of Notch1, Jagged1, and, Dll4 genes in the Notch pathway was examined in breast cancer cells treated with resveratrol.

In numerous cancer models, resveratrol exhibits a variety of beneficial benefits, including antioxidant, anti-inflammatory, and anticancer activities [32]. In the modulation of multiple angiogenesis pathways of human umbilical vein endothelial cells by Dll4 and Notch, Dll4 level was up-regulated [33]. We can tentatively confirm that these results are close to the outcomes of this experiment when combined with the aforementioned investigations. Resveratrol may inhibit the growth and proliferation of breast cancer cells by regulating the Notch1, Dll4, and Jagged-1 elements of the Notch pathway. Resveratrol is anti-tumorous in several studies, however, its ability to target these specific NOTCH targets in breast cancer has not been reported. The specific biological mechanism underlying resveratrol inducing cell death by targeting crucial deregulated notch components in breast cancer is still unknown. The current research work would aid the existing knowledge of how resveratrol causes anticancer activity in human breast cancer cells at the molecular level. Intracellular ROS are essential for maintaining homeostasis and cell signaling 34]. Depending on the cell type, drug concentration, and other experimental findings, we may conclude that resveratrol has both antioxidant and pro-oxidant characteristics.

In the current work, resveratrol increased ROS levels in MCF-7 cells, while the antioxidant NAC suppressed the antiproliferative ability of resveratrol leading to cell death. These findings strongly imply that ROS generation is linked to resveratrol-induced cell death. Similar outcomes in other female cancer cells treated with resveratrol have been documented [35]. In a variety of cancer cells, notch signaling encourages cell proliferation, migration, invasion, and death. There are numerous processes involved in the complex relationship between ROS production and Notch1 signaling [36]. Conversely, ROS production controls Notch1 signaling whereas Notch1 decreases ROS production. In our investigation, resveratrol appeared to inhibit Notch1 signaling by generating ROS. Treatment with NAC inhibited the down-regulation of Notch-1 caused by resveratrol. These findings imply that ROS formation occurs prior to resveratrol-induced alterations in the Notch1 signaling pathway.

The link between the unregulated cell cycle and carcinogenesis is widely known [37]. Cell cycle research has amply shown that resveratrol effectively stopped the growth of MCF-7 cancer cells in the G0/G1 phase, supporting our earlier findings. These findings thus demonstrated that resveratrol not only induces apoptosis but also the expression of CDK4 and cyclinD1 mRNA (cell cycle markers).

During the execution stage of different types of apoptosis, caspase-3 plays a crucial role [39]. Caspase-3 exists as an inactive pro-enzyme that is cleaved by proteolysis to become active. This cleavage is started by the ligands of several cell surface receptors in a complex linked to the cytoplasmic death domain, which causes the release of cytochrome c from mitochondria. Apoptotic protease activation factor 1 and cytochrome C interact to activate caspase-9, which then cleaves caspase-3 [40]. Furthermore, caspase-3 is induced, and Z-DEVD-FMK, a specific caspase-3 inhibitor, reduces the cell death caused by resveratrol. In conclusion, the current work showed that resveratrol increased human breast cancer cell death through ROS-dependent Notch1 signaling. Altogether these research findings deciphered a mechanism involved behind the inhibitory efficacy of resveratrol against Notch signaling that can further be utilized for developing potent therapeutics for breast cancer management.

5. Conclusion

The occurrence of breast cancer is influenced by a number of variables, including genetics and food. Many molecules and different signaling pathways, including oxidative stress, apoptosis, and inflammation, are implicated in its occurrence and progression. Its pathophysiology is also varied. The potential effects of resveratrol in a variety of human malignancies have been supported by numerous research. Several features of this polyphenol molecule include antioxidant, anti-inflammatory, apoptotic inducer, and anti-angiogenesis activity. Resveratrol is proposed as a potential treatment agent for malignancies as a result of these important benefits. The maintenance of proliferative signaling, tumour suppressor escape, activation of telomerase, induction of angiogenesis, and metastasis are all key aspects of how deregulated Notch signaling has been significantly engaged in the advancement of breast cancer from the very beginning. Our findings strongly demonstrated that resveratrol could induce apoptosis in a dose-dependent manner via targeting three crucial components of the notch pathway including Notch1, Jagged1 and Dll4 which has been reported with the progression of breast cancer while posing minimal toxic effects on normal cells. Altogether, targeting Notch1, Jagged1 and Dll4 with resveratrol could provide an effective and safe therapeutic approach for the management of breast cancer.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed consent

The authors declare that no patients were used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request

Author contributions

Pratibha Pandey, Seema Ramniwas, Fahad Khan : Research design and Writing; Sara Seifeldin, Khalid Alshaghdali, Talal Alharazi, Tolgahan Acar, Vijay Jagdish Upadhye, Nishesh Sharma, Amir Saeed: Supervison, Data Analysis, Funding, Review and editing. All authors have read and agreed to the published version of the manuscript.

Funding
This research has been funded by the Scientific Research Deanship at the University of Ha'il-Saudi Arabia through project number RG-21 160.

Acknowledgements
This research has been funded by the Scientific Research Deanship at the University of Ha'il-Saudi Arabia through project number RG-21 160.

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