Research Article

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Systems biology based elucidation and experimental validation of anticancer effects of warfarin in vulvar cancer

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Abstract

Objectives: To evaluate the efficacy of warfarin in inhibiting vulvar cancer cell growth and explore its molecular mechanisms, including effects on immune-infiltration and pathways utilizing both computational and experimental approaches.

Methods: The pharmacokinetic and toxicity profiles of warfarin were computationally evaluated to confirm drug-likeness and safety. Biological targets were then predicted, and the resulting genes were analyzed using a systems biology framework to construct a protein–protein interaction network, identifying key hub-genes. These genes underwent survival analysis to assess prognostic significance and were further analyzed for immune cell infiltration, indicating possible immunomodulatory effects. Molecular docking simulations examined warfarin's binding affinity to the PD-1/PD-L1 immune checkpoint complex, suggesting immune regulatory potential. Experimental validation using SW-962 (vulvar-cancer) and HaCaT (normal) cell lines assessed cytotoxicity, oxidative stress, nuclear morphology, and cell cycle dynamics.

Results: Warfarin has promising physicochemical properties: high GI-absorption, BBB-permeability, and drug-likeness. Its predicted targets overlap with 20-vulvar cancer-related genes and form a PPI-network of key cancer-related pathways. GO and KEGG analyses reveal that the genes' involvement in stress response, structural components, and cancer pathways. The top hub genes include AKT1 and CASP3, both of which have poor survival, and studies on immune infiltration show that warfarin interacts with immune cells. The docking analysis shows a strong binding affinity to the PD-1/PD-L1-complex, whereas cytotoxicity tests show selective action against cancer cells through oxidative stress, apoptosis, and G0/G1-cell cycle arrest.

Conclusions: Warfarin demonstrates promising anticancer effects by promoting apoptosis, cell cycle arrest, ROS-generation, and immune modulation, therefore warranting further clinical studies.

Keywords: warfarin; drug repositioning; gene ontology; immune infiltration; differential gene expression; apoptosis

Introduction

Vulvar cancer, while rare, remains a serious gynecologic malignancy with approximately 7,480 new-cases and 1,770 deaths projected in the United States in 2025 [1]. The five-year relative survival rate significantly declines with disease progression – from 86 % in localized stages to 53 % with regional spread, and just 19 % for distant metastasis [2]. Current FDA-approved treatments for vulvar cancer are limited. Gardasil and Gardasil 9 are prophylactic HPV vaccines approved by the FDA to prevent vulvar and other anogenital cancers caused by high-risk HPV types [3]. While highly effective in reducing the risk of HPV-related lesions, these vaccines have no therapeutic effect on existing

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malignancies. For treatment, Bleomycin Sulfate is one of the few FDA-approved chemotherapeutic agents specifically indicated for vulvar cancer [4]. It exerts its effect by inducing DNA strand breaks, leading to apoptosis of cancer cells. However, its clinical use is often limited by toxicities such as pulmonary fibrosis, skin reactions, and mucositis. Other chemotherapeutic options like cisplatin, 5-fluorouracil, and paclitaxel are used off-label, often in combination with radiation, but carry significant side effects and modest efficacy in advanced disease [5]. The limited number of approved agents, significant toxicity profiles, and poor survival in late-stage vulvar cancer underscore a critical need for novel, targeted, and less toxic therapeutic strategies to improve clinical outcomes.

Given this therapeutic gap, there is a pressing need to explore novel agents that are both mechanistically distinct and clinically feasible. Warfarin, marketed as Coumadin, is an FDA-approved anticoagulant widely used for the prevention and treatment of thromboembolic disorders such as deep vein thrombosis, pulmonary embolism, and stroke in atrial fibrillation [6, 7]. Recent studies have suggested that warfarin may exert anti-tumor effects through inhibition of vitamin K-dependent pathways that play a role in tumor progression, angiogenesis, and metastasis [8]. Although warfarin is not FDA-approved for cancer treatment, these emerging findings justify investigation into its potential repurposing as an anticancer agent. The rationale for studying warfarin in vulvar cancer lies in its established safety profile, widespread clinical use, and its mechanistic potential to interfere with pathways critical to tumor survival and spread. Incorporating warfarin into preclinical or clinical studies for vulvar cancer could offer a cost-effective and innovative therapeutic avenue, especially in context of the current limitations in approved treatment options and the ongoing need to improve survival outcomes.

Network pharmacology, a new field in drug discovery and development, allowed correlations between drugs and diseases being studied through the complex web of biological systems [9]. This approach helps identify potential treatment targets and pathways that are affected by bioactive molecules or drugs, aiming to improve cancer treatment effectiveness. Network pharmacology enables the use of computational and bioinformatics methods to gather an all-inclusive view of the relationships between drugs, genes, and drug characteristics, which makes creating multi-target treatment solutions possible [10]. Network pharmacology in drug development is one key technique that included molecular docking, allowing researchers the ability to assess the relationship between pharmaceuticals and proteins at the molecular scale [11]. Molecular docking may elucidate the interactions between pharmaceuticals and target proteins, offering insights into the processes by which they may exhibit anticancer effects and aiding in the identification of the most promising targets for future exploration. The use of network pharmacology in cancer research facilitates the identification of novel targets and clarifies how warfarin may influence critical biological mechanisms.

This study integrates network pharmacology investigation, docking analysis, immune infiltration assessment using TIMER, differential gene expression evaluation, survival analysis via GEPIA2, and molecular docking techniques (Figure 1). This study aims to evaluate the efficacy of warfarin in modulating critical apoptotic pathways in cancer cells, reactive oxygen species, and cell cycle control pathways in vulvar cancer. This study investigates novel therapeutic targets and pathways in vulvar cancer through the comprehensive integration of bioinformatics and computational technologies, potentially leading to safe and effective treatments in the near future.

Materials and methods

Physicochemical property analysis

To investigate the physiochemical properties of warfarin, including drug-likeness, bioavailability and toxicity, SwissADME [12], Molsoft, and Protox-II [13] databases were employed. **SwissADME** (https://www.swissadme.ch/) provides insights into parameters such as bioavailability, water solubility, pharmacokinetics, and synthetic accessibility. **Molsoft** (https://www.molsoft.com/mprop/mprop.cgi) offered data on molecular properties such as polar surface area and drug score, further contributing to the selection of potential drug candidates with favourable profiles. Additionally, **Protox-II** (https://tox.charite.de/protox3/) was used to assess the toxicity profiles of warferin, predicting acute toxicity and safety classifications.

Gene target prediction and intersection

We applied the SwissTargetPrediction tool to predict the probable target genes of warfarin [14]. SwissTargetPrediction (http://www.swisstargetprediction.ch/) makes use of a chemical similarity-based approach for mapping chemicals onto targets by similarity towards known ligands, contributing towards the discovery of novel possible biological targets. The targets were selected based on a probability threshold of ≥0.1. Additionally, **GeneCards** (https://www.genecards.org/) was used to retrieve gene targets specifically related to vulvar

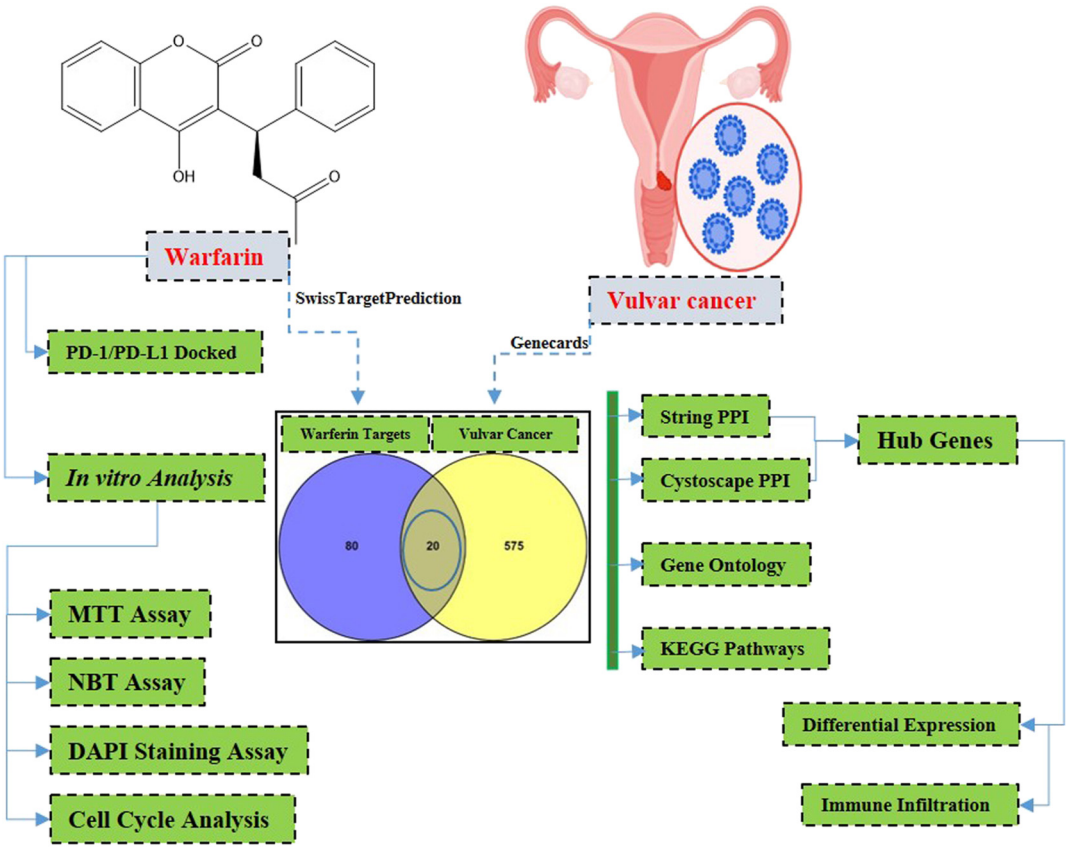


Figure 1: Workflow diagram.

cancer, offering a comprehensive gene database containing detailed genomic, proteomic, and functional information. The vulvar cancer-related targets from the database were selected based on a Gifts score of ≥60. The intersection between warfarin targets and vulvar cancer targets was identified with **Venny** 2.0.2.

Protein-protein interaction (PPI) network construction

PPI network was constructed with the **STRING** database and analyzed with the help of **Cytoscape** software [15]. The STRING database (https://string-db.org/) integrates PPI data from diverse sources to ensure reliability and coverage. Experimental data from BioGRID, IntAct, and DIP provide validated physical and functional interactions. Curated pathway databases like KEGG, Reactome, and BioCyc contribute known functional links. Text mining extracts interactions from PubMed literature, while co-expression data from GEO and Expression Atlas suggest functional associations. Gene fusion and co-occurrence studies, using comparative genomics, find genes that probably interact because they have a similar evolutionary background or are found together in the genome. Together, these sources build a comprehensive PPI network. Herein, the PPI data generated for intersecting targets between warfarin and vulvar cancer was set at a confidence threshold of ≥0.400. The use of a Cytoscape (Version 3.10.2) plug-in named **CytoHubba** identifies central, highly interacting proteins within the network. Hub genes, which are highlighted and may have critical roles in the pathogenesis of vulvar carcinoma, were indicated.

Functional gene-ontology and KEGG analysis

The SRPLOT (https://www.bioinformatics.com.cn/en) online tool was used to conduct GO and KEGG pathway enrichment analyses of the hub genes. This web resource enables researchers to easily visualise enriched biological functions and pathways. The results were obtained as bar plots for GO terms of Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) and as bubble plots for KEGG pathways by querying the hub genes in SRPLOT.

Differential expression and survival analysis

The clinical significance of warfarin-targeted hub genes was determined using **GEPIA2** for differential gene expressions and survival analysis. GEPIA2 (http://gepia2.cancer-pku.cn/) is an interactive web-server which allows cross-comparison of gene expression between tumor and normal samples based on the TCGA and GTEx databases, deriving box plots and Kaplan-Meier survival plots useful in determining the prognostic value of certain genes.

Immune infiltration analysis

The **TIMER** (Tumor Immune Estimation Resource) platform was used to assess the association of hub gene expression with infiltration of immune cells, which are B cells, CD4+ and CD8+ T cells, macrophages, neutrophils, and dendritic cells [16]. The TIMER (http://timer.cistrome.org/) is an efficient online tool to predict the infiltration of the immune microenvironment in the tumors and to provide mutual interplay between the tumor cells. This tool could assess the landscape of immunology for numerous forms of cancer and demonstrate the means by which potential pharmaceutical interventions might affect immune response in different ways.

Molecular docking studies

Molecular docking studies were performed to explore the interaction between warfarin and the PD-1/PD-L1 checkpoint complex using **CB-Dock2** (https://cadd.labshare.cn/cb-dock2/index.php) [17]. CB-Dock2 is an efficient, automated, and user-friendly tool ideal for blind docking tasks, combining cavity detection and Vina-based docking with accessible visualization. It significantly lowers the barrier for structural bioinformatics and drug discovery studies. The structure of the PD-1/PD-L1 complex was downloaded in.pdb from the **RCSB PDB** database with PDB ID 7DY7 [18] and warfarin from the **PubChem** database. The PD-1/PD-L1 complex was prepared for docking using BIOVIA Discovery Studio Visualizer, 2024, by removing water molecules, crystallized ligand and the addition of polar hydrogens and saved in.pdb format. Then both warfarin and PD-1/PD-L1 complex were submitted to the CB-Dock2 platform, which identified 5 CurPockets (binding sites) and we used the blind docking option to dock warfarin into these CurPockets.

Cell culture

Vulvar cancer cells (SW-962, ATCC, Rockville, MD, USA) and keratinocyte cells (HaCaT, ATCC, Rockville, MD, USA) were cultured in complete DMEM supplemented with 10 % FBS and 1 % penicillin-streptomycin under standard conditions at 37 °C in a 5 % CO2 atmosphere. Cells were seeded in 96-well plates for *in vitro* assays and allowed to adhere overnight.

MTT assay

To check the cytotoxic effects of warfarin on SW-962 and HaCaT cells, an MTT assay was performed 24 and 48 h after the treatment. The treated cells with control, 15, 30, 60, and 120 mg/mL concentrations of warfarin were incubated with the solution of MTT for a period of 4 h. After that, DMSO was used for dissolving the formazan crystals, and a microplate reader measured their absorbance at 570 nm. This assay measured the metabolic activity of treated cells relative to untreated controls and, hence, the possibility of observing dose-dependent cytotoxicity and cell viability at every time point.

NBT assay

To establish the level of oxidative stress induced by warfarin, the NBT (Nitro Blue Tetrazolium) assay was performed on SW-962 cells. After incubating the cells with control, 15, 30, 60, and 120 mg/mL warfarin for 24 h, the cells were exposed to NBT solution so that it could be reduced into formazan, a dark-blue precipitate. The amount of formazan produced, which represents ROS, was determined spectrophotometrically at 560 nm.

DAPI assay

For nuclear staining and apoptosis analysis, SW-962 cells treated with warfarin (control, 15, 60, and 120 mg/mL) were fixed in paraformaldehyde after 24 h and stained with DAPI (4′,6-diamidino-2-phenylindole). DAPI binds to DNA and allows visualization of nuclear morphology under a fluorescence microscope. Condensed or fragmented nuclei in treated cells were identified as signs of apoptosis.

Cell cycle assay

Flow cytometry was utilized to determine the effect of warfarin on cell cycle distribution in SW-962 cells. The cells were fixed and stained with PI, and then the cells were analyzed using flow cytometry after treatment with control, 15, 60, and 120 mg/mL warfarin. PI binds to DNA; thus, cells can be detected at different cell cycle phases, namely G0/G1, S, and G2/M. Cell cycle distribution changes were evaluated relative to the controls that were not treated.

Results

Physiochemical properties of warfarin

Studies by SwissADME, Molsoft, and Protox-II give hints about the physicochemical qualities that make warfarin a promising pharmacological candidate. There are no lead-likeness violations, and the drug-likeness model score moderately high, which is one property of conventional drugs. It has a significant bioavailability score along with ease in synthesis, passage through BBB, and substantial GI absorption suggesting extensive tissue distribution (Figure 2A). Lipophilicity and solubility enhance the therapeutic usefulness as well as appropriate hydrogen bond acceptor and donor. Hepatotoxicity, neurotoxicity, cardiotoxicity, carcinogenicity, immunotoxicity, and mutagenicity are inactive for warfarin, which proves its safety (Supplementary Table 1).

PPI network and hub genes

SwissTargetPrediction returned 100 targets for warfarin, while GeneCards returned 595 gene targets for vulvar cancer. The overlap of 20 common targets between the predicted targets of warfarin and the genes related to vulvar cancer was found using Venny 2.0.2 (Figure 2B and Supplementary Table 2). These targets were then submitted to the STRING database, which returned a PPI network of 20 nodes and 72 edges, with an average node degree of 7.2 and a highly significant PPI enrichment p-value of 3.11e-15 (Supplementary Figure 1). Further analysis in Cytoscape revealed an extended network with 19 nodes and 144 edges (Figure 2C). Using the CytoHubba plugin, we found that some key hub genes are AKT1, CASP3, EGFR, PTGS2, PARP1, KDR, MAPK1, MAPK8, CHEK1, and PIK3CB; these genes might be important in how warfarin affects vulvar cancer pathways (Figure 2D).

Gene-ontology and KEGG pathways for warfarin targeted hub genes in vulvar cancer

GO bar plot results for BP showed enrichments in responses to UV light, light stimuli, environmental and chemical stress, as well as metal ions indicating the hub genes being a part of cellular stress responses (Figure 3A). CC was showing gene enrichment in membrane rafts, microdomains, endosomes, nuclear envelopes, and telomeric regions, suggesting involvement of such genes in cellular structure and signaling (Figure 3A). The hub genes were attributed to protein kinase and MAP kinase activity, phosphatase binding, transmembrane receptor kinase activity, histone deacetylase binding, growth factor binding, and integrin binding in MF, hence suggesting involvement in regulatory and signaling purposes (Figure 3A).

KEGG pathway bubble plot indicated the enrichment for pathways such as colorectal cancer, TNF signalling, apoptosis, MAPK signalling, VEGF signalling, pancreatic cancer, EGFR tyrosine kinase inhibitor resistance, and focal adhesion pathways (Figure 3B). In addition, hub genes of apoptosis (Supplementary Figure 2) and cell cycle control (Supplementary Figure 3) were annotated; thus, functional relevance to cancer-related processes was evidenced. A combined network assembling warfarin, hub genes, and enriched pathways was generated in Cytoscape, comprising 25 nodes and 71 edges (Figure 4A). Their strong predicted associations with hub genes and relevant pathways demonstrated the potential mechanisms through which warfarin might exert therapeutic effects in vulvar cancer.

Differential expression and survival outcomes associated to hub genes

GEPIA analysis revealed six highly interacting hub genes differentially expressed in the PPI network. AKT1 and EGFR were partially overexpressed in normal tissues, whereas CASP3, PTGS2, PARP1, and MAPK1 were greatly overexpressed in cancer tissues, suggesting they are more active in carcinogenesis (Figure 4B). EGFR was inconsistent with OS (Figure 5A) and DFS (Figure 5B) survival study, although greater expressions of CASP3, PTGS2, PARP1, and MAPK1 were associated with poor survival. Their overexpression in cancer tissues is linked to poor clinical outcomes and low OS. Higher expressions also compromise the DFS, increasing the risk of recurrence. These findings suggest that overexpression of CASP3, PTGS2, PARP1, and MAPK1 may be cancer prognostic biomarkers, affecting cancer growth and patient survival.

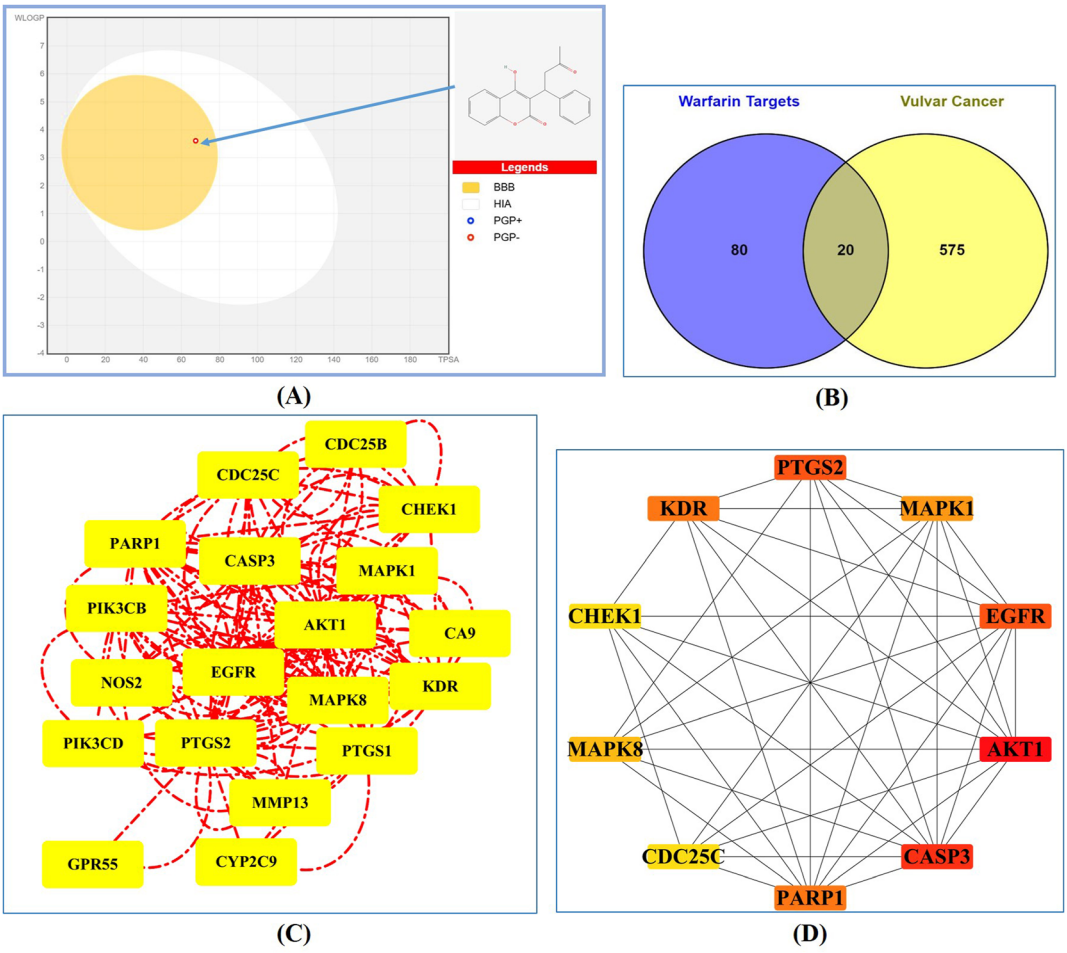


Figure 2: Multi-level systems analysis of warfarin targets and networks in vulvar cancer. (A) Physicochemical properties of warfarin supporting its potential as a drug candidate, showing high GI absorption and BBB permeability using a boiled egg model. (B) Venn diagram showing the overlap of 20 common gene targets between warfarin and vulvar cancer-related genes, identified using Venny 2.0.2. (C) PPI network analysis in cytoscape showing 19 nodes and 144 edges, revealing more interactions among warfarin targets in vulvar cancer pathways. (D) identification of top hub genes (e.g., AKT1, CASP3, EGFR) in the PPI network using CytoHubba, indicating central nodes in the vulvar cancer pathway.

Immune infiltration analysis for the hub gene expression

The association between hub genes – AKT1, EGFR, CASP3, PTGS2, PARP1, and MAPK1 – and immune cell infiltration was analysed using the TIMER platform, using p-values to denote the strength of these associations. All the correlations of hub genes with different immune cells compared to tumor purity are shown in Supplementary Figure 4. AKT1 has the strongest association with macrophages (p=9.47e-01), indicating that its expression may be correlated with macrophage presence; nevertheless, the majority of other relationships are quite moderate. EGFR has a substantial correlation with dendritic cells (p=3.40e-05) and macrophages (p=6.79e-04), suggesting potential interactions with these immune cell types. CASP3 has a substantial correlation with CD4+ T cells (p=2.56e-03) and neutrophils (p=3.44e-03), suggesting its involvement in the existence of these cell types. The correlation of PTGS2 with neutrophils was significant (p=9.19e-01) and with CD4+ T cells (p=3.42e-05), suggesting an association and potential regulatory role with these immune cells. PARP1 had a weak association with B cells (p=1.26e-03) and with neutrophils (p=3.12e-02). Finally, MAPK1 had a strong correlation with CD8+ T cells, p=8.22e-01, and B cells, p=6.40e-01.

Docking affinity of warfarin with immune checkpoint protein complex

The CB-Dock2 algorithm used for docking analysis identified five unique CurPockets sites in the PD-1/PD-L1 complex with

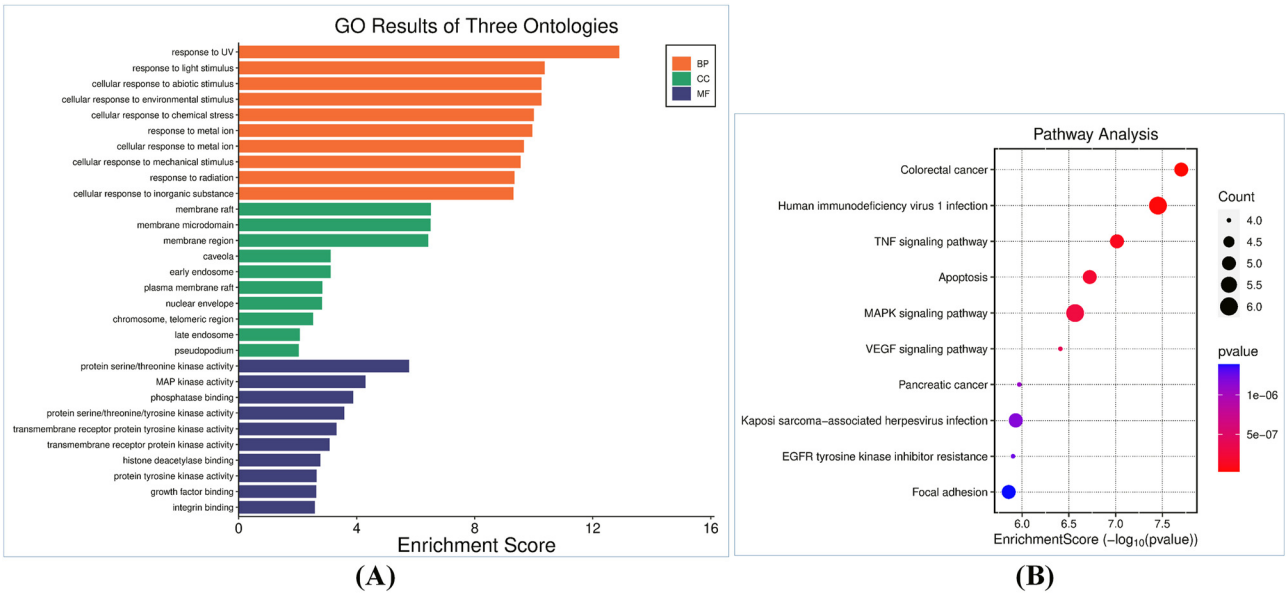


Figure 3: Functional enrichment of warfarin-associated hub genes (GO and KEGG analysis). (A) GO analysis showing enrichment of hub genes in biological processes related to cellular stress, structural components, and molecular functions related to protein binding. (B) KEGG pathway analysis indicating pathways associated with cancer, TNF signaling, MAPK signaling, and apoptosis among warfarin targets in vulvar cancer.

potential warfarin binding. These five unique sites were ranked according to their achieved binding affinities, and the highest docking score obtained was that of CurPocket C2 (Figure 6A), being −8.0, which indicates the highest affinity toward warfarin binding (Figure 6B). Other binding sites showed a sequence of decreasing affinities; the values were −6.4 for C1, -6.0 for C3, -5.7 for C5, and -5.5 for C4 (Supplementary Table 3). These scores are calculated binding energy between warfarin and the PD-1/PD-L1 complex in each pocket, with higher negatives signifying stronger interaction.

Some residues involved in the binding have been found to be common across two chains of the complex: in Chain B, ILE54, TYR56, GLU58, ASN63, GLN66, ASP73, VAL76, and MET115 are interacting with warfarin. Involved residues were PHE19, ALA121, ASP122, TYR123, and LYS124 (Figure 6C). A 2D interaction study on all the pockets was done in Discovery Studio, and noncovalent interactions that had participated in the warfarin binding with the described pockets were confirmed. These are types of interactions: van der Waals, charge attractions, hydrogen bonding, pi-donor hydrogen bonds, pi-pi stacking, and pi-alkyl (Figure 6D). Altogether, these types contribute to warfarin stability in binding at the PD-1/PD-L1 interface. Such diverse interactions suggest the presence of distinct binding modes for warfarin to the protein complex and possibly an influence on the pathway inhibition by PD-1/PD-L1.

Selective cytotoxicity of warfarin

The MTT assay revealed that the viability of the SW-962 cells is drastically reduced in a dose- and time-dependent manner by warfarin. The viability of HaCaT cells was not altered at 24 (Figure 7A) and 48 h (Figure 7B) after treatment. Such a differential effect on SW-962 cells indicates that warfarin specifically targets cancerous cells and spares normal cells (HaCaT). Differential sensitivity among cell types shows warfarin could be a selective therapeutic agent.

Warfarin triggers oxidative stress in SW-962 cells

NBT assay shows dose-dependent elevation of ROS in SW-962 cells. The concentration of warfarin directly correlated directly to the increase in optical density, thereby a relative increase in ROS (Figure 7C). Increase in ROS is related to oxidative stress, causing damage and apoptosis in cancerous cells. This finding suggested that warfarin's cytotoxic action may be partly mediated through promoting oxidative stress through ROS generation.

Warfarin triggers apoptosis in SW-962 cells

Results of DAPI staining showed that cells treated with low concentrations of warfarin were similar to control cells, with

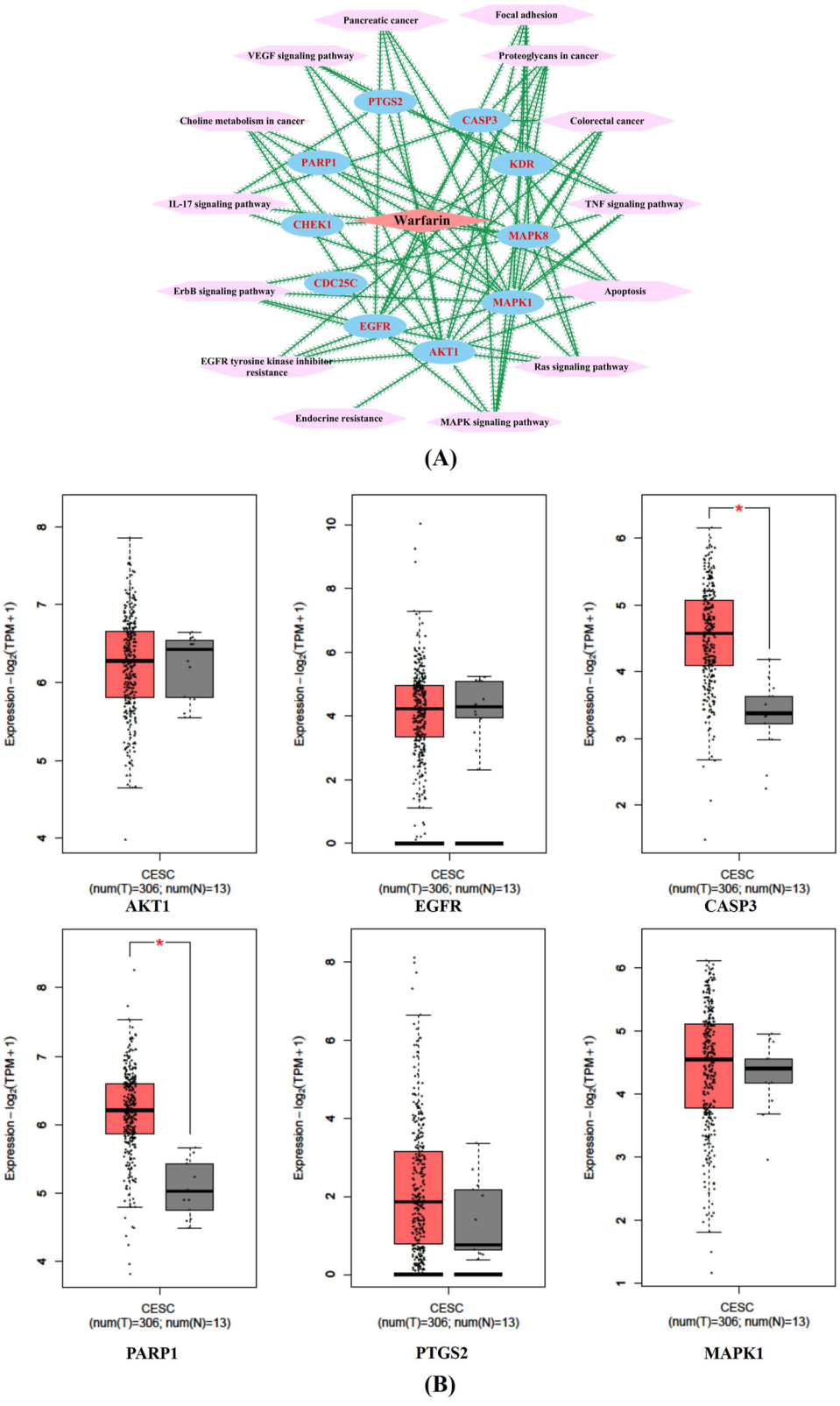
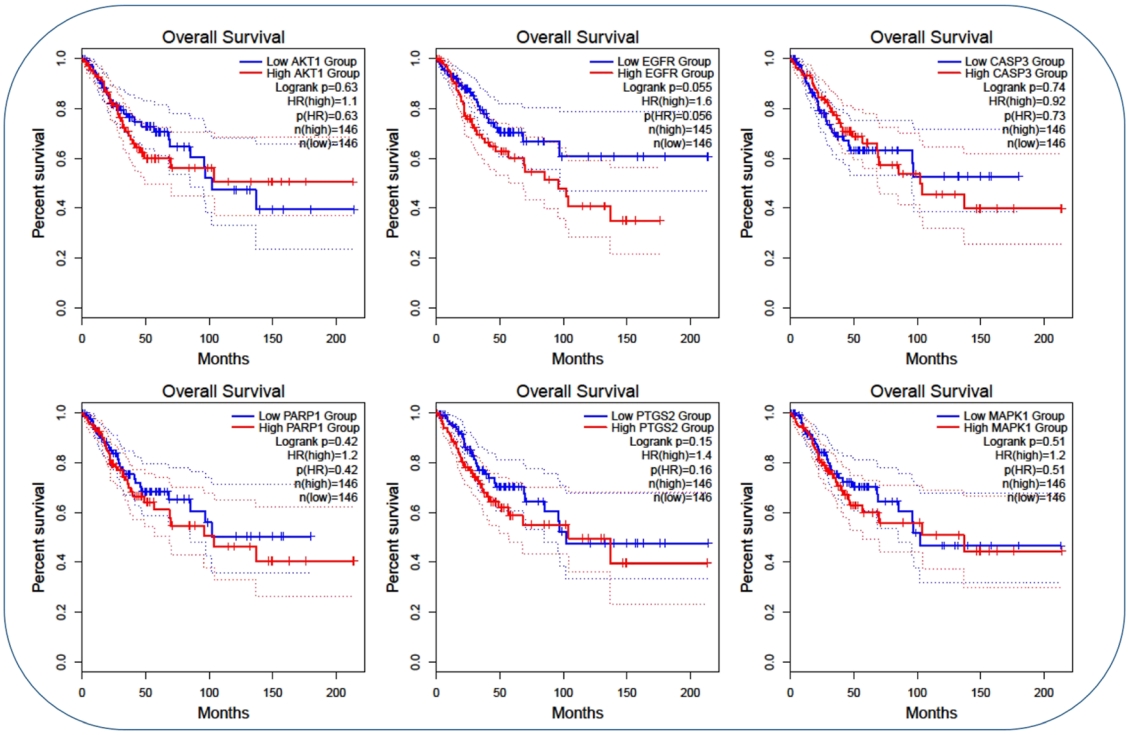
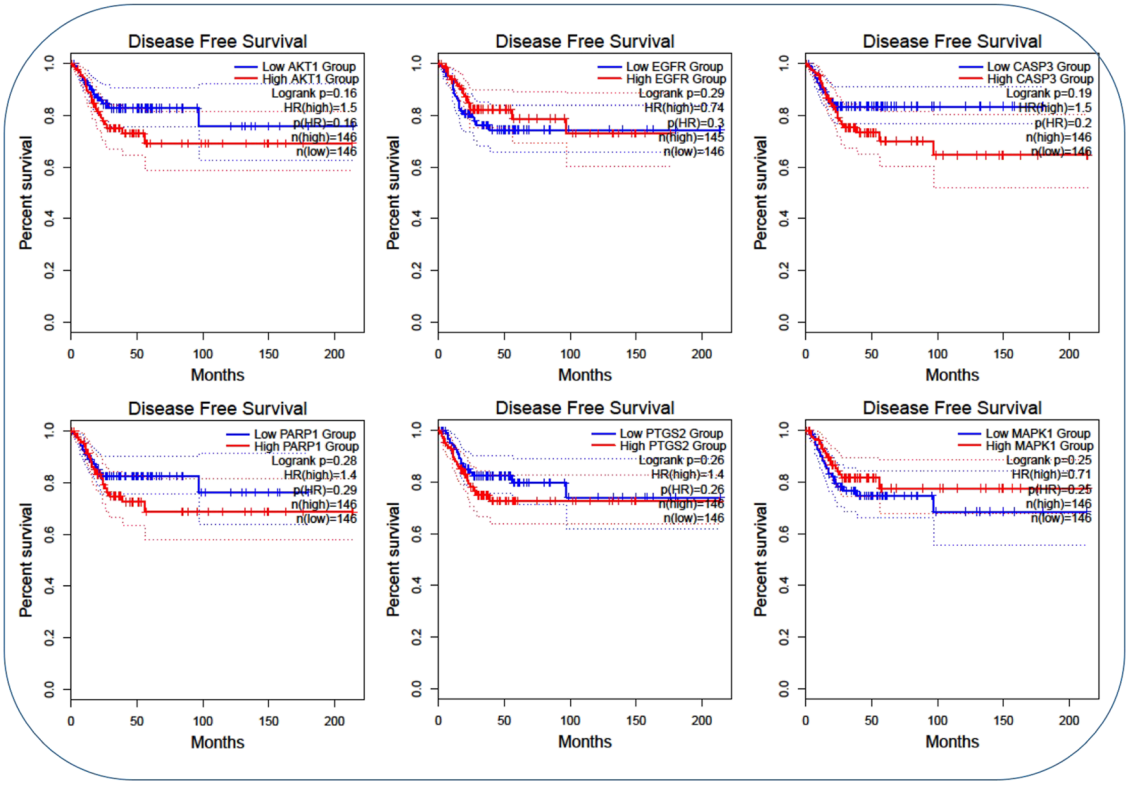


Figure 4: Integrated network and differential expression of hub genes in vulvar cancer. (A) Combined cytoscape network of warfarin, hub genes, and enriched pathways, illustrating potential therapeutic mechanisms for vulvar cancer. (B) Differential expression of six hub genes in cancer vs. normal tissues using GEPIA, with overexpression in cancer suggesting oncogenic roles.



(A)



(B)

Figure 5: Prognostic impact of warfarin-associated hub genes on patient survival and recurrence. (A) OS analysis of hub genes, with higher expressions of CASP3, PTGS2, PARP1, and MAPK1 linked to reduced survival. (B) DFS analysis of hub genes, showing associations between high expression levels and increased recurrence risk.

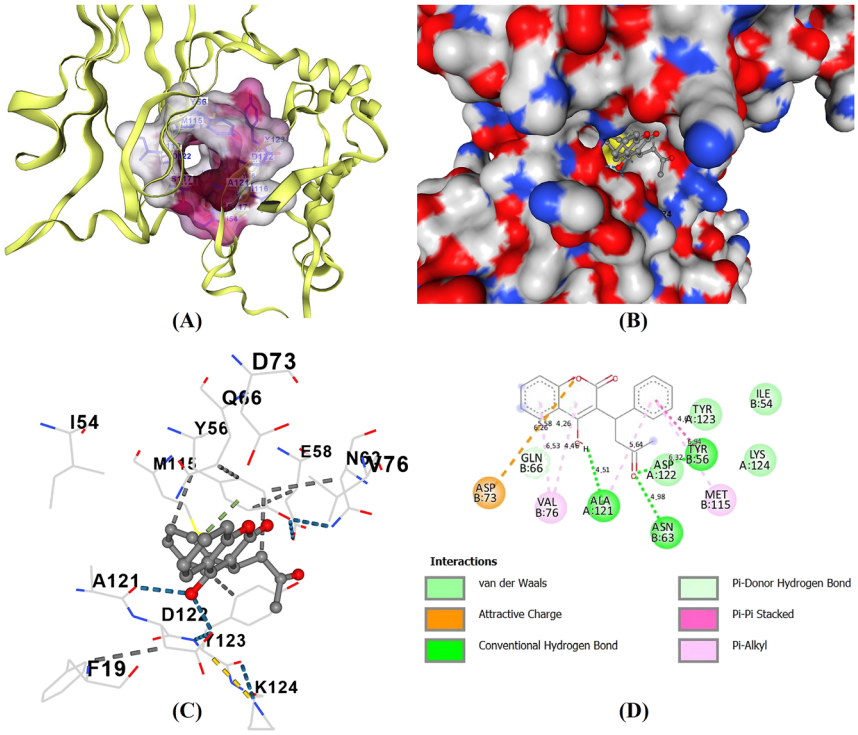


Figure 6: Molecular docking of warfarin with PD-1/PD-L1 complex and key binding interactions. (A) Docking of warfarin in PD-1/PD-L1 complex showing highest binding affinity at CurPocket C2 with a score of −8.0. (B) Docking affinities for five binding sites on PD-1/PD-L1, indicating warfarin's strongest interaction with CurPocket C2. (C) Identification of interacting residues in PD-1/PD-L1 complex chains, showing key noncovalent interactions with warfarin. (D) visualization of 2D noncovalent interactions between warfarin and PD-1/PD-L1 complex, highlighting van der waals and hydrogen bonds.

no apparent changes in nuclear morphology, whereas high concentrations caused marked apoptotic features such as nuclear fragmentation (Figure 8A). Morphological evidence of apoptosis at high doses demonstrates that the effect of warfarin on SW-962 cells is dose-dependent and higher concentrations can actively promote apoptotic cell death.

Warfarin triggers G0/G1 phase arrest in SW-962 cells

Analysis by cell cycle showed a gradual increase in the G0/G1 cell population as the concentration of warfarin increased compared to controls (Figure 8B). This indicates cell cycle arrest. The G0/G1 phase arrest means that warfarin prevents SW-962 cells from moving forward in the cell cycle, which limits their growth. The concentration-dependent cell cycle arrest further supports the role of warfarin in the inhibition of cancer cell growth.

Discussion

AKT1 is essential for cell survival, proliferation, and metabolism and is implicated in cancer pathogenesis [19, 20]. This study indicates that AKT1 expression is slightly elevated in normal tissues, suggesting a potential balance in noncancerous cells. The presence of AKT1 in the PPI network and its association with pathways such as MAPK and VEGF signalling suggest its significance in cancer cell survival and angiogenesis [21]. This finding is further supported by the hypothesis that warfarin indirectly influences AKT1-driven immune evasion in cancer cells through its affinities for the PD-1 and PD-L1 complex. Analysis of macrophage infiltration in relation to AKT1 demonstrated a significant correlation, suggesting that AKT1 may play a role in modulating the immune landscape established by the tumour microenvironment. This data may suggest a potential target for warfarin therapy in vulvar cancer with implications for modulating tumour cell survival and immune response.

CASP3 is a key executor in apoptosis that cleaves specific cellular substrates during cell death [22, 23]. The significantly higher expression of CASP3 in cancer tissues observed in this study correlates well with its apoptotic function, especially under conditions of stress caused by treatments such as warfarin. This finding for CASP3 indicated central placement within the apoptotic pathway through the PPI network. We could confirm our original finding that the *in vitro* DAPI assay showed an increase in nuclear fragmentation, a hallmark of apoptosis, through this pathway. The immunity-related analysis also correlated with CD4+ T cells and neutrophils and thus indicated a participation of CASP3 not just in cell death pathways in the tumor microenvironment, but also in an interface of immune interactions. This dual role of CASP3 in apoptosis and immune response makes it a potential therapeutic target, especially when combined with

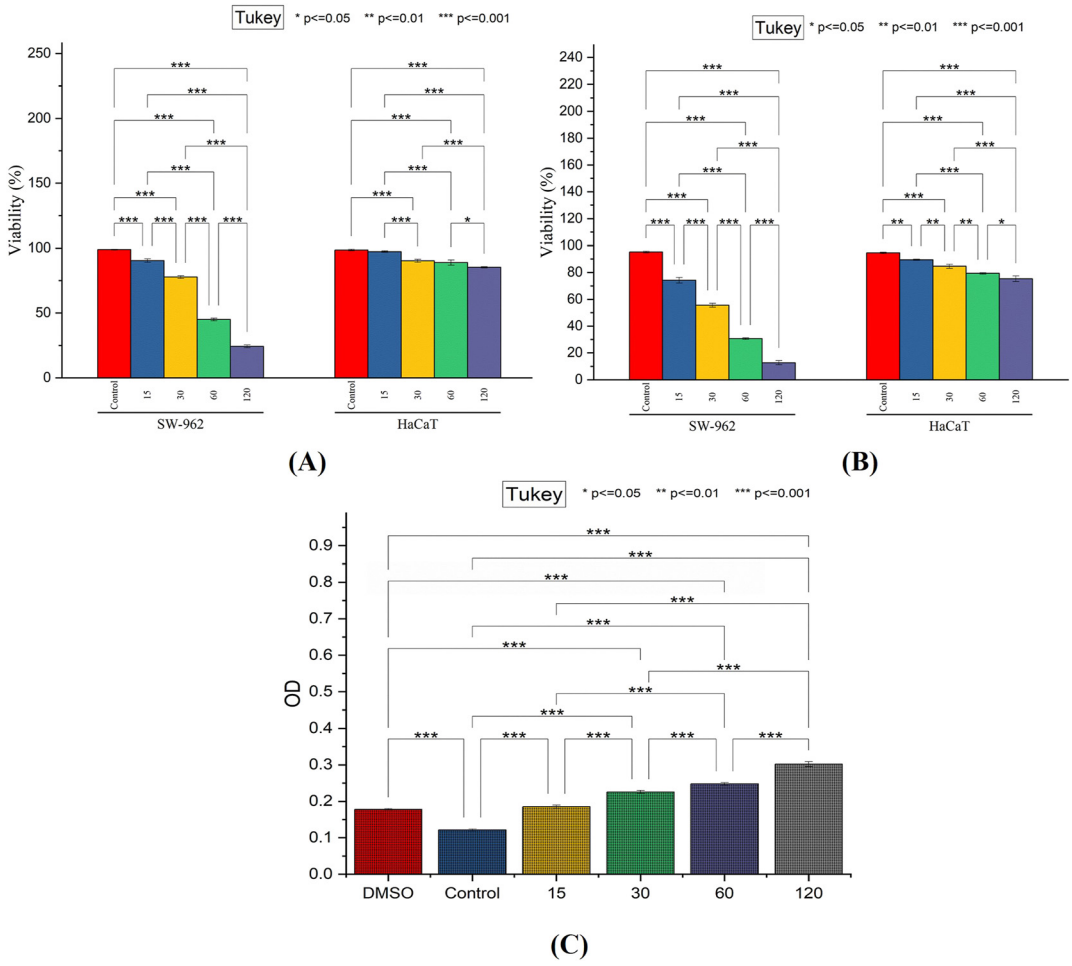


Figure 7: Selective cytotoxic and pro-oxidant effects of warfarin in vulvar cancer cells. (A) MTT assay showing selective cytotoxicity of warfarin on SW-962 cancer cells at 24 h, with no effect on normal HaCaT cells. (B) MTT assay results at 48 h confirming selective warfarin cytotoxicity in cancer cells while sparing normal cells. (C) NBT assay indicating dose-dependent ROS generation in SW-962 cells treated with warfarin, suggesting oxidative stress (*p<*0.05*, **p<*0.01 and* ***p<*0.001*).

drugs such as warfarin that induce apoptosis in cancer cells by producing ROS and cell cycle arrest.

EGFR is a transmembrane receptor that plays an important role in cell proliferation and differentiation and is usually overexpressed in cancers [24, 25]. Though the expression was higher in normal tissues, the factor's involvement in vulvar cancer progression is evident from its central position in our PPI network, where it interacts with pathways such as EGFR tyrosine kinase inhibitor resistance and focal adhesion pathways that suggest the influence of EGFR on cell proliferation and metastasis. The interaction with dendritic cells and macrophages that were proven to be involved in immune infiltration analysis indicates that this signaling could play a role in affecting the immune response within the tumor microenvironment. Warfarin targeting EGFR arrested the cell cycle in SW-962 cells and would result in controlled unchecked proliferation within vulvar cancer.

PTGS2, also known as COX-2, is expressed in the inflammation and the tumorigenesis resulting from the synthesis of prostaglandin [26, 27]. As a result of increased expression of PTGS2, the pro-inflammatory role leads to the growth of the tumor but inhibits the immune system [28]. On the PPI network, PTGS2 was related to the signaling pathway TNF, given its inflammation role in promoting cancerous progression. This invasion study also revealed that PTGS2 was associated with neutrophils, which may serve to promote an inflammatory tumor environment. Since warfarin was demonstrated to induce ROS in SW-962 cells, the combination of PTGS2 inhibition with warfarin should increase oxidative stress and inhibit inflammation while supporting the growth of tumors to facilitate cell death.

Highly overexpressed in many cancers, PARP1 helps the survival of cancer cells by repairing the DNA damage [29, 30]. We discovered that PARP1 is significantly overexpressed in

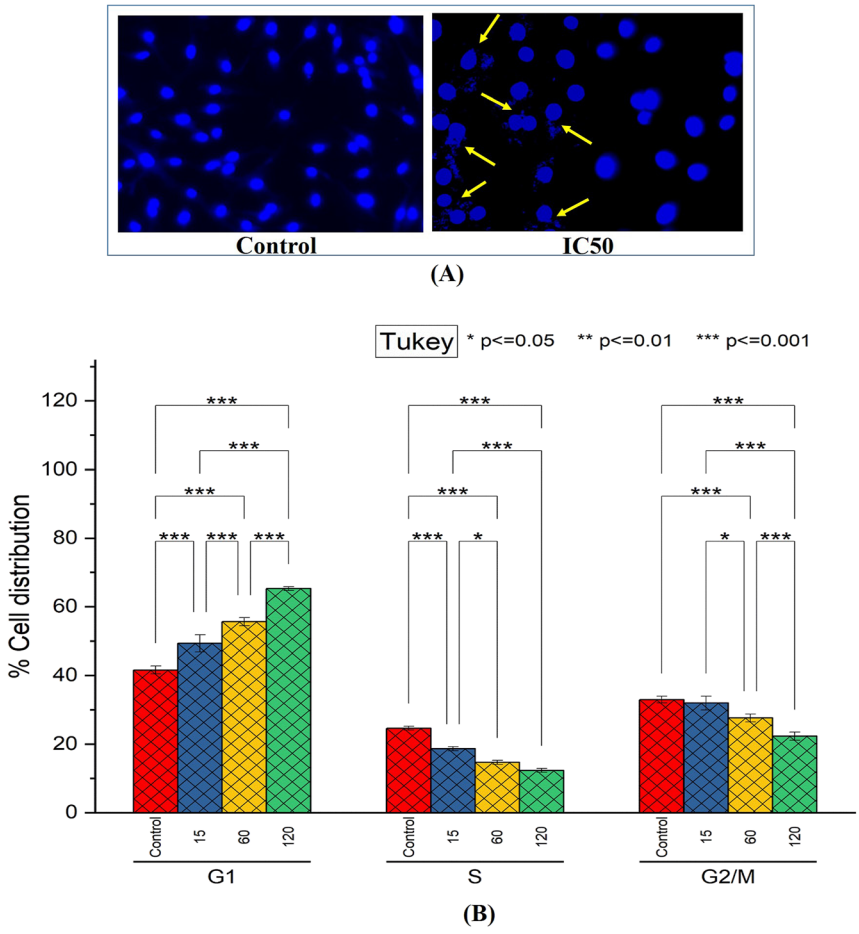


Figure 8: Warfarin-induced apoptosis and cell cycle arrest in SW-962 vulvar cancer cells. (A) DAPI staining demonstrating apoptotic nuclear changes in SW-962 cells at high warfarin concentrations. (B) Cell cycle analysis showing increased G0/G1 arrest in SW-962 cells with increasing warfarin concentration, inhibiting cell proliferation (*p*<0.05, **p*<0.01 and ***p*<0.001).

cancer tissues and acts as a central hub in DNA repair pathways and cell cycle regulation. From network analysis, PPI indicated that PARP1 is correlated with apoptosis and cell cycle pathways. This finding aligns with our *in vitro* results, where we observed G0/G1 cell cycle arrest following PARP inhibition treatment in SW-962 cells. Also, the significant correlation of PARP1 with B cells and neutrophils in the results of the immune infiltration study may imply its role as being involved in the immune contexture that may help these tumor cells to escape killing by the immune system. Further enhancement of warfarin-induced cell cycle arrest and apoptosis in the SW-962 cells would be achieved by disruption of PARP1-mediated DNA repair to support the survival of cancer cells.

A key player in the MAPK signaling pathway is the gene MAPK1, which regulates cell growth, differentiation, and apoptosis [31, 32]. The overexpression of this gene in cancer tissues identified in our study is thus in accordance with its oncogenic potential. PPI network showed MAPK1 to be participating in pathways such as TNF signaling and VEGF signaling that contribute towards the survival of cancer cells and angiogenesis [33]. *In vitro* studies demonstrated that warfarin induces apoptosis and cell cycle arrest in SW-962 cells through the modulation of the MAPK1 signaling pathway. In addition, strong association of MAPK1 with CD8+ T cells was found to result from the analysis of immune infiltration, which may therefore influence the immune interactions which occur within the tumor microenvironment. Targeting MAPK1 with warfarin could both inhibit tumor-promoting signaling pathways and enhance immune infiltration to improve the treatment outcome of vulvar cancer.

Conclusions

Our integrated network analysis and *in vitro* assays show that warfarin could exert therapeutic effects in vulvar cancer by targeting critical hub genes involved in cancer progression, immune modulation, and apoptosis. The interaction of AKT1, CASP3, EGFR, PTGS2, PARP1, and MAPK1 with immune cells and their involvement in cancer-related pathways suggests that warfarin may disrupt the survival mechanisms of cancer cells, induce apoptosis, and potentially increase immune infiltration. This study showed the possibility of repurposing warfarin as a targeted therapeutic in vulvar

cancer. Subsequent molecular studies on pathways in which warfarin acts may open up the possibility of new ways for this drug to be used with greater efficacy in cancer treatments.

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