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Original article

# Epidermal growth factor-induced prostate cancer (PC3) cell survival and proliferation is inhibited by quercetin, a plant flavonoid through apoptotic machinery

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ABSTRACT

Epidermal growth factor (EGF) plays a key role in epithelial malignancies by enhancing cancer cell proliferation, survival, invasion, and metastasis. The aberrant expression of epidermal growth factor receptor (EGFR) by tumors typically confers a more aggressive phenotype and is often predictive of poor prognosis. Quercetin is an anti-oxidative flavonoid widely distributed in fruits and vegetables and have attracted much attention as potential anti-carcinogens. Prostate cancer is the most common cause of cancer related deaths in men. In the present study, we examined the effects of quercetin on EGF induced signaling molecules involved in proliferation, survival and apoptosis in PC-3 cells. EGF-stimulated EGFR, Akt, PI3 K, PDK1 and ERK1/2 protein levels were inhibited by quercetin. The inhibitory effects of quercetin on EGF induced signaling were compared with PI3 K inhibitor (LY294002) and MAPK inhibitor (PD98059). Quercetin down-regulated EGF induced Bcl-2 expression and upregulated Bax protein levels. Caspase-3 activity was significantly increased by quercetin treatment. Acridine orange and ethidium bromide staining showed that quercetin was able to induce apoptosis even in the presence of EGF. To conclude, the present study showed that quercetin inhibits EGF induced cell survival, proliferation and induced apoptosis in PC-3 cells.



## 1. Introduction

Prostate adenocarcinoma is one of the most common cancers, and the second leading cause of death among men in USA [1]. The major cause of death is due to metastasis to the bone and lymph nodes. The metastatic prostate cancer resists conventional therapies like androgen-deprivation therapy [2]. Hence, novel therapies are on demand for the treatment of malignant forms of prostate cancer. Prostate cancer commonly overexpresses several growth factors and their receptors, including epidermal growth factor (EGF) and its receptor (EGFR).

EGFR plays a critical role in tumor growth, and the prostate tissue becomes more susceptible to the growth-promoting actions of EGF family growth factors during androgen withdrawal. Progression from androgen-responsive tumor to hormone-refractory carcinoma is a multistep process, usually accompanied by the upregulation of growth factor receptors, their ligands, and down-regulation of tumor suppressor gene products [3,4]. EGFR ligands, such as EGF, HB-EGF, and TGF (transforming growth factor), are expressed in the prostate and prostatic carcinomas [5,6]. The major EGF/EGFR signaling pathways include PI3 K/Akt and MAPK kinase (MEK)/extracellular-related kinase (ERK) [7,8]. There is substantial evidence validating the importance of Raf and ERK in cancer progression [9]. The importance of this pathway in oncogenesis was first suggested by the initial identification of Raf as potent retrovirus oncogenes [10]. Raf kinases phosphorylate and activate the MEK1 and MEK2 dual-specificity protein kinases. MEK1/2 (MAPKK) then phosphorylates and activates the ERK1 and ERK2 MAPKs. Activated ERKs can translocate to the nucleus, where they phosphorylate and regulate various transcription factors, such as Ets family transcription factors (e.g., Elk-1), ultimately leading to changes in gene expression [11,12].

*Abbreviations:* EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; PDK1, Phosphoinositide dependent kinase 1; ERK, Extracellular-signal-regulated kinases; PI3K, Phosphoinositide 3-Kinase; mTOR, mammalian target of rapamycin; PARP, Poly (ADP-ribose) polymerase; PSA, Prostate specific antigen; PTEN, phosphatase and tensin homolog; TSC, Tuberous sclerosis protein; GSK-3β, Glycogen synthase kinase-3-β.

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Inhibition of tyrosine kinase signaling pathways provides therapeutic advantage against prostate cancer metastasis [13]. Therefore, inhibiting the activation of growth factor receptors, especially EGFR, may be a promising strategy for the treatment of prostate cancer. A novel therapeutic approach should be to target the epidermal growth factor receptor (EGFR), which is often mutated and/or overexpressed in many tumors and regulates proliferation, apoptosis, angiogenesis, tumor invasiveness, and metastasis [14,15].

Apoptosis is a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. The ability to modulate the life or death of a cell is recognized for its immense therapeutic potential [16]. The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins. The Bcl-2 family of proteins governs mitochondrial membrane permeability and can be either pro-apoptotic (e.g., Bad) or antiapoptotic (e.g., Bcl-2) [17]. The main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome c release from the mitochondria via alteration of mitochondrial membrane permeability. Caspases are proteases that activate cytoplasmic endonucleases, which degrade nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3, caspase-6, and caspase-7 function as effector or "executioner" caspases, cleaving various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others, that ultimately cause the morphological and biochemical changes seen in apoptotic cells [18].

Several dietary cancer chemopreventive agents could provide promising strategies for reducing the incidence of prostate cancer [19]. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) belongs to an extensive class of polyphenolic flavonoid compounds almost ubiquitous in plants and plant food sources. Their cancer-preventive effects have been attributed to various mechanisms including their anti-oxidative activity, the inhibition of enzymes that activate carcinogens, the modification of signal transduction pathways, and interactions with receptors and other proteins. Quercetin has also been shown to have potential activity against prostate cancer. It can attenuate the function of the androgen receptor (AR), inhibiting AR-mediated expression of prostate specific antigen (PSA) [20], up-regulating tumor suppressor genes while down-regulating oncogenes and cell cycle genes [21]. PC-3, cells are androgen insensitive and are excellent models for studying EGFR signaling in hormonal-refractory prostate cancer.

We therefore examined the effect of quercetin on EGF induced signaling molecules involved in survival, proliferation and apoptosis in PC-3 cells for the potential treatment and prevention of prostate cancer.

## 2. Materials and methods

### 2.1. Chemicals

Quercetin, epidermal growth factor, DMEM (Dulbecco's Modified Eagle Medium), 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Aldrich Chemicals Pvt Ltd (USA). Primary antibodies for EGFR, p-EGFR, Akt, P-Akt, PI3 K, p-PDK1, p-ERK1/2, mTOR, p-mTOR, p-PTEN, GSK-3ß, p-GSK-3ß (rabbit monoclonal) were purchased from Cell Signaling, USA. Bcl-2, Bax, Caspase-3, cyclin D1 primary antibodies were purchased from Santa Cruz USA. ß- Actin (mouse monoclonal antibody) was purchased from Sigma Aldrich Chemicals Pvt Ltd (USA). The secondary antibodies, horse radishperoxidase (HRP) conjugated, rabbit- antimouse IgG and goat-anti rabbit IgG were obtained from Genei, Bangalore. Caspase-3/CPP32 Colorimetric Assay Kit was purchased from Biovision, USA. Polyvinylidinedifluoride (PVDF) membrane was purchased from Millipore, USA. 100X antibiotic & anti-mycotic mixtuter, fetal bovine serum (FBS) and trypsin-EDTA were obtained from Gibco (Invitrogen, USA). All other chemicals were obtained from Sisco Research Laboratories (SRL), Pvt Ltd, India.

### 2.2. Cell culture

Human prostate cancer cell line (PC-3) was obtained from NCCS, Pune, India and grown in t-75 culture flask, containing DMEM medium supplemented with 10% FBS, 1% penicillin–streptomycin, under 5% $CO_2$, 95% $O_2$ at 37 °C. Upon reaching 80% confluency, the cells were trypsinized and passaged.

### 2.3. EGF, PI3K inhibitor, MAP kinase Inhibitor and quercetin treatment

Human prostate cancer cells were cultured under recommended conditions. The cells were maintained at 37 °C and 5% $CO_2$ in a humid environment. At 60% confluence, cultures were switched to serum-free medium for overnight and treated with 50 ng/mL of EGF for different time durations 0 h, 10 m, 30 m, 1 h, 3 h, 6 h, 12 h and 24 h. EGFR, p-EGFR, and Akt protein levels were analyzed by western blotting. For inhibitor treatment, once the cells reached 60% confluency, cultures were switched to serum-free medium overnight and pre-treated with 10 μM PI3K inhibitor (LY294002), 25 μM MAPK inhibitor (PD98059) for 1 h. Quercetin, PI3K inhibitor (LY294002) and MAPK inhibitor (PD98059) doses were selected from previous studies [22,23]. After 24 h, the cells were lysed with RIPA buffer and protein was quantified.

### 2.4. Western Blot analysis

Cells were plated on 100 mm petri plates at density of $1 \times 10^6$ cells per plate, allowed to attach for 24 h and then treated with control (DMSO) (0.01%), EGF (50 ng/mL), EGF (50 ng/mL) + quercetin (100 μM), quercetin (100 μM), LY294002 (10 μM), EGF (50 ng/mL) + LY294002 (10 μM), PD98059 (25 μM), and EGF (50 ng/mL) + PD98059 (25 μM) for 24 h. After 24 h treatment period, PC-3 cells were harvested and washed with PBS three times. One millilitre of RIPA (Radio-immuno-precipitation assay buffer) containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% Sodium deoxycholate, 0.1% NP-40 with 1 × protease, phosphatase inhibitor cocktails were added to the cells and ultrasonicated for 5s, followed by centrifugation at 10,000 g for 15 min Protein concentrations were determined by Lowry's method [24]. The samples were stored at −80 °C for further study. The cell lysate (30 μg) samples were mixed with 6 × sample buffer, boiled for 5 min and were electrophoresed in 10% SDS polyacrylamide gel and then transferred onto PVDF membranes. Next the membrane was blocked in PBS containing 5% BSA for 3 h at room temperature. The membranes were incubated with primary antibodies against EGFR, Akt, mTOR, Casp-3, Bax, Bcl-2, Cyclin D1, GSK-3-β (1: 2000) and p-EGFR, p-PI3 K, p-Akt, p-ERK, p-PTEN, p-GSK-3-β, p-mTOR (1: 1000) in Tris-buffered saline at 4 °C overnight. After washing, the membranes were incubated with HRP conjugated antimouse IgG (1: 5000) or HRP conjugated goat-antirabbit IgG (1: 5000) secondary antibodies at room temperature. Protein bands were detected using chemiluminescence system (ECL kit) and quantified in Chemi Doc XRS Imaging System, Bio-Rad (USA).