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Effects of Quercetin Induced Cell Death on a Novel Gene *"URG4/URGCP"* Expression in Leukemia Cells

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Abstract

The present study aimed to investigate anti-proliferative and apoptotic effects of quercetin on human leukemia cells and effects of quercetin-induced cell death on a novel gene Up-regulated gene 4/upregulator of cell proliferation (*URG4/URGCP*), in leukemia cells. *URG4/URGCP* expression is determined by using RT-PCR. IC_{50} of quercetin was determined as 25 microM in CCRF-CEM, HL–60 and K562 cells. In IC_{50} dose group, *URG4/URGCP* expression was decreased 99% in HL-60 cells, 90% in CCRF-CEM cells, and 52% (24 hour) - 99% (72 hour) in K-562 cells. *URG4/URGCP* may play important roles in the development of leukemia, and might be a useful molecular marker for predicting the prognosis of leukemia via quercetin treatment.

Keywords: Quercetin; *URG4/URGCP;* Leukemia cells

Introduction

Various hypotheses exist about having higher cancer risk reduction by consuming fruits and vegetables rich in terms of phytochemicals. Although they are the frequently consumed compounds of human diet, biological and pharmacological features of flavonoids are yet to be defined. Major effects of flavonoids are reported to occur in consequence of release of radicals. Another possible mechanism is the activation of flavonoids over various enzyme systems. Cellular activities of these chemicals are not well known as, along with protecting cells from oxidative stress, flavonoids can contribute apoptosis and geno-toxicity of tumor cells by their pro-oxidant features [1].

Anti-cancer mechanisms, anti-oxidants, anti-inflammatory and anti-proliferative activities of flavonoids are associated with inhibition of bioactive enzymes and induction of detoxification enzymes [2]. Quercetin is a type of plant-based chemical flavonoid, shown to have anti-inflammatory and antioxidant properties. It has been promoted as being effective against a wide variety of diseases, including cancer and reported to affect cell cycle kinetics, proliferation and induction of apoptosis [3,4]. There is limited data related with the antagonistic or synergistic interactions among polyphenols in fruits and vegetables. Characterization of potential interactions among these compounds can be effective in determination of the effects of nutrients contain polyphenol in prevention of cancer development [5].

Up-regulated gene 4/Upregulator of cell proliferation *(URG4/URGCP),* GeneBank NM_017920), a novel gene located on 7p13 and originally identified by Satiroglu-Tufan NL, encodes 922 amino acid in cytoplasm. Over-expression of *URG4/URGCP* in HepG2 cells promoted hepatocellular growth and survival in tissue culture and in soft agar, and accelerated tumor development in nude mice [6]. *URG4/URGCP* is strongly expressed in hepatocellular carcinoma, gastric cancer and osteosarcoma. Hence, *URG4/URGCP* may be a putative oncogene that contributes importantly to multistep carcinogenesis and cell cycle regulation. The present study examined the expression of *URG4/URGCP* in leukemia cells and possible association of *URG4/URGCP* and leukemogenesis has been analyzed for the first time.

This study aimed to investigate anti-proliferative and apoptotic effects of quercetin on human leukemia cells and effects of quercetin-induced cell death on a novel gene *URG4/URGCP* expression in leukemia cells.

Material and Methods

Chemicals and reagents

Quercetin (Sigma Chemical Co., St Louis Missouri) is diluted in 0.5% dimethylsulphoxide (DMSO) for the assays. Cell proliferation assay (XTT) was supplied from Roche Diagnostics. For determination of *URG4/URGCP* gene expression, TaqMan Master Kit (Roche Diagnostics) was obtained from Roche Applied Science, Mannheim, Germany, respectively. All other tissue culture supplies were obtained from Corning Incorporated (USA) unless otherwise specified.

Cell lines and cultures

Human T-cell acute lymphoblastic leukemia cell line (CCRF-CEM; ATCC number: CCL-119), Human promyelocytic leukemia cells (HL-60; ATCC number: CCL-240), Human erythromyeloblastoid leukemia (K-562; ATCC number: CCL-243) cell lines were used as model cell lines in this study which were provided by ATCC.

Cell culture and preparation of cytotoxicity experiments

All tumor cell lines grown in RPMI-1640 medium containing 2mM L-glutamine supplemented with 10% fetal bovine inactivated serum

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Gene	ForwardPrimer	Reverse Primer	Probe (Roche)
URG4/URGCP	cgaacagctgtggccatt	ccataacggaactcgcaatc	Ggaaggag
GAPDH	gaaggtgaaggtcggagtc	gaagatggtgatgggatttc	caagcttcccgttctcagcc

Table 1: Primers and probes of genes.

and 1% penicillin/streptomycin were maintained at a density of $5x10^5$ cells/ml in a standard cell culture incubator at 37°C, humidified 95% air, and 5% CO_2 atmosphere. Prior to any experiment, cells were split at 5 $x10^5$ cells/ml in the RPMI 1640 medium and cell suspensions was aliquoted into flasks for subsequent treatments. Quercetin diluted in RPMI 1640 was used in treatments of 12.5 μM, 25 μM, 50 μM, 75 μM, 100μM.

Analysis of cell viability and cytotoxicity

Cytotoxic assays and determination of IC_{50} doses of quercetin in leukemia cells were performed by using trypan blue dye exclusion and XTT assay [15-17] as indicated in manufacturers' instruction.

Apoptosis was determined morphologically by fluorescence microscopy following the staining with acridine orange and ethidium bromide. Cells were washed in cold PBS and adjusted to the cell density to $1x10^6$ cells/ml in PBS. Acridine orange and ethidium bromide (1:1) (v/v) were added to the cell suspension in final concentrations of 100 μg/ml and then cells were incubated for 30 min. The cellular morphology was evaluated by fluorescent microscopy (Olympus, Japan). Apoptotic cells were essentially characterized by nuclear condensation of chromatin and/or nuclear fragmentation. Three hundred cells were evaluated for apoptosis and/or necrosis for each sample. When more than 50% of the pre-apoptotic plus apoptotic to total cell ratio were positive, the result was accepted positive for apoptosis [16,17].

Cytotoxicity (%) = (1-A of experiment well/A of positive control well) x 100

Relative quantification of *URG4/URGCP*

Real-time quantitative RT-PCR analyses of *URG4/URGCP* gene was performed with Light cycler instrument and software (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH* "housekeeping" gene) was chosen as a standard to control the variability in amplification. PCR was performed by using TaqMan Master Kit (Roche Diagnostics) according to the instructions of the manufacturer. Studied genes target probe was labeled at the 5' end with the reporter dye molecule 6-carboxyfluorescein (FAM). The *GAPDH* target probe was labeled with 6-carboxyfluorescein. Both probes were labeled with the quencher fluor 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. To quantify genes mRNA from cell culture, we constructed a calibration curve (Error: 0.100 Efficiency: 1,790) using copy number (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10) variations of *GAPDH* gene. Relative ratio (RR) of gene expressions was calculated using the formula:

RR= Copy number of gene/Copy number of *GAPDH* x 1000.

Results

Quercetin exerts its cytotoxic effect on HL-60, CCRF-CEM and K562 cells in a time- and dose-dependent fashion. We determined the relative reduction in the cell number that evolved in cultures of three cell lines. Assays were performed to determine CCRF-CEM, HL-60 and K562 leukemia cells viability, *URG4/URGCP* gene expression and changes occurring in this parameter in quercetin-treated samples.

These experiments aimed to assess the best concentration of quercetin to be used in CCRF-CEM, HL-60 and K562 leukemia cell lines, at different concentrations, ranging from 12.5 μM to 100 μM. Cell viability, evaluated by trypan blue exclusion test, was determined after 24, 48, 72 h exposure to quercetin and are shown in Figure 1. The cell numbers (using trypan blue dye exclusion) as well as cell metabolic rate (by using XTT assay) were also determined. The IC_{50} of quercetin was found to be 25 mM revealed by trypan blue dye exclusion test as well as it was confirmed by XTT assay (Figure 1, Figure 2). On the other hand, quercetin induced apoptosis in HL-60 and K562 cell lines, correlated with IC_{50} dose, were increased as 70% and 65%, respectively (Figure 3).

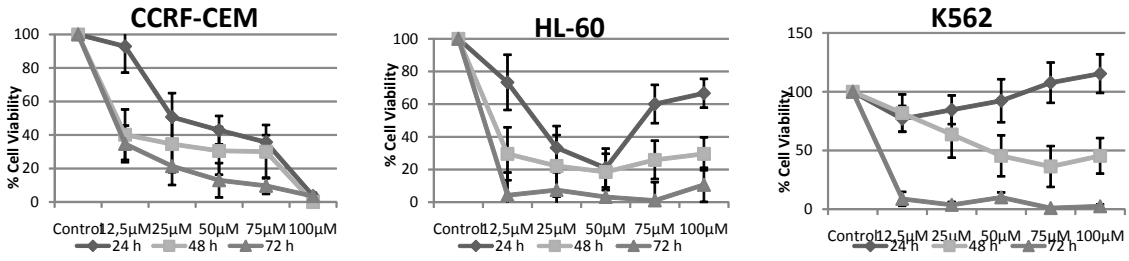

Figure 1: Results of Tyripan Blue Dye Assay.

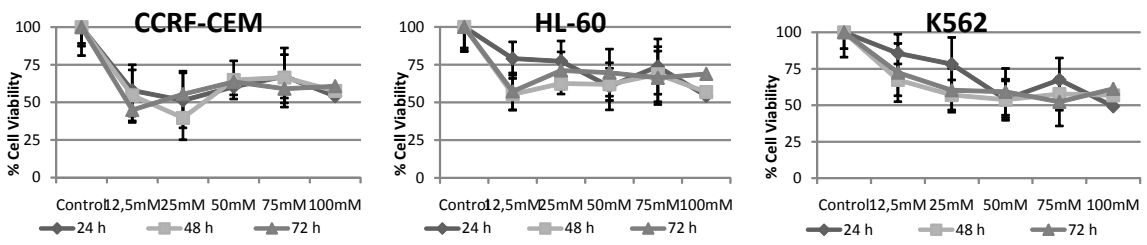

Figure 2: Results of XTT assay.

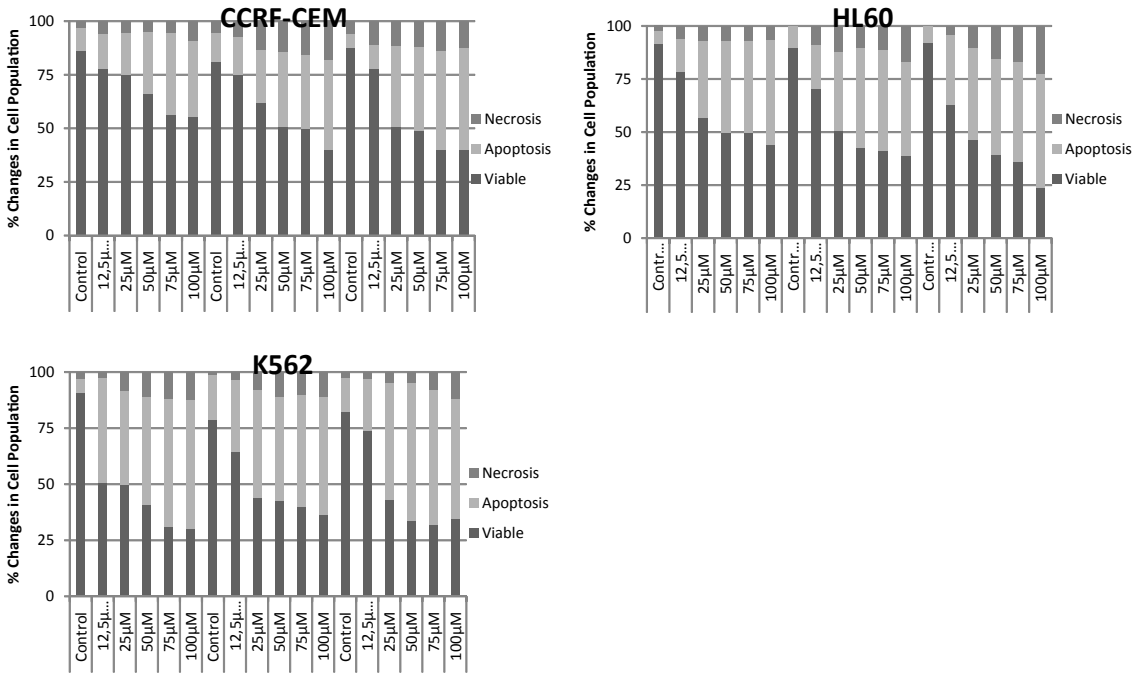

Figure 3: Apoptosis ratio of Ethidium Bromide/Acridine Orange assay.

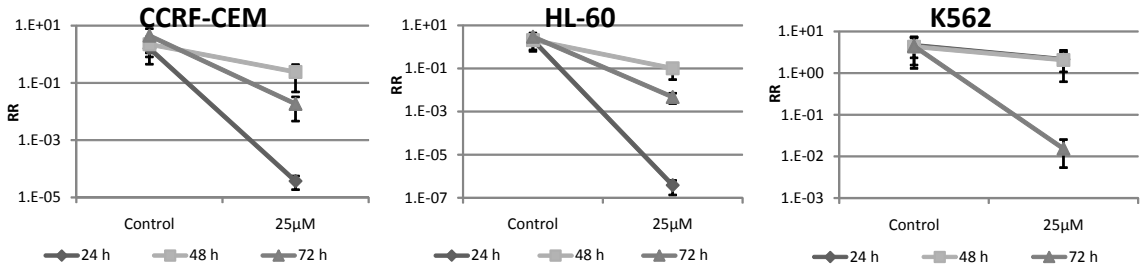

Figure 4: *URG4/URGCP* mRNA expression ratio.

Expression of *URG4/URGCP* mRNA results showed that there was slight decrease in a time-dependent manner on all three cell lines. The averages of *URG4/URGCP* mRNA gene expression in control groups of HL-60, K-562 and CCRF-CEM cells were found 2.73, 4.48 and 2.76, respectively. In IC_{50} dose group, *URG4/URGCP* gene expression was decreased 99% in HL-60 cells, 90% in CCRF-CEM cells, and 52% (24 hour) - 99% (72 hour) in K-562 cells (Figure 4).

Discussion

Quercetin has found to affect some types of cancer cells in several studies done in cell cultures. In a previous work described by Shen et al., it is demonstrated that quercetin was able to induce apoptosis in HL-60 human leukemia cell line. They reported that IC_{50} is found to be 80 µM for viability of the cells. They thought that the induction of apoptosis by quercetin is consistent with the activation of apoptotic machinery including activating the caspase 3 cascade, but is independent on its pro-oxidant activity (ROS: Reactive Oxygen Species).

Wei et al. [7] and Schroeter et al. [8] both have showed that induction of apoptosis by quercetin but could not confirm the apoptotic mechanism. Caspase cascade has been shown to be involved in the action of apoptosis. Caspase 3 is an executioner caspase, and exists in the cytoplasm as an inactive pro-caspase 3 that becomes proteolytically activated by multiple cleavages in apoptotic cells.

Quercetin has been shown to reduce cell proliferation, causing cell-cycle arrest in the G0/G1 phase, the S phase, and the G2/M phase in *in vitro* experiments with various cell lines [9-11]. Rusak et al. [12] suggested that the different effects off flavonoids on the cycle and proliferation of cells is cell-type specific. Their data indicate that *Lactucaindica L.* induces a G0/G1 block in HL-60 cells.

In the past decade, a very large number of proto-oncogenes and tumor suppressor genes have been found. Recently, up regulated gene 4 (*URG4/URGCP*), a novel gene up regulated by HBxAg in human hepatocellular carcinoma, has been identified (Gen Bank accession no. NM_017920). Previous data suggested that overexpression of *URG4/URGCP* in HepG2 cells promoted hepatocellular growth and survival in tissue culture and nude mice. Hence, *URG4* maybe an oncogene operating in hepatocarcinogenesis [6].

The mechanism of *URG4/URGCP* biologic activity in normal and

malignant cells is not yet fully understood. Song et al. [13] showed that *URG4/URGCP* was up regulated in human gastric cancer tissues and also in gastric cancer cell lines and overexpression of *URG4/URGCP* could promote cell proliferation.

Huang et al. [14] described in their data *URG4/URGCP* was highly expressed in 40 of 46 (86.96%) osteosarcoma specimens with cytoplasmic staining, and also increased in the specimens with recurrence ($p<0.05$) and metastasis ($p<0.05$). They thought that *URG4/URGCP* may play important roles in the development of osteosarcoma, and might be a useful molecular marker for predicting the prognosis of osteosarcoma.

In this study, we investigated anti-proliferative and apoptotic effects of quercetin on human leukemia cells and effects of quercetin induced cell death on a novel gene *URG4/URGCP* expression in leukemia cells. It was found that quercetin diminishes *URG4/URGCP* gene expression in leukemia cell lines. Our ongoing studies include the detailed investigation of URG4's molecular function in oncogenic signaling pathways and cell cycle regulation. Based on our results of growth inhibition and apoptosis induction data, URG4 gene expression is suppressed by quercetin and also this correlation is inhibited cell cycle and induced apoptosis by quercetin. *URG4/URGCP* plays an important role in the development of leukemia, and might be a useful molecular marker for predicting the prognosis of leukemia via quercetin treatment. Controlled clinical trials are needed to show whether quercetin has a positive effect on cancer treatment [15-17].

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