

Effects of Quercetin and Curcumin Combination on Signal Transduction Pathways in Chronic Myeloid Leukemia Cells [†]

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Abstract: Flavonoids have chemo-preventive and chemotherapeutic properties against different human cancers including chronic myeloid leukemia. Quercetin and curcumin are two polyphenols with potential anti-carcinogenic and pro-apoptotic properties. We have previously demonstrated the synergistic protective effect of quercetin and curcumin on chronic myeloid leukemia cells (K562) cells. Anti-proliferative and apoptotic effects of these polyphenols were examined by apoptosis and cell viability assays. Oxidative status of the cells was analyzed by determining the level of reactive oxygen species, mitochondrial permeability and intracellular glutathione. Obtained data showed that quercetin and curcumin had beneficial and synergistic effects on K562 cells. On the basis of the above-mentioned data, herein we aimed to clarify signaling pathways involved in synergistic combination of quercetin and curcumin on K562 cells. Normal peripheral blood mononuclear cell line was used as controls. The mRNA and protein expressions of the signaling pathways were detected by Human Signal Transduction Pathway Finder-RT2 PCR Array system and Western blotting, respectively. The results of PCR array were evaluated by DAVID v6.8 and database for KEGG pathways. Our data revealed that synergistic combination of curcumin quercetin was effective on genes that were particularly related to P53, NF and TGF. We believe that our findings will lead to new research in this area and will contribute to the chronic myeloid leukemia treatment protocols.

Keywords: chronic myeloid leukemia (CML); quercetin; curcumin; apoptosis; signal transduction

1. Introduction

Chronic myeloid leukemia (CML) is the most studied human cancer because of its chromosomal abnormality and has been termed as Philadelphia Chromosome in 1960 [1]. Various methods like chemotherapy, radiation therapy and use of tyrosine kinase inhibitors have been used to combat this disease. In recent years, some plant-derived compounds have been shown to be highly effective as anticancer agents. Research has revealed that quercetin and curcumin have antioxidant, anticancer, antiviral, anti-inflammatory, apoptosis-inducing, cell cycle modulatory and angiogenesis inhibitory

effects. It has also been reported that quercetin suppresses tumor growth of various cancer cell lines, including breast, colorectal, stomach, head and neck, lung, ovarian, melanoma, leukemia and thyroid cancer cells at various concentrations [2,3]. In our previous study we have demonstrated the synergistic and apoptotic effect of quercetin and curcumin on K562 cells [4]. However, the synergistic signaling mechanisms of the combinational treatment are still unclear. In the present study we have investigated affected signaling pathways by Human Signal Transduction Pathway Finder-RT2 PCR Array system and evaluated the obtained results by bioinformatics and protein expression analysis.

2. Materials and Methods

Stock solutions of quercetin (500 mM) and curcumin (200 mM) were prepared in DMSO and stored at -20°C . Solutions were diluted with RPMI-1640 before being used in various assays. Final concentration of DMSO was always kept below 0.1%. We have used Human Signal Transduction Pathway Finder-RT2 PCR Array system (BioRad, USA) for the investigation of various signaling pathways. Real-time PCR was performed with CFX Real Time PCR Detection System. Analyses were based on the $\Delta\Delta\text{Ct}$ method with normalization of raw data to five housekeeping genes. DAVID v6.8, which is a well-known function/annotation method, was used to analyze and visualize the results of the microarray experiments. KEGG database was used for pathway analysis. Functional and localization information about the genes were retrieved from the cancer cells. Antibodies used in Western blot analyses were purchased from Cell Signaling Technologies. All experiments were performed with four groups (Control-chronic myeloid leukemia cell line (K562), Combination (K562-Quercetin-Curcumin), Control human mononuclear cell line (HMN), human mononuclear cell line-combination (HMN-Quercetin-Curcumin). In our previous study, synergistic combination doses of quercetin and curcumin were found as 11.39 mM and 2.85 mM respectively [4]. All groups were treated with stated dosage of quercetin-curcumin, total RNA were isolated and reverse transcribed for microarray analysis. The concentration and purity of total RNA were determined by measuring absorbance at 260/280 nm. The resulting cDNA was added to the RT2 Real-TimeTM SYBR Green/ROX PCR master that contained real-time PCR buffer, a high-performance HotStart DNA Taq polymerase, nucleotides, SYBR Green dye, and the ROX reference dye. Gene expressions were measured by RT2-PCR using BioRad Light Cycler Syst using SYBR Green Master. Aliquots of the mixture were placed into each well of a 96-well plate that included primer sets for specific genes plus housekeeping genes. The relative expression levels of the genes were calculated by the $2^{-\Delta\Delta\text{Ct}}$ methods after normalization to expression of beta actin (ACTB), beta-2-microglobulin (b2M), glucuronidase-beta (GUSb), hypoxanthine phosphoribosyl transferase 1 (HPRT1), phosphoglycerokinase (PGK), peptidylprolyl isomerase A (PPIA). Combination regulated signaling networks and functional pathways were found by bioinformatics and related proteins of the genes were confirmed by Western blotting. After treatment, cells were harvested and total cell lysates were prepared by lysis using nuclear and cytoplasmic extraction kit containing protease inhibitors. Protein (40 μg) was separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with specific antibodies. Detection was performed using the Western blotting luminol reagent and the ChemiDoc MP System. All experiments were carried out three times and the results were expressed as mean \pm SD. Statistical significance was determined using Kolmogorov-Smirnov test with a p value of less than 0.05 considered to be significant.

3. Results

Different signaling pathways and networks are regulated by quercetin and curcumin combination in K562 cells. We have evaluated our gene expression results and studied some of these genes that are related to p53, TGF β and NF κ B signaling pathways (Table 1). These genes changed at least two-fold in combination group compared to control group of K562 cells. Three genes were up-regulated and three genes were down-regulated. They were related to cell cycle, inflammation and especially apoptosis. On the other hand, we observed that these genes were not changed in HMN cells.

Table 1. Gene expression analysis results for signal pathways in quercetin and curcumin treated K562 and HMN cells. Fold-changes of studied genes are given.

Symbol *	K562 Cells			HMN Cells		
	Quercetin	Curcumin	Combination	Quercetin	Curcumin	Combination
BTG2	31,760	−22,824	188,450 ↑	−11,231	−10,652	−10,132
CDKN1A	−19,672	−16,941	236,884 ↑	21,202	12,575	−11,049
CDKN1B	119,082	68,659	65,105 ↓	26,284	−17,185	−21,345
FAS	−39,617	−11,625	158,101 ↑	13,325	29,395	−10,202
AKT1	−13,484	−24,293	−21,643 ↓	−22,697	−25,218	−10,759
IF γ	11,255	−31,468	−29,702 ↓	--	--	--

* HGNC (Human Genome Organization [HUGO] Gene Nomenclature Committee (<http://www.genenames.org>)).

Protein expression levels of p21/waf1/cip1, pAKT, FasL and p27/Kip1 proteins were evaluated by Western blotting 48 h after each treatment. Out of the proteins we have studied, pAKT and IF- γ were down-regulated, and p21/waf1/cip1, FasL, Fas, btg2 and p27/Kip1 were up-regulated in K562 cells (Figure 1), whereas p21/waf1/cip1 and p27/Kip1 protein expressions were not changed in HMN cells (data not shown).

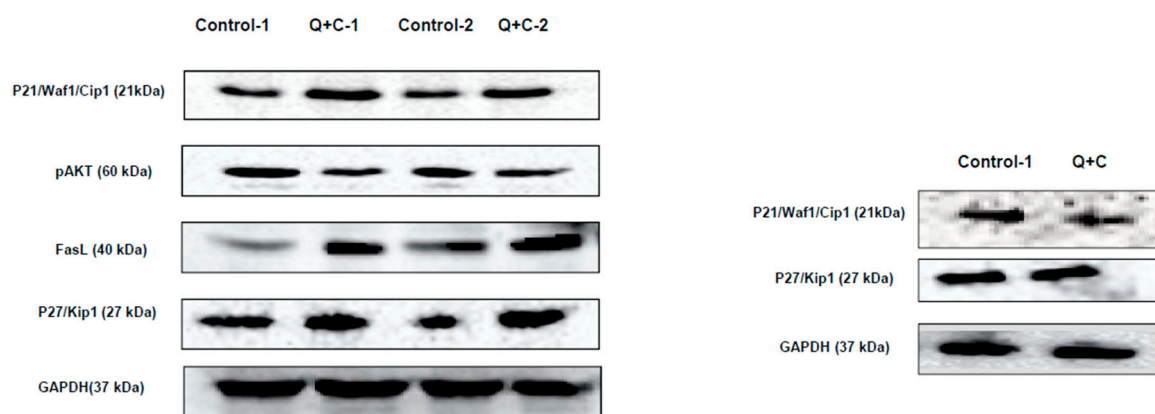


Figure 1. Representative Western blot analysis results for the protein levels of p21/waf1/cip1, p-AKT, FasL, P27/Kip1, btg2, Fas, IF γ in K562 cells.

4. Discussion

Several biological effects of quercetin and curcumin including anti-inflammatory, anti-apoptotic and anti-cancer activities have been previously described [5]. We have previously reported the synergistic effect of the polyphenols quercetin and curcumin on apoptosis induced in chronic myeloid leukemia (K562) cells [4]. In the present study, we investigated the effect of combination treatment in K562 and HMN cells on 84 genes of different signaling pathways. Quantitative RT²-PCR assays indicated that quercetin and curcumin combination augmented the expression of apoptosis-related genes (FAS, FasL), genes involved in cell cycle arrest (p21/waf1/cip 1 and p27/kip1) and inflammatory gene (IF γ) [6,7]. In K562 cells, from the 84 genes that were studied 10 mRNAs were induced and 22 were reduced by combination treatment. The pathways were TGF- β , NFK- β , p53, oxidative stress and hypoxia [8]. The KEGG pathway analysis disclosed that cell cycle, p53 signaling pathway, leukemia pathways were also involved. Functional analysis demonstrated that cell cycle, p53 signaling pathway, leukemia and pathways of cancer were enriched in direct and indirect target genes.

Some of the genes we have identified have been previously implicated in tumors or oncogenic signaling pathways and their cross-talk. Differential expression of several direct and indirect target genes of signal pathway were observed following treatment with combination of curcumin and

quercetin. These findings not only advance our comprehension of the importance of mechanisms involved in leukemia, but also provide clues for future development of therapeutic strategies.

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