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Upregulation of Extrinsic Apoptotic Pathway in Curcumin-Mediated Antiproliferative Effect on Human Pancreatic Carcinogenesis

Mahmoud Youns[1,2]* and Gihan Mahmoud Fathy[3]

[1]*Department of Functional Genome Analysis, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 580, 69120Heidelberg, Germany*

[2]*Faculty of Pharmacy, Department of Biochemistry and Molecular Biology, Helwan University, Cairo, Egypt*

[3]*Faculty of Pharmacy, Department of Pharmaceutics and Industrial Pharmacy, Helwan University, Cairo, Egypt*

ABSTRACT

Pancreatic cancer is one of the most lethal human cancers, with almost identical incidence and mortality rates. Curcumin, derived from the rhizome of Curcuma longa, has a long history of use as coloring agent and for a wide variety of disorders. Here, the antiproliferative activity of curcumin and its modulatory effect on gene expression of pancreatic cancer cell lines were investigated. The effect of curcumin on cellular proliferation and viability was monitored by sulphurhodamine B assay. Apoptotic effect was evaluated by flow cytometry and further confirmed by measuring amount of cytoplasmic histone-associated DNA fragments. Analysis of gene expression was performed with and without curcumin treatment using microarray expression profiling techniques. Array results were confirmed by real-time PCR. ingenuity pathway analysis (IPA) has been used to classify the list of differentially expressed genes and to indentify common biomarkergenes modulating the chemopreventive effect of curcumin. Results showed that curcumin induces growth arrest and apoptosis in pancreatic cancer cell lines. Its effect was more obvious on the highly *COX-2* expressing cell line. Additionally, the expression of 366 and 356 cancer-related genes, involved in regulation of apoptosis, cell cycle, metastasis, was significantly altered after curcumin treatment in BxPC-3 and MiaPaCa-2 cells, respectively. Our results suggested that up-regulation of the extrinsic apoptotic pathway was among signaling pathways modulating the growth inhibitory effects of curcumin on pancreatic cancer cells. Curcumin effect was mediated through activation of *TNFR*, *CASP 8*, *CASP3*, *BID*, *BAX*, and down-regulation of *NFκB*, *NDRG 1*, and *BCL2L10* genes. J. Cell. Biochem. 114: 2654–2665, 2013.

KEY WORDS: CURCUMIN; PANCREATIC CANCER; MICROARRAY; APOPTOSIS AND COX-2

Pancreatic cancer is among the most deadly cancer forms, with poor prognosis and increasing incidence. It is the fourth cause of cancer deaths in Western countries [Chiang et al., 2012; Dempe et al., 2012], with a survival rate that is among the worst of any solid cancers. The annual incidence rate is almost identical to mortality and the 5-year survival rate is still less than 5% [Li et al., 2004a; Siegel et al., 2013]. To date, surgery is the selected treatment option, although it is only applicable to 9–15% of patients due to the tumor's late presentation and rapid progression [Mielgo and Schmid, 2013; Siegel et al., 2013; Tjensvoll et al., 2013]. Although clinical outcome after gemcitabine treatment is rather small, it is generally recommended as the first-line chemotherapeutic treatment for pancreatic cancer [Tjensvoll et al., 2013]. The disappointing performance and/or safety of available treatments, underscore an urgent need for novel safer agents for pancreatic cancer treatment.

Previous studies reported that regular consumption of natural products including fruits, vegetables, and tea is associated with lower incidences of many chronic diseases, including cancers. A recent review reported that bioactive compounds found in natural products can either activate or deactivate multiple molecular signaling cascades by targeting small molecules in cancer cells [Lee et al., 2011; Chung et al., 2013] indicating the enormous impact that natural compounds may have.

Curcumin (diferuloylmethane) is a natural yellow pigment polyphenol extracted from the rhizome of Curcuma longa. Curcumin

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*Correspondence to: Mahmoud Youns, PhD, Faculty of Pharmacy, Department of Biochemistry, Helwan University, Uni-Campus, Ain Helwan, Cairo, Egypt. E-mail: mahmoud_younis@pharm.helwan.edu.eg, dr.mahmoudyouns@yahoo.com

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is widely consumed as a dietary additive to enhance coloring and flavoring of food and food products. Numerous health benefits were reported for curcumin, including different pharmacological activities, such as anti-inflammatory, antioxidative, and chemopreventive properties. The safety and chemopreventive efficacy of curcumin in preclinical studies were previously reported [Hidaka et al., 2002; Martin-Cordero et al., 2003; Goel and Aggarwal, 2010; Lee et al., 2011; Qiao et al., 2013].

It was documented that curcumin possesses multiple anticancer effects. Curcumin inhibited proliferation and angiogenesis, induced apoptosis, and inhibited DNA topoisomerase II [Jobin et al., 1999; Plummer et al., 1999; Chidambara Murthy et al., 2013; Masuelli et al., 2013; Yallapu et al., 2013; Zhang et al., 2013]. Various mechanisms have been proposed as mediators of curcumin effects. Among implicated theories are, inhibition of activator protein 1 (AP-1), c-Jun N-terminal kinase, Akt, and NF-κB signaling pathways [Plummer et al., 1999; Hartojo et al., 2010]. The inhibition of NF κB activation was reported to be mediated through suppressing the IκB kinases (IKKs) [Jobin et al., 1999; Plummer et al., 1999]. It was also reported that curcumin modulates an array of cellular processes such as inhibition of epidermal growth factor (EGF) receptor kinase C activity, down-regulation of cyclooxygenase-2, and matrix metalloproteinase-2 expressions, down-regulation of DNA methyltransferase 1 protein kinase C activity and production of reactive oxygen species [Kakar and Roy, 1994; Lu et al., 1994; Brouet and Ohshima, 1995; Ramachandran et al., 2005; Yu et al., 2013]. Results on tumor models of animals suggested that curcumin is a potent inhibitor of mutagenesis and chemically-induced carcinogenesis [Subramanian et al., 1994; Kawamori et al., 1999].

Here, the effect of curcumin on human pancreatic cancer cell growth and proliferation were investigated. Two pancreatic adenocarcinoma cell lines with different differentiation stages and different COX-2 expression levels were used. Our results showed that curcumin inhibited cellular proliferation and induced apoptosis, mainly through up-regulation of the extrinsic apoptotic pathway, in both cell lines. The high COX-2 expressing BxPC-3 cells were more sensitive to the growth inhibitory and apoptotic effects of curcumin. Moreover, we showed that curcumin effect was mediated through activation of TNFR, CASP 8, CASP3, BID, BAX, and down-regulation of NFκB, NDRG 1, and BCL2L10 genes.

MATERIALS AND METHODS

CELL LINES AND MATERIALS

BxPC-3 and MiaPaCa-2 human pancreatic cancer cell lines were used in our study. Cells were cultures and treated as previously described in our previous study [Youns et al., 2009b]. Briefly, BxPC-3 and MiaPaCa-2 cells were maintained in RPMI 1640, and DMEM (Invitrogen, Carlsbad, CA) medium, respectively, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS) (Invitrogen). Both cell lines were tested with the Venor Mycoplasma detection kit (Minerva Biolabs, Berlin, Germany) and proved to be free of mycoplasma as we mentioned previously [Alhamdani et al., 2012]. The purity of curcumin powder (Sigma–Aldrich, Hamburg, Germany) was ≥98%.

EFFECT OF CURCUMIN ON CELLULAR PROLIFERATION AS DETERMINED BY SRB ASSAY

To investigate the effect of curcumin on cellular proliferation, the sulforhodamine B (SRB) assay was used (Sigma–Aldrich) as previously described [Youns et al., 2009b]. Briefly, cells were grown with or without increasing concentrations of curcumin for 24, 48, and 72 h. Medium was aspirated, and 10% TCA was added. After fixing cells, 0.4% (w/v) and addition of SRB, cells were incubated at room temperature for 30 min. After washing unbound dyes, tris-base solution (10 mM) was added to dissolve bound SRB dyes. Absorbance intensity was measured using a precision microplate reader at 570 and 650 nm (Molecular Devices, Sunnyvale, CA).

APOPTOTIC EFFECT OF CURCUMIN AS DETERMINED BY FLOW CYTOMETRY

Flow cytometry experiment has been carried out as previously described [Youns et al., 2009b]. After curcumin treatment for 24, 48, and 72 h, cells were harvested and washed with phosphate-buffered saline (PBS, Invitrogen) and resuspended in hypotonic fluorochrome solution (50 μg/ml PI, 0.1% w/v sod. citrate, and 0.1% v/v Triton X-100). The cells were incubated at 4°C overnight before FACS analysis. The PI fluorescence of individual nuclei was measured using FACS-Calibur (BD Biosciences, Heidelberg, Germany). Data analysis was carried out with the CellQuess Pro V5.2.1 software (BD Biosciences).

APOPTOTIC EFFECT OF CURCUMIN AS DETERMINED BY MEASURING HISTONE ASSOCIATED DNA FRAGMENTS

In order to further confirm the apoptotic effect of curcumin in pancreatic cancer cells, the Cell Death Detection ELISA kit^{plus} assay (Roche Diagnostics GmbH, Germany) was used according to the manufacturer's protocol. Briefly, cells were treated with various concentrations of curcumin. Twenty-four hours later, the cytoplasm of control and treatment groups was transferred to a streptavidin coated 96 well plate. After incubation with biotinylated histone antibody and peroxidase-tagged mouse anti-human DNA, the absorbance was measured using a precision microplate reader (Molecular Devices) at 405 nm. Results were expressed as the ratio of optical density of treated sample to that of the untreated control.

QUANTITATIVE DETERMINATION OF PROSTAGLANDIN E2

In order to investigate the possible inhibitory effect of curcumin on prostaglandin E2 (PGE2) production, the PGE2 Monoclonal EIA Kit (Cayman Chemicals, Michigan) was used as described in our previous work [Mulyaningsih et al., 2010]. Briefly, 24 h after curcumin treatment, BxPC-3 cells were stimulated with arachidonic acid. Prostaglandin E2 levels were estimated by a competitive enzyme immunoassay and calculated relative to DMSO treated control.

TRANSCRIPT PROFILING USING MICROARRAY TECHNIQUES

Cells were either treated with 25 μM curcumin or with solvent (control) for 48 h. RNA extraction, purification, and microarray processing has been carried out as previously described [Youns et al., 2009b, 2011]. Briefly, total-RNA was isolated and the RNA integrity was checked on an Agilent Bioanalyser 2100 (Agilent Technologies, Palo Alto). Fluorescently labelled cDNA samples were then prepared and a well-defined subset of some 7,000 genes highly

associated with the occurrence of pancreatic carcinoma was spotted on our microarray chips. Spotted genes included those related to apoptotis, cell cycle, growth factors, angiogenic, and housekeeping genes. After hybridization, fluorescence signals detection was done with a confocal ScanArray 5000 scanner (Packard Bioscience, USA). GenePix Pro 6 (Axon Instruments, Union City, USA) software was used for signal analysis.

QUANTITATIVE REAL-TIME RT-PCR ASSAYS

The same RNA samples used for microarray experiment were used for quantitative real-time (RT-PCR) experiment using QuantiTect SYBR Green PCR Kit (Qiagen) and Light Cycler 480 instrument (Roche Diagnostics). As previously described [Youns et al., 2009b, 2011], PCR was done by initial denaturation at 95°C, strand separation at 94°C, annealing at 56°C, and extension at 72°C. Target genes expression levels were normalized relative to β-actin internal control.

IDENTIFICATION OF CANONICAL PATHWAYS AND NETWORKS OF INTERACTING GENES

Here, we used the ingenuity pathway analysis software (IPA) (Mountain View, USA) to identify networks of genes and other canonical pathways modulated after curcumin treatment of pancreatic cancer cells. Using IPA tool, the microarray result genes were grouped and classified based on their biological functions and pathways involved in their effects. A cut-off value of 1.5 was used to define genes. Only genes with expression level equal to or more than 1.5 were included in our pathways and networks.

DATA ANALYSIS

Microarray quality, normalization, and correspondence cluster analysis were performed as described in our previous studies [Youns et al., 2009b, 2011].

RESULTS

CURCUMIN INHIBITS CELLULAR VIABILITY AND PROLIFERATION OF PANCREATIC CANCER CELL LINES

Curcumin has been previously shown to possess potent antiproliferative activity against many tumor types. In order to investigate the effect of curcumin on pancreatic cancer cell growth, BxPC-3 and MiaPaCa-2 cells were cultured and treated with gradient concentrations of curcumin for 24, 48, and 72 h. The rate of cellular proliferation was assessed using SRB assay. Curcumin showed growth inhibitory effects in a concentration and time-dependent manner (Fig. 1). With regard to differentiation stage and COX-2 expression, the highly COX-2 expressing and moderately differentiated BxPC-3 cells (Fig. 1A) were more sensitive to curcumin treatment compared to the poorly differentiated, non COX-2 expressing MiaPaCa-2 cells (Fig. 1B).

THE APOPTOTIC EFFECT OF CURCUMIN IS TIME AND DOSE-DEPENDENT

Apoptotic induction is an important mechanism of cancer chemoprevention [Khan et al., 2007]. To analyze the apoptotic effect of

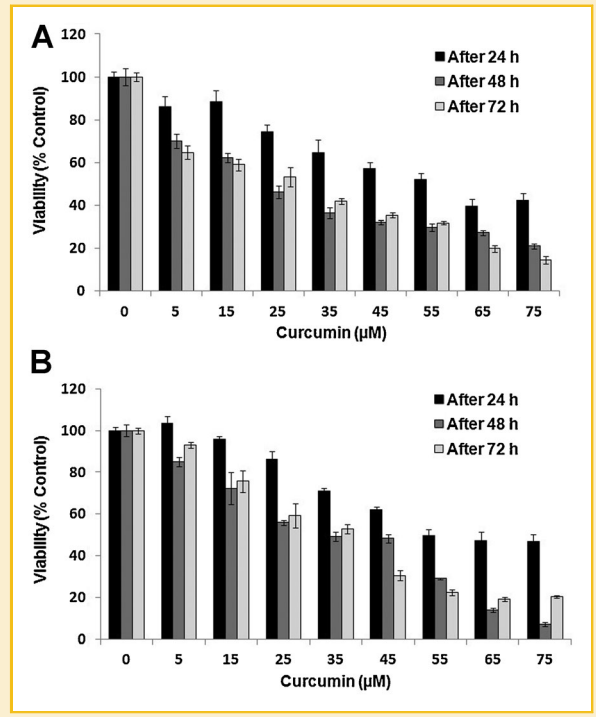

Fig. 1. Curcumin inhibits growth of BxPC-3 and MiaPaCa-2 pancreatic cancer cells. Cultures of exponentially growing BxPC-3 cells (A) and MiaPaCa-2 (B) were cultivated and treated with curcumin in 96-well plates for 1, 2, and 3 days. The SRB assay was performed to assess cellular proliferation. The data are represented as the mean value ± S.E.M.

curcumin in pancreatic cancer cells, flow cytometry analysis after 24, 48, and 72 h treatment with curcumin has been investigated. Our results showed that curcumin treatment increased the percentage of cells with hypo-diploid (sub-G1) peaks. The apoptotic induction was a concentration and time dependant (Fig. 2A). Curcumin induced apoptosis was more obvious with the highly COX-2 expressing cells (Fig. 2B (I)) compared to non COX-2 expressing ones (Fig. 2B (II)). The apoptotic effect of curcumin was further confirmed by evaluating cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death, where exposure of both cell lines to curcumin for 24 h, significantly increased the amount of apoptotic cells (Fig. 2C).

CURCUMIN INHIBITS PROSTAGLANDIN E2 PRODUCTION

In order to assess the inhibitory effect of curcumin on COX-2 enzyme activity via inhibition of PGE2 production, the PGE2 levels produced by BxPC-3 cells after curcumin treatment were determined. Our results showed that curcumin significantly inhibited PGE2 production in a dose dependant manner relative to untreated controls (Fig. 3).

IDENTIFICATION OF MOLECULAR TARGETS AND PATHWAYS INVOLVED IN THE GROWTH INHIBITORY EFFECT OF CURCUMIN ON PANCREATIC CANCER CELLS

For the identification of molecular targets, networks, and pathways mediating the growth inhibitory effect of curcumin; BxPC-3 and

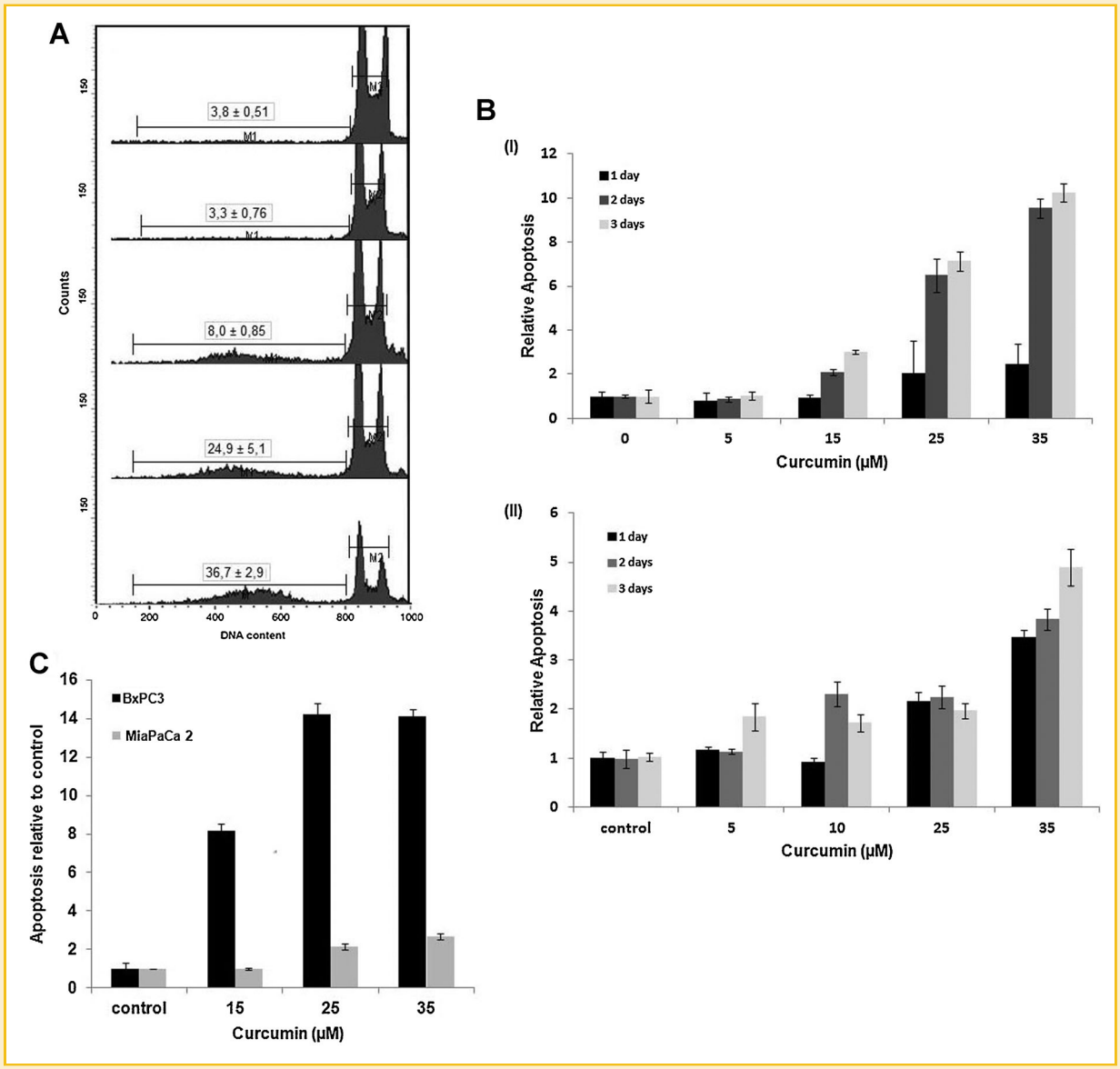

Fig. 2. The apoptotic effect of curcumin as shown by flow cytometry and by the Cell Death Detection ELISA kit, pancreatic cells were treated with gradient concentrations of curcumin and flow cytometry analysis were carried out as described in materials and methods part. A: Flow-cytometry histogram of BxPC-3 cells showing sub-G1 cells (the hallmark of apoptosis). B: Flow-cytometry results for effect of curcumin on both cell lines. C: Apoptotic effect induced by curcumin relative to untreated controls as measured by Cell Death Detection ELISA ^{plus} kit. Results are presented as the mean value ± S.E.M. of three independent experiments.

MiaPaCa-2 cells were treated with 25 μM curcumin or with DMSO. Forty-eight hours later, gene expression analysis has been carried out as previously described [Youns et al., 2009b, 2011]. Microarray results showed that curcumin treatment significantly up-regulated 176, 293 genes and down-regulated 190, 63 genes in BxPC-3 and MiaPaCa-2 cells, respectively ($P < 0.05$). Significantly regulated genes included those apoptotic, cell cycle, growth factors, and tumor suppressor genes.

VALIDATION OF MICROARRAY GENE EXPRESSION RESULTS WITH Rt-PCR

Expression of some significantly regulated genes after microarray data analysis was then analyzed by quantitative real-time RT-PCR, and normalized to ß-actin internal control. Rt-PCR results were matching those of microarray results (Fig. 4).

PATHWAY ANALYSIS AND CLASSIFICATION OF SIGNIFICANTLY REGULATED GENES

In order to get insight into canonical pathways and mechanisms involved in the modulatory effect of curcumin on pancreatic cancer cell growth, the IPA tool (www.ingenuity.com) was utilized as previously described [Youns et al., 2009b]. The top ten canonical pathways, regulated after curcumin treatment, included aryl hydrocarbon receptor signaling, p53 signaling, death receptor signaling, apoptosis signaling, and molecular mechanisms of cancer (Fig. 5). Additionally, connective tissue development, cell cycle,

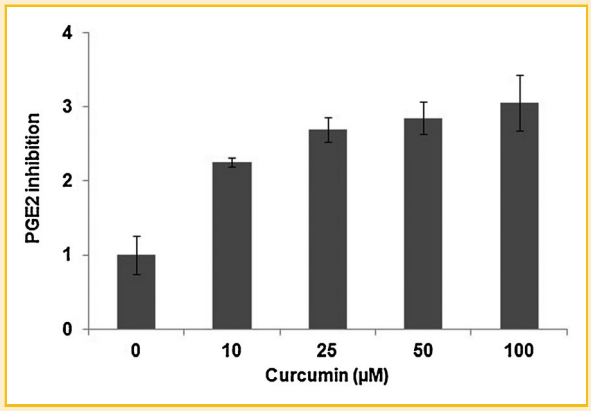

Fig. 3. Inhibition of prostaglandin E2 (PGE2) after curcumin treatment in BxPC-3 cells. Data shown are the mean value ± S.E.M. of three independent experiments.

cellular function, and gastrointestinal diseases were among the top ten regulated networks after curcumin treatment (data not shown). Biomarker analysis showed that 141 common biomarker genes were regulated in both cell lines after curcumin treatment (Fig. 6). Seventy-one unique genes were specifically regulated in BxPC-3 compared to 59 unique genes in case of MiaPaCa-2 cells (Fig. 7). It was obvious that the extrinsic apoptotic pathway was specifically modulated after curcumin treatment. Many genes involved in extrinsic apoptotic pathway were significantly altered in their expression following curcumin treatment, either up-regulated such as TNFR, CASP 8, CASP 3, BID, BAD genes, or down-regulated such as NFκB and BCL2L10 genes (Fig. 8).

DISCUSSION

The deleterious side effects of current clinically used chemotherapeutic agents augment cancer mortality and morbidity and

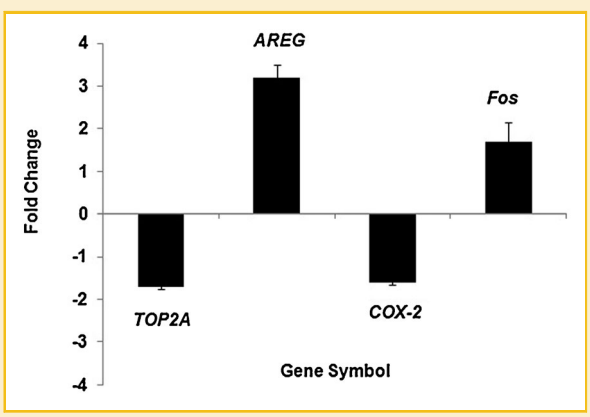

Fig. 4. Rt-PCR verification of microarray data. The same RNA samples from BxPC-3 cells used for microarray experiment were used for Rt-PCR validation of results. Expression changes were expressed relative to internal control (β actin). Data represented here are mean ± S.D. of three independent experiments.

underscore a desperate need for safer therapies. The long-term consumption of natural products present in fruits and spices, with proven safety, favoring their use in cancer chemoprevention [Park et al., 2013]. To date, natural products and their derivatives comprising many approved anticancer drug candidates [Youns et al., 2009a,b, 2010].

Numerous natural products have been previously investigated for their potential as cancer chemo-preventative agents. Among these agents, curcumin is a highly promising drug candidate.

It has previously shown that curcumin down-regulates the Notch signaling pathway, inhibits cell growth, and induces apoptosis in BxPC-3 and PANC-1 pancreatic cancer cells [Wang et al., 2006; Stan et al., 2010]. Additionally, curcumin was reported to upregulate the expression of miRNA-22 and down-regulates miRNA-199a, miRNA-27a, miRNA-20a, and miRNA-17-5p [Sun et al., 2008; Gandhy et al., 2012]. It was reported that up-regulation of miRN-22 was associated with down-regulation of two of its target genes, the transcription factor SP1, and the estrogen receptor 1 protein (ESR1), which is also expressed by pancreatic cancer cells [Sun et al., 2008]. As a combination therapy, curcumin has been reported to sensitize pancreatic cancer cells to gemcitabine [Kunnumakkara et al., 2007; Lev-Ari et al., 2007], paclitaxel [Wang et al., 1999], celecoxib [Lev-Ari et al., 2005], garcinol [Parasramka and Gupta, 2012], aspirin and sulforaphane [Thakkar et al., 2013]. It was concluded that curcumin (1 g/kg, once daily by mouth) is able to potentiate the anticancer activity of gemcitabine (25 mg/kg, twice weekly, intraperitoneal injection), and the effect was mediated through inhibiting cellular proliferation, angiogenesis and NF-kB-regulated gene products (cyclin D1, Bcl-2, Bcl-xL, c-myc, COX-2, and survivin) in an orthotopic animal model with human pancreatic cancer cells (MiaPaCa-2) implanted in the pancreas of athymic nude mice [Kunnumakkara et al., 2007]. The synergistic effect of curcumin with omega-3 fatty acids in pancreatic cancer xenograft model of BxPC-3 has been reported through down-regulation of proinflammatory proteins COX-2, 5-LOX, iNOS, and up-regulation of cyclin-dependent kinase inhibitor p21 [Swamy et al., 2008].

Curcumin has been shown to inhibit constitutive STAT3 phosphorylation in human pancreatic cancer cell lines and down-regulates survivin/BIRC5 gene expression [Glienke et al., 2010]. Recently, it was shown that Curcumin is able to reverses the epithelial-mesenchymal transition of pancreatic cancer cell line (panc1) by inhibiting the Hedgehog signaling pathway [Sun et al., 2013].

In spite of the great efforts that have been done, the exact mechanism of action of curcumin on pancreatic cancer cell growth is still unknown and needs to be further elucidated. The powerful anticancer activity and the higher therapeutic index of curcumin encourage us to further investigate the molecular pathways modulating its effect on two pancreatic cancer cell lines with different differentiation stages and different COX-2 expression levels. Moreover, we carried out a microarray gene expression analysis study to point out the global genes, pathways and molecular targets modulating the effect of curcumin on BxPC-3 and MiaPaCa-2 pancreatic cancer cell lines. Our gene expression analysis results could be used as starting point for the generation of hypotheses on candidate genes and for a more detailed dissection of the functional

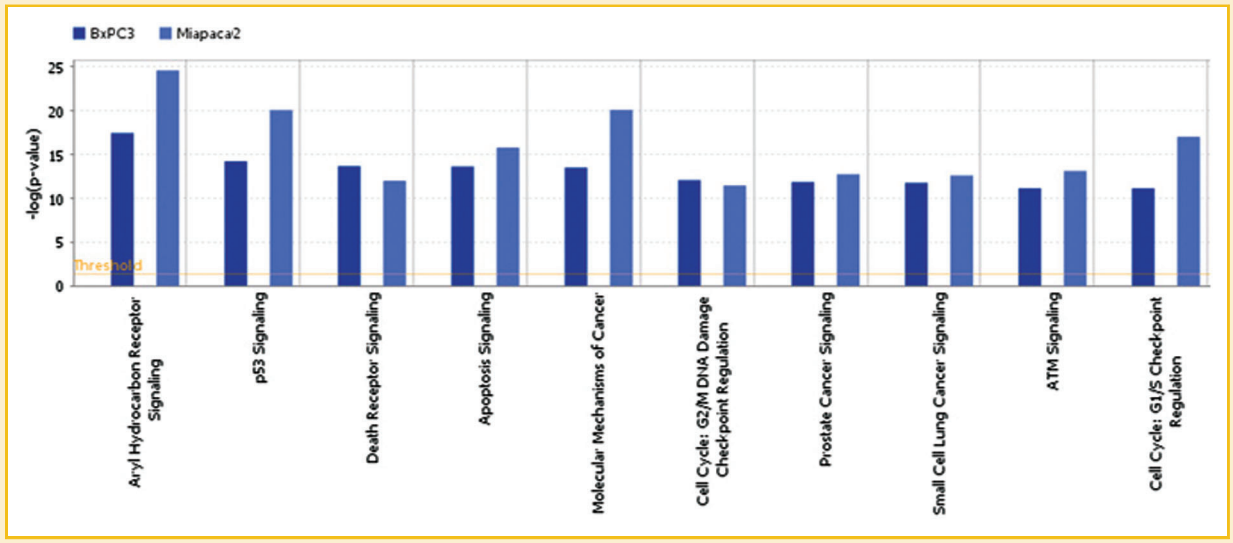

Fig. 5. Top 10 canonical pathways significantly regulated in pancreatic cancer cells after curcumin treatment.

role of individual genes for the activity of curcumin in pancreatic cancer cells. Additionally, our results provide new insights into curcumin-related signaling activities, which may facilitate the development of curcumin-based anticancer strategies and/or combination therapies.

Here, the anti-proliferative effect of curcumin on pancreatic cancer cells was investigated. Two pancreatic cancer cell lines with different differentiation stages and different COX-2 expression levels were used in our study in order to monitor whether the inhibitory effect of curcumin on pancreatic cancer cell growth is mediated only through Cox-2 effect or other molecular targets could be involved. BxPC-3 cells with moderate differentiation and high COX-*2* expression and the poorly differentiated MiaPaCa-2 cells with no COX-*2* expression were treated with and without gradient concentrations of curcumin. The cytotoxic effect of curcumin was monitored by SRB cell proliferation assay. The apoptotic effect was investigated by flow cytometry and confirmed by evaluating the amount of histone-associated DNA fragments released into cytoplasm after curcumin-induced cell death.

Results presented here, showed that curcumin inhibited cellular viability and induced apoptosis of pancreatic cancer cells. The high COX-2 expressing BxPC-3 cells were more sensitive to curcumin treatment where curcumin down-regulated COX-2 expression by threefolds compared to untreated control. Our results suggested that COX-2 inhibition is among molecular mechanisms modulating curcumin effect on pancreatic cancer cells.

Phase I clinical trials have shown that curcumin is safe even at high doses of 12 g/day for 3 months in humans without showing dose-limiting toxicities [Anand et al., 2007; Goel et al., 2008]. Oral administration of 4–8 g of curcumin results in peak plasma levels of 0.41–1.75 μmol/L. Although free plasma levels may not reflect tissue levels, in vitro studies suggest that microgram levels are required for activity in pancreatic cancer [Goel et al., 2008; Stan et al., 2010].

It is well documented, that a compound that is able to target different signaling pathways in tumor cells is the drug of choice to overcome multiple signaling pathways used by cancer cells to escape host defense mechanisms [Ramachandran et al., 2005]. Here, we showed that curcumin inhibited pancreatic cancer cell growth through regulating multiple signaling pathways suggesting its valuable role as anticancer agent.

Activation of the intrinsic and/or extrinsic apoptotic signaling pathways in cancer cells is involved in most anticancer strategies [Makin and Dive, 2001; Fulda and Debatin, 2004, 2006]. Morphological characters of apoptosis include cell shrinkage, nuclear DNA fragmentation and membrane blebbing [Makin and Dive, 2001]. Our results confirmed that curcumin induced apoptosis in pancreatic cancer cell lines, through induction of sub-G1 phase (Fig. 2A,B), increasing the number of histone-associated DNA fragments released into the cytoplasm after cell death (Fig. 2C) and up-regulation of important apoptotic and proapoptotic genes including TNF, TNFR, BAX, BID, BIM and CASP3, and CASP8 [Fulda and Debatin, 2006]. It was obvious that the apoptosis induction after curcumin treatment was mediated through involvement of the extrinsic apoptotic pathway starting from activation of death receptor TNFR and ending with activation of CASP3 and hence apoptosis (Fig. 8).

Among significantly regulated genes after curcumin treatment of both pancreatic cancer cell lines is the X chromosome-linked IAP (XIAP). Inhibitors of apoptosis protein (IAPs) are cellular guardians that are important for controlling prodeath proteins such as different caspases, Smac, and HtrA2. Previous studies have shown that XIAP can directly bind to and inhibit caspases, proteases that execute apoptotic cell death. This protein also inhibits at least two members of the caspase family of cell-death proteases, caspase-3, and caspase-7 [Nakatani et al., 2013; Zhuang et al., 2013]. Down-regulation of XIAP in both cell lines could explain, in part, the apoptotic effect induced by curcumin on human pancreatic cancer cells.

The finding, that curcumin induced apoptosis in pancreatic cancer cells in a time and dose dependent manner, is in agreement with a previous study in human leukemia cells [Kuo et al., 1996].

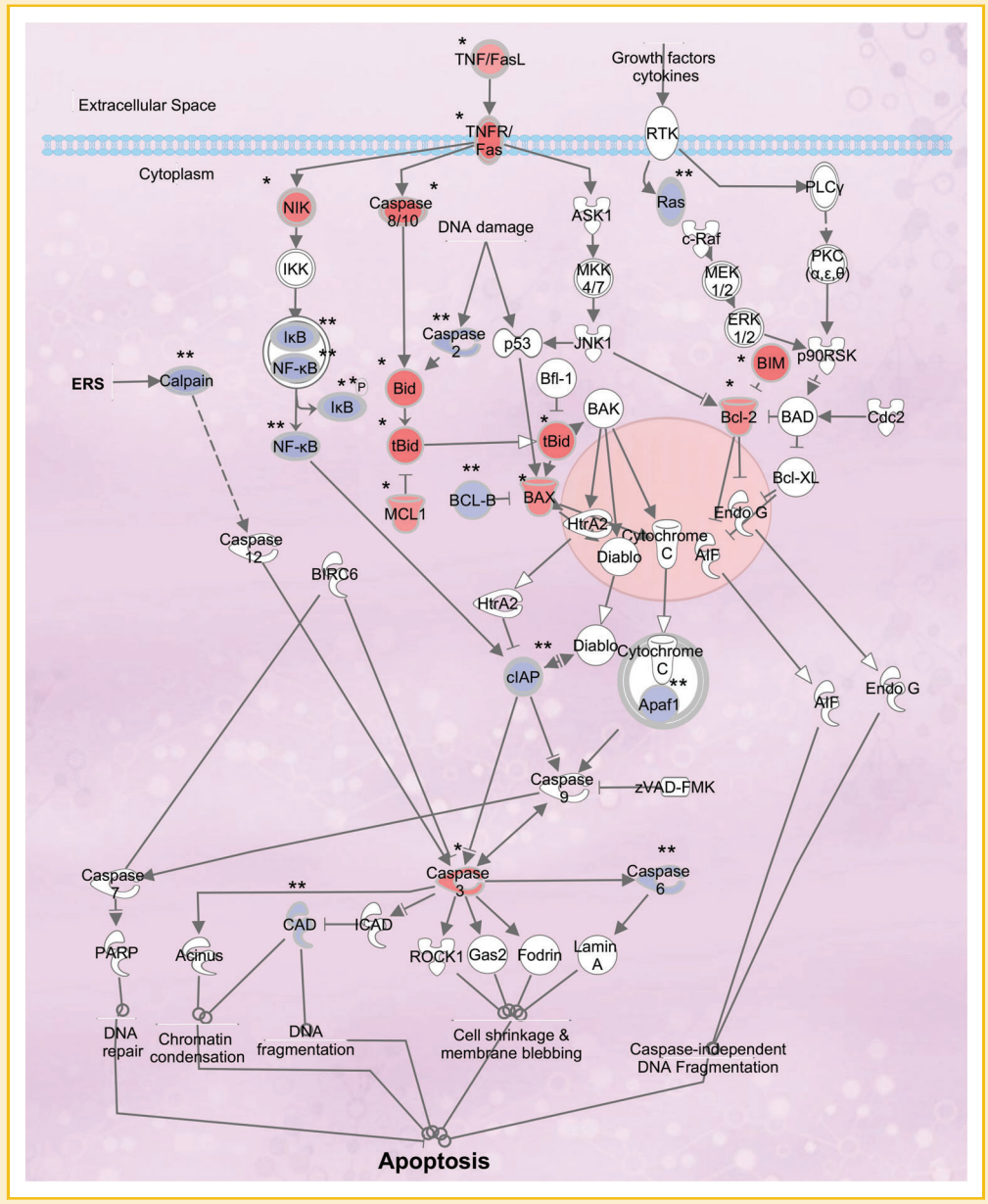

Fig. 6. Apoptotic signaling pathway after curcumin treatment of BxPC-3 cell line, Genes with red color (single asterisk in case of black and white image) indicates up-regulated genes, and genes with blue color (double asterisks in case of black and white image) indicate down-regulated genes. (Image was generated from IPA software using path designer tool).

Additionally, results presented here are in complete accordance with a very recent study reported that curcumin induces growth arrest via induction of apoptosis, and the apoptotic effect was mediated through activation of BAX in human malignant testicular germ cells [Cort et al., 2013]. The decrease in BCL2 expression after curcumin treatment were previously reported on different tumor types such as small cell lung cancer, myeloid leukemia cells, and human tongue squamous cell carcinoma [Ip et al., 2011; Rao et al., 2011; Yang et al., 2012; Cort et al., 2013].

It is well known that topoisomerase II-inhibitors, including etoposide and doxorubicin are widely used anticancer drugs [Berger et al., 1996; Schmidt et al., 2012]. Previous results showed that curcumin is a powerful DNA topoisomerase II poison [Martin-Cordero et al., 2003; Ketron et al., 2013]. Here, we showed that curcumin inhibited m-RNA transcription of topoisomerase II (TOP2A) enzyme in BxPC3 cells and that could be responsible, in part, for the growth inhibitory effect of curcumin on BxPC-3.

Cyclooxygenases (COXs) are rate-limiting enzymes involved in the conversion of phospholipase A2-mobilized arachidonic acid into prostaglandins and thromboxanes. COX-2 is a key mediator of inflammation during both physiologic and pathologic responses to endogenous stimuli and infectious agents. Its over-expression has

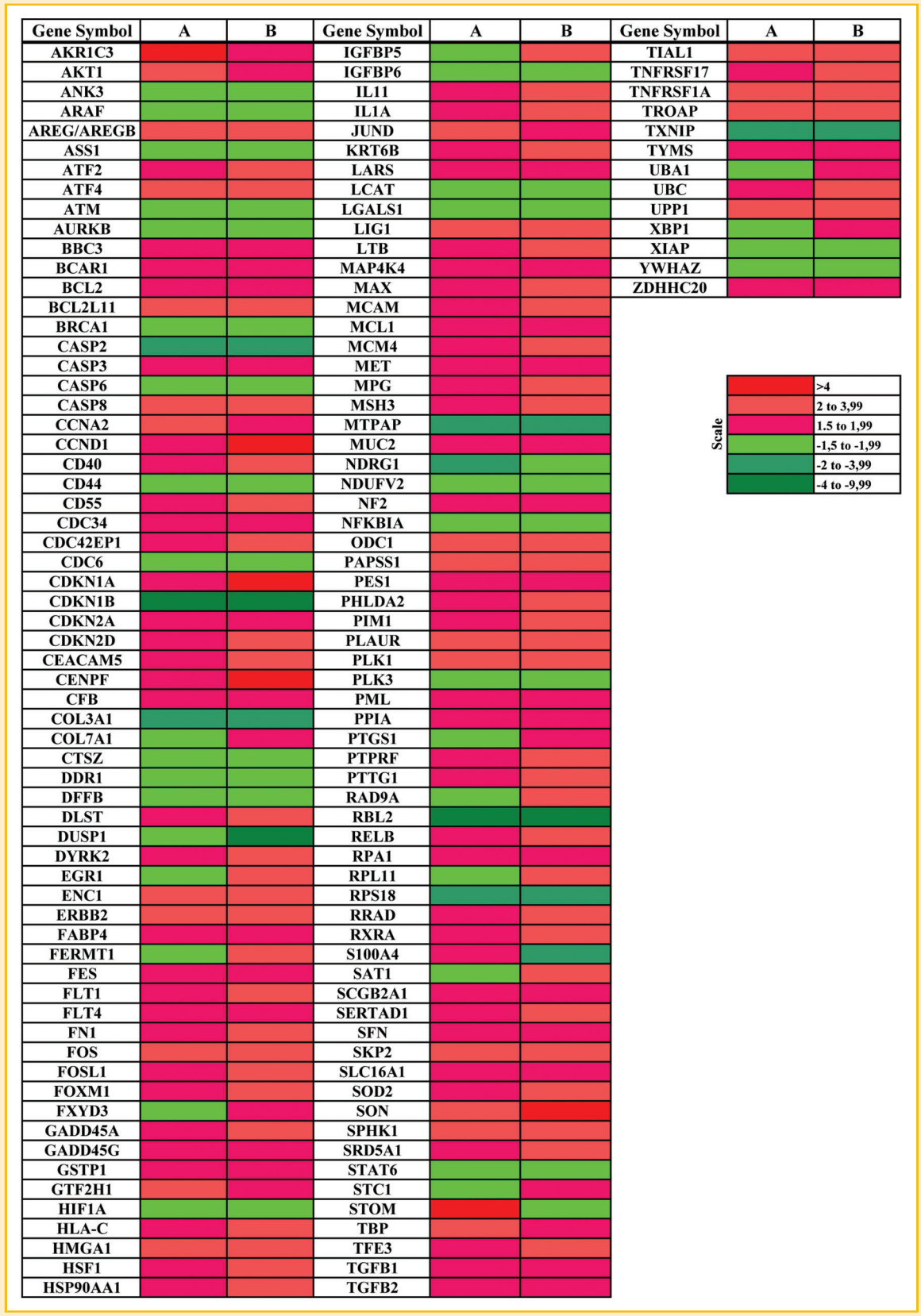

Fig. 7. Common genes that were significantly regulated after curcumin treatment in both BxPC-3 (A) and MiaPaCa-2 (B) cells (Common Biomarkers between both treated cell lines).

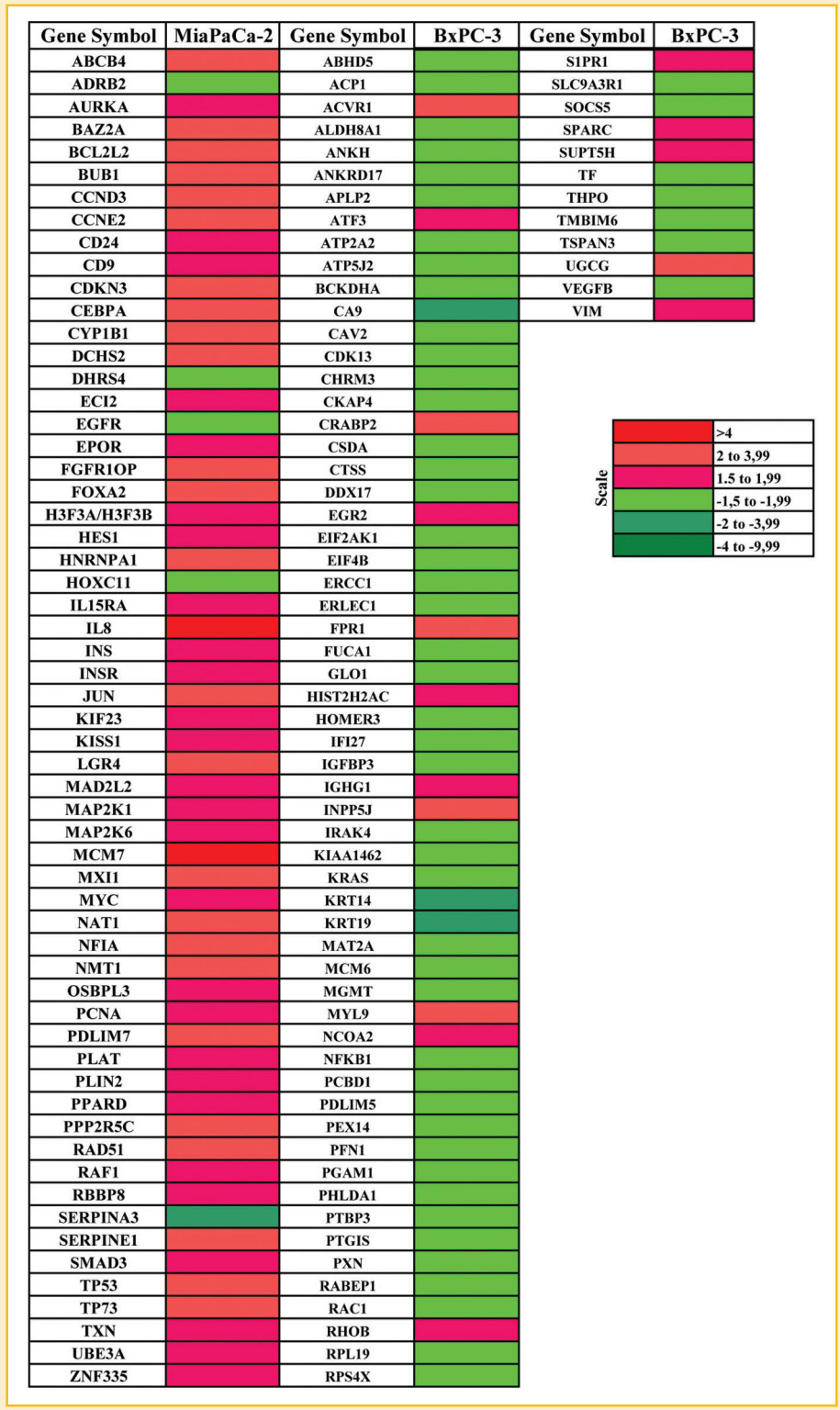

Fig. 8. Genes specifically regulated in each cell line after curcumin treatment of BxPC-3 and MiaPaCa-2 cells (Unique biomarkers of each cell line).

been detected in different cancers, including that of the pancreas [Fujimura et al., 2006; Stasinopoulos et al., 2007]. At present, there are clinical reports of the effectiveness of combining selective COX-2 inhibitors with chemotherapy to treat digestive tract tumors [Fujimura et al., 2006]. Our results are in complete agreement with a very recent study, showing that curcumin attenuates COX-2 expression via inhibition of the NF-κB pathway [Hu et al., 2013], that was also confirmed in our study through down-regulation of

COX-2, NF-κB genes, and inhibition of PGE2 release after curcumin treatment.

Previous studies reported that among causes of pancreatic cancer resistance to apoptosis is the activation of nuclear transcription factor-κB (NF-κB) [Wang et al., 1999]. Other reports showed that the higher expression of NF-κB regulated gene products, including cyclooxygenase-2, IκB, prostaglandin E2, is responsible for the proliferative and metastatic potential of pancreatic cancer [Yip-Schneider et al., 2000; Li et al., 2004b]. Therefore, it was concluded that NF-κB and COX-2 inhibitors could be important targets for pancreatic carcinoma treatment [Li et al., 2004b; Park et al., 2013]. Our results clearly demonstrated that curcumin decreased the expression of NF-κB and its regulated gene products, cyclooxygenase-2.

N-myc down-stream regulated 1 gene (NDRG1) is a stress responsive protein and has been reported as a marker of tumor progression and enhancer of cellular differentiation. The protein encoded by this gene is a cytoplasmic protein involved in stress responses, hormone responses, cell growth, and differentiation [Ellen et al., 2008]. A recent study reported that digoxin inhibited tumor cell growth through down-regulation of NDRG1 [Wei et al., 2013]. Our gene expression results provide clear evidence that NDRG1 was among significantly down-regulated genes in both cell lines after curcumin treatment suggesting that NDRG1 inhibition plays a role in the chemopreventive effect of curcumin on pancreatic cancer cells.

CONCLUSION

The present study clearly demonstrated that curcumin inhibited pancreatic cancer cellular proliferation and up-regulated the extrinsic apoptotic pathway regardless of cell type. Curcumin inhibitory effect was mainly mediated through activation of TNFR, CASP 8, CASP3, BID, BAX, and down-regulation of NFκB, NDRG1, and BCL2L10 genes. Curcumin-induced growth arrest was dependent, in part, on COX-2 expression level being more effective with highly COX-2 expressing cells. Moreover, it was shown that curcumin is able to regulate multiple signaling pathways in human pancreatic cancer cells. Our results and the expression data presented here could be used to as a tool for further development of curcumin-based anticancer strategies and/or combination therapies.

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