Tropical Journal of Pharmaceutical Research November 2019; 18 (11): 2355-2361
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v18i11.18

Original Research Article

Curcumin inhibits gastric cancer growth via down-regulation of zinc finger protein, ZNF139

Heng Xu[1], Wenhui Yu[2], Wenbo Yu[3], Meijun Zhang[2], Yingying Ma[2], Duoxiang Wu[2], Qifan Zhang[4]*

[1]Department of Oncology, The Forth Clinical Medical College, Harbin Medical University, Harbin 150081, [2]Department of Vascular Surgery, The First Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine, Harbin, Heilongjiang 150040, [3]Department of Clinical Laboratory, Harbin First Hospital, Harbin, Heilongjiang 150070, [4]Department of Oncology Surgery, Harbin Medical University Tumor Hospital Harbin, Heilongjiang 150081, China

For correspondence: **Email: axinlang666@sina.com*

Sent for review: 20 May 2019 *Revised accepted: 26 October 2019*

Abstract

Purpose: *To investigate the effect of curcumin on gastric cancer cell proliferation and the mechanism of action involved.*
Methods: *Viability of gastric cells following curcumin treatment was determined by 3 (4,5 dimethyl thiazol 2 yl) 2,5 diphenyl 2H tetrazolium bromide (MTT) assay. Flow cytometry was used for the assessment of apoptosis induction in SGC 7901 cells. Reverse transcriptase polymerase chain reaction (RT-PCR) and western blotting assay were used for the analysis of Znf139, survivin and Bcl 2 protein expressions.*
Results: *The results showed that curcumin treatment reduced the viability of gastric cancer cell line SGC 7901 cells at 30 μM concentration to 29.67 % after 48 h compared to 99.78 % for control culture. Apoptotic cell population increased significantly ($p < 0.05$) following treatment with curcumin. Zinc finger protein-139 mRNA and protein expression decreased significantly ($p < 0.05$) on treatment with curcumin. Furthermore, curcumin suppressed the levels of B cell lymphoma 2 (Bcl 2) and survivin protein. In the mice model of gastric cancer, treatment with 50 mg/kg dose of curcumin inhibited tumor growth and development significantly, compared to the untreated group ($p < 0.05$).*
Conclusion: *The results demonstrate that curcumin treatment inhibits gastric cancer cell proliferation via down-regulation of zinc finger protein-139. It also suppresses tumor growth in mice. Therefore, curcumin is a promising gastric cancer inhibitor and should be further investigated for the management of gastric cancer.*

Keywords: *Curcumin, Finger protein, Survivin, Gastric cancer, Inflammation, Anti-oxidant*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Gastric cancer is one of the most commonly diagnosed carcinomas throughout the world and ranks second cause of deaths associated with cancer [1]. Cancer statistics has revealed that in 2008 there were 990,000 new cases of gastric cancer detected and 740, 000 deaths were caused by gastric cancer [2]. The data showed 8% increase in new cases and 10% rise in the

deaths because of gastric cancer compared to the previous record [2]. Gastric cancer incidence rate has been found to be higher in southern America, Eastern Europe and Asia whereas it is rarely detected in North America and almost throughout Africa [3]. At present gastric cancer treatment strategy makes use of both chemotherapy as well as surgical resection, however, despite advancement in technology the rate of 5 year survival for patients is less than 20% [4]. Thus, there is urgent need for the development of efficient treatment strategy for gastric cancer because of its most lethal nature.

Zinc finger proteins are distributed widely in the cells of eukaryotic organisms where they play an important role in maintaining various cellular functions [5]. Studies have shown that some zinc finger proteins are involved in the development of tumor and its metastasis to distant organs [6,7]. In the development of urinary bladder cancer the zinc finger protein ZNF165 is involved where as in colorectal and ovarian cancers ZNF217 has been found to play vital role [6,7]. Another member of the family ZNF139 which contains transcription factor has been found to be linked to the development of gastric cancer [8,9].

Natural products isolated from various sources like plants, fungi, insects, etc. have shown significant anti-proliferative activity against several types of cancer cells [10]. The advantage of using natural products for the development of cancer treatment strategy is that they do not induce any harmful effect in the body [10]. Curcumin because of various pharmacological activities and with almost no side effects has been used in traditional medicine over long times [11]. Studies have revealed that curcumin acts as a potent candidate for the treatment of infection, inhibition of inflammation, as anti-oxidant, lipid reducing and anti-fibrotic agent [11-13]. Moreover, curcumin treatment prevents myocardial injury and inhibits tumor growth [11-13]. Inhibition of inflammation related gene expression by curcumin plays a vital role in the protection of myocardial injury through NF-κB pathway [14]. In the present study the effect of curcumin on the viability of gastric cancer cells and development of gastric cancer in mice model was investigated. The study aimed to investigate the inhibitory effect ofcurcumin on gastric cancer cell proliferation *in vitro* and *in vivo*.

EXPERIMENTAL

Cell lines and culture

SGC-7901 andMKN28 cell lines were obtained from the Cell Bank belonging to the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Culture of the cells was performed in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) mixed with 10% FCS at 37˚C under5% CO_2 and 95% air. The medium also contained penicillin (10,000 units) and streptomycin (10 mg).

MTT assay. SGC-7901 andMKN28 cells were seeded into the96-well microtiter plates at $3x10^6$cells/mL concentration and incubated for 12 h. The cells were then transferred into a fresh mediummixed with 5, 10, 15, 20, 25 and 30 µM concentrations of curcumin. Following 48 h of exposure, 10 µL volume of MTT solution was put into each of the well and cells were incubated for additional 4 h. DMSO (100 µL) was then added to each well for dissolving any formazen crystal formed and the absorbance measurements were recorded three times for each well at 572 nm using a microplate reader.

Assessment of apoptosis

SGC-7901 cells at 2 x 10^5cells/mL were exposed for 48 h to 10, 20, and 30 µM concentrations of curcumin or DMSO alone (control). Then cells collected were subjected to two times washing with cold PBS and subsequently put into the binding buffer (200 µL). The cells were then treated for 20 min at room temperature with 5 µl each of Annexin V-FITC PI in the dark. Apoptosis induction in SGC-7901 cells was analysed using flow cytometry (from Quanta SC, Beckman Coulter).

Western blot analysis

SGC-7901 cells were exposed to 5, 10, 15, 20, 25 and 30 µM concentrations of curcumin for 48 h. The cells were treated with lysis buffer followed by IP (Beyotime Institute of Biotechnology) to obtain lysates. Centrifugation of the lysates was performed for 25 min at 12,000 x g to remove the cell debris to collect the supernatant which was subsequently boiled for 10 min with the sample buffer. The protein resolution from the concentrated supernatant was achieved by electrophoresis on 12 % sodium dodecylsulphate [SDS]-polyacrylamide gel. The proteins were subsequently transferred onto polyvinylidene fluoride (PVDF) membranes which was blocked on treatment with 3% (w/v) non-fat milk in TBST.

Incubation of the blots was carried out overnight with primary antibodies at 4˚C. The blots after washing were subjected to incubation with horseradish peroxidase-conjugated goat anti-

rabbit secondary antibodies for 2 h at room temperature. The blot visualization was performed using the ECL detection kit (Thermo, USA) in accordance with the manual protocol. The antibodies used were against ZNF139 (rabbit polyclonal anti-mouse; dilution 1:900; catalog number ab126124), Bcl-2 (monoclonal; dilution 1:900; catalog number ab32124), survivin (polyclonal; dilution 1:900; catalog number ab469) or β-actin (polyclonal; dilution 1:1000; catalog number ab8227).

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analysis

SGC-7901 cells were exposed to 5, 10, 15, 20, 25 and 30 μM concentrations of curcumin for 48 h. The RNA extraction from cells was performed by RNeasy Plus Mini kit (Qiagen, TX, USA) in accordance with the manual instructions. The 2 μg RNA samples were subjected to reverse transcription using the SuperScript Reverse transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) to obtain the complementary DNA (cDNA). The SYBR Premix Ex Taq II (Takara Bio, Inc., Otsu, Japan) on an ABI-7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was employed for carrying out the RT-qPCR. The level of gene expression was detected in relation to GAPDH taken as internal control. The PCR was performed by an initial 3 min incubation at 53˚C, then denaturation for 10 min at 92˚C and 48 cycles of 93˚C for 20 s and for 2 min at 62˚C.

Animals

Seven to eight-weeks old male BALB nude mice were supplied by the Center for Experimental Animals of Wuhan University (Wuhan, China). The mice were placed in autoclaved, microisolator cages with filtered tops in the animal house under filtered air at 25 ^{o}C and 55% humidity. All mice were provided free access to water and food *ad libitum*. All the procedures involving mice were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [15]. Animal study approval was obtained from the Committee on Animal Experimentation of Wuhan University Wuhan, China (no. WUW/16/0002).

Establishment of mouse model of gastric cancer

Sixty mice were separated randomly into six groups of 10 each, normal control, untreated, 10, 20, 25 and 30 mg/kg treatment groups. Initially few mice were injected with 5 x 10^5 SGC-7901 cells subcutaneously on the dorsal side and kept under sterilize conditions for 21 days. After the tumor size reached 1 cm diameter, the mice were sacrificed by cervical dislocation under pentobarbital sodium (6%) anaesthesia. The tumors were excised and then cut into sections of 1 x 1 x 1 mm dimensions which were then implanted into the mice belonging to untreated and three treatment groups (except normal control group) following pentobarbital sodium anaesthesia. The implantation procedure involved making a 5 - 10 mm incision in the abdomen of mice to carefully expose the wall of the stomach. Then tumor was implanted into the stomach through a cut made in the serosa of the greater curvature. The stomach was sealed using OB glue and the abdominal cavity was sutured using silk thread. After 1 h of tumor implantation the mice in the four treatment groups were administered 10, 20, 25 and 30 mg/kg doses of curcumin intragastrically. The mice were sacrificed on day 30 to excise the gastric tumors for determination of Bcl-2 and survivin expression. The tumor weight was measured and the body weight of all mice was recorded on alternate days during the study.

Statistical analysis

The data presented are the mean ± SD of three experiments carried out independently. Differences were evaluated statistically using Student's t-test or one-way analysis of variance (ANOVA). $P < 0.05$ was taken to represent statistically significant difference.

RESULTS

Curcumin reduced viability of SGC-7901 and MKN28 cells

The cells after 48 h of exposure to 5, 10, 15, 20, 25 and 30 μM concentrations of curcumin were subjected to MTT assay (Figure 1). Exposure of SGC-7901 and MKN28 cells to curcumin reduced cell viability in a concentration dependent manner. The viability of SGC-7901 cells was decreased to 92.78, 84.21, 71.66, 59.54, 37.22 and 29.67%, respectively on exposure to 5, 10, 15, 20, 25 and 30 μM concentrations of curcumin in comparison to 99.78% in control cultures. Exposure to 5, 10, 15, 20, 25 and 30 μM concentrations of curcumin decreased MKN28 cell viability to 93.41, 86.77, 74.12, 63.88, 41.42 and 32.59%, respectively in comparison to 100% in control cultures.

Curcumin exposure induced apoptosis in SGC-7901 cells

The cells were exposed to 10, 20, and 30 μM

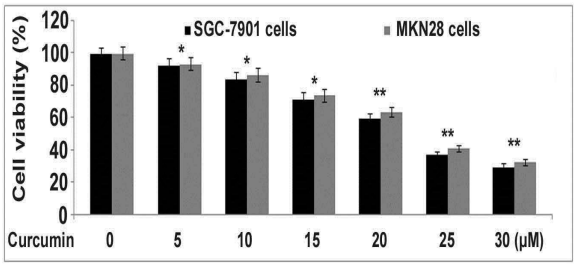

Figure 1: Reduction of SGC-7901 andMKN28 cell viability by curcumin. The cells were exposed to 5, 10, 15, 20, 25 and 30 μM concentrations of curcumin for 48 h and then evaluated by MTT assay. The presented values are the mean ± SD. *P<0.05 and **P<0.02 vs. cells exposed to culture medium alone

concentrations of curcumin for analysis of apoptosis by flow cytometry (Figure 2). The apoptotic cell population increased significantly (p<0.05) in SGC-7901 cell cultures on exposure to curcumin for 48 h. The population of apoptotic cells increased to 11.36, 18.54, 29.65 and 59.09%, respectively on exposure to 5, 10, 20, and 30 μM concentrations of curcumin. In control SGC-7901 cell cultures the percentage of apoptotic cells was 2.23 %.

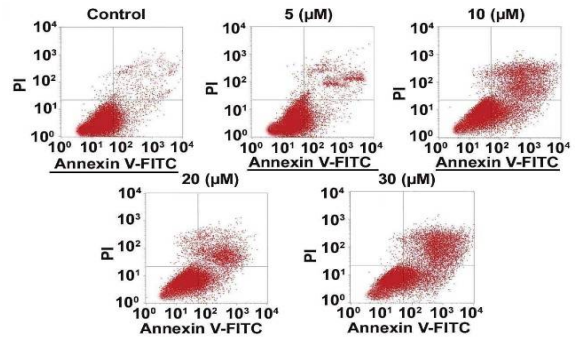

Figure 2: Analysis of apoptosis induced by curcumin in SGC-7901 cells. The cells exposed to 5, 10, 20, and 30 μM concentrations of curcumin were subjected to flow cytometry using Annexin V/PI staining

Curcumin down-regulated Znf-139 expression in gastric cancer cells

In SGC-7901 cells, changes in Znf-139 expression on exposure to 5, 10, 15, 20, 25 and 30 μM concentrations of curcumin were measured (Figure 3). A significant reduction in Znf-139 mRNA and protein expression was caused in SGC-7901 cells at 48 h of exposure to curcumin. The expression of Znf-139 mRNA and protein was decreased to a maximum level on exposure to 30 μM concentration of curcumin. These findings suggest that curcumin inhibits gastric cancer cell viability by down-regulating Znf-139 expression.

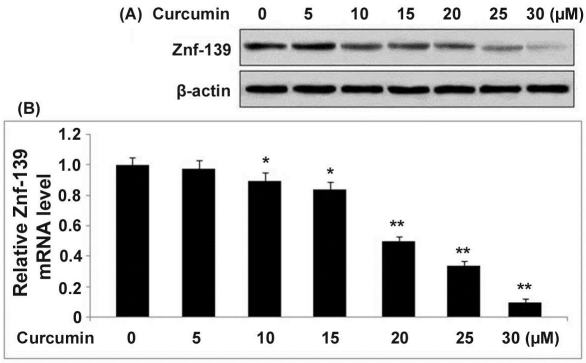

Figure 3: Curcumin down-regulated ZNF139 expression in SGC-7901 cells. The cells exposed for 48 h to 5, 10, 15, 20, 25 and 30 μM concentrations of curcumin were examined for expression of Znf139 (A) protein by western blotting and (B) mRNA by RT-PCR assays. *P < 0.02 and **p < 0.01 vs. control cells

Effect of curcumin on Bcl-2 and survivin

RT-PCR and western blotting were used to determine the effect of 10, 20, 25 and 30 μM concentrations of curcumin on the expression of Bcl-2 and survivin (Figure 4). Curcumin exposure led to a significant (p<0.05) decrease in the level of mRNA and protein corresponding to both Bcl-2 and survivin in SGC-7901 cells. The inhibitory effect of curcumin on the expression of both Bcl-2 and survivin was maximum at 30 μM concentration at 48 h.

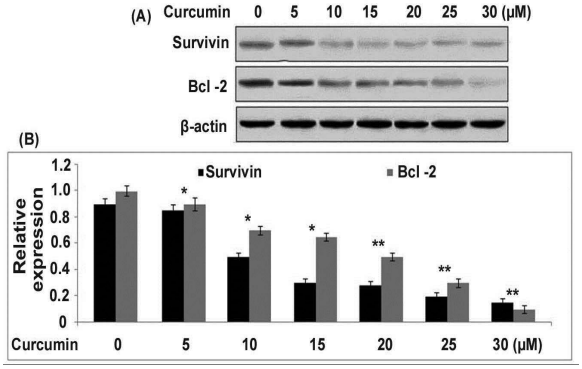

Figure 4: Bcl-2 and survivin expression inhibition by curcumin. SGC7901 cells were exposed to 10, 20, 25 and 30 μM concentrations of curcumin for 48 h to assess the level of Bcl-2 and survivin (A) proteinand (B) Mrna expression. *P<0.05 and **p<0.02 vs. cells exposed to culture medium alone

Curcumin treatment suppressed Bcl-2 and survivin in mice

The mice treated with 10, 20, 25 and 30 mg/kg doses of curcumin were sacrificed on day 30th of tumor implantation. The growth of tumor was reduced significantly in the mice treated with curcumin than those in the untreated group (Figure 5). In comparison to the untreated mice

metastasis of tumor was markedly inhibited in the curcumin treatment groups. The level of Bcl-2 and survivin in the tumor excised from the curcumin treated mice were significantly lower than those in the untreated group.

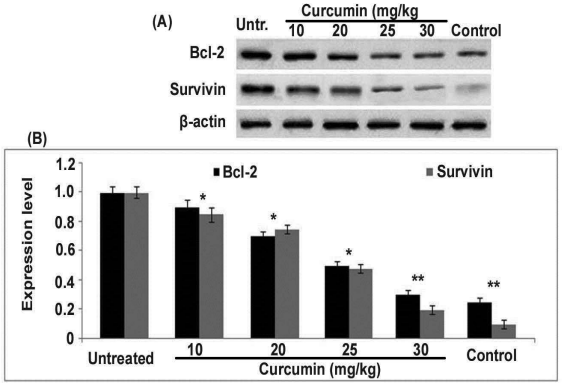

Figure 5: Inhibition of Bcl-2 and survivin by curcumin *in vivo*. In the mice model of gastric cancer level of Bcl-2 and survivin was assessed by (A) Western blot (B) RT-PCR assays on day 30th of tumor implantation. **P*<0.05 and ***p*<0.02 vs. untreated mice

DISCUSSION

The present study investigated the effect of curcumin on gastric cancer cells *in vitro* and on tumor growth *in vivo* in the mice model. The study demonstrated that curcumin reduced gastric cancer cell viability and caused apoptosis induction. Its exposure led to suppression of ZNF139, Bcl-2 and survivin expression in the gastric carcinoma cells. Treatment of the gastric cancer mice with curcumin inhibited tumor growth possibly through inhibition of Bcl-2 and survivin expression.

Gastric cancer is a commonly detected digestive tract carcinoma throughout the world with very high mortality rate [16]. Investigation of the etiology of gastric cancer is of immense significance for the development effective treatment strategy [16]. Apoptosis is a highly regulated and programmed natural process for elimination of undesired cells from the body of an organism [17,18]. Therefore, induction of apoptosis in carcinoma cells by therapeutic agents is considered to be of vital importance for cancer treatment. In the present study the effect of curcumin on gastric cancer cell viability and apoptotic changes were investigated. The results showed that curcumin exposure significantly led to reduction in the viability of the tested cancer cells. Flow cytometry revealed that in gastric cancer cell cultures addition of curcumin caused a marked enhancement in the proportion of apoptotic cells. These findings suggested that curcumin reduced viability of gastric carcinoma cells by induction of apoptosis. ZNF139 belongs to the family of zinc finger proteins and plays an important role in the regulation of transcription of various genes involved in tumor progression [19].

It has been reported that ZNF139 expression is markedly higher in the adenocarcinoma present at the junction between oesophagus and stomach junction [19]. ZNF139 is believed to promote gastric cancer by enhancing the level of fascin as well as ribonucleoproteins A2/B1 [20]. The expression of ZNF139 has been found to be markedly higher in gastric tumors which suggests its association with development and progression of gastric carcinoma [20]. ZNF139 is responsible for failure of many treatment strategies by increasing multidrug resistance protein 1 (MDR1) and MRP1 [21]. In the present study curcumin exposure significantly decreased ZNF139 mRNA and protein level in the gastric cancer cells. This provides an evidence that curcumin inhibits gastric carcinoma cell viability by down-regulating the expression of ZNF139.

The anti-apoptotic potential of carcinoma cells is enhanced by an increase in the expression of Bax through regulation of thiolredox state in the mitochondria [22]. Another factor inhibiting cell apoptosis by preventing activation of the caspases is the survivin [23]. In the present study effect of curcumin on Bcl-2 and survivin expression in the gastric cancer cells was examined. The results revealed that exposure of gastric cancer cells to curcumin significantly decreased the expression of both Bcl-2 and survivin. In the mice model of gastric carcinoma effect of curcumin was investigated on the in vivo tumor development.

CONCLUSION

The findings of this study indicate that curcumin inhibits gastric cancer growth *in vitro* as well *in vivo* in mice via down-regulation of ZNF139, Bcl-2 and survivin expression. It suppresses tumor development in mice by inhibiting Bcl-2 and survivin expression. Thus, curcumin has a significant potential for development as a treatment strategy for gastric cancer.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Qifan Zhang designed the study and wrote the paper. Heng Xu, Wenhui Yu, Wenbo Yu and Meijun Zhang performed the experimental work, Yingying Ma and Duoxiang W carried out the literature study and compiled the data. Heng Xu, Wenhui Yu, Yingying Ma and Duoxiang W performed literature survey, analyzed the data and compiled the data. The research article was thoroughly read by all the authors before commination for the consideration of publication.

Open Access

REFERENCES

1. *Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 13: 2893-2917.*
2. *Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 13: 69-90.*
3. *Hartgrink HH, Jansen EP, van Grieken NC, van de Velde CJ. Gastric cancer. Lancet 2009; 13: 477-490.*
4. *Wagner AD, Grothe W, Haerting J, Kleber G, Grothey A, Fleig WE. Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data. J Clin Oncol 2006; 24: 2903-2909.*
5. *Wei S, Zhang L, Zhou X, Du M, Jiang Z, Hausman GJ, Bergen WG, Zan L, Dodson MV. Emerging roles of zinc finger proteins in regulating adipogenesis. Cell Mol Life Sci 2013; 70: 4569 4584.*
6. *Singh PK, Srivastava AK, Dalela D, Rath SK, Goel MM, Bhatt ML. Frequent expression of zinc finger protein ZNF165 in human urinary bladder transitional cell carcinoma. Immunobiol. 2015; 220: 68 73.*
7. *Li P, Maines Bandiera S, Kuo WL, Guan Y, Sun Y, Hills M, Huang G, Collins CC, Leung PC, Gray JW, Auersperg N. Multiple roles of the candidate oncogene ZNF217 in ovarian epithelial neoplastic progression. Int J Cancer 2007; 120: 1863 1873.*
8. *Li Y, Tan BB, Zhao Q, Fan LQ, Wang D, Liu Y. ZNF139 promotes tumor metastasis by increasing migration and invasion in human gastric cancer cells. Neoplasma 2014; 61: 291 298.*
9. *Nie H F, Li Y, Li Z X, Mu J X, Wang J S. Effects of ZNF139 on gastric cancer cells and mice with gastric tumors. Oncol. Lett 2016; 12: 2550-2554.*
10. *Gupta SC, Kim JH, Prasad S. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. Cancer Metastasis Rev 2010; 29: 405-434.*
11. *Qian Y, Zhong P, Liang D, Xu Z, Skibba M, Zeng C, Li X, Wei T, Wu L, Liang G. A newly designed curcumin analog Y20 mitigates cardiac injury via anti inflammatory and anti oxidant actions in obese rats. PLoS One 2015; 10: e0120215.*
12. *Patel PB, Thakkar VR, Patel JS. Cellular effect of curcumin and citral combination on breast cancer cells: Induction of apoptosis and cell cycle arrest. J Breast Cancer 2015; 18: 225 234.*
13. *Jang EM, Choi MS, Jung UJ, Kim MJ, Kim HJ, Jeon SM, Shin SK, Seong CN, Lee MK. Beneficial effects of curcumin on hyperlipidemia and insulin resistance in high fat fed hamsters. Metabolism 2008; 57: 1576 1583.*
14. *Yeh CH, Chen TP, Wu YC, Lin YM, Jing Lin P. Inhibition of NFkappaB activation with curcumin attenuates plasma inflammatory cytokines surge and cardiomyocytic apoptosis following cardiac ischemia/reperfusion. J Surg Res 2005; 125: 109 116.*
15. *Guide for the Care and Use of Laboratory Animals: National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. 8th edition. National Academies Press, Washington (DC), 2011.*
16. *Fock KM. Review article: The epidemiology and prevention of gastric cancer. Aliment Pharmacol Ther 2014; 40: 250 260.*
17. *Arends MJ, Wyllie AH. Apoptosis: mechanisms and roles in pathology. Int Rev Exp Pathol 1991; 32: 223-254.*
18. *Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell 1997; 88: 347-354.*
19. *Van Dekken H, Tilanus HW, Hop WC, Dinjens WN, Wink JC, Vissers KJ, van Marion R. Array comparative genomic hybridization, expression array, and protein analysis of critical regions on chromosome arms 1q, 7q, and 8p in adenocarcinomas of the gastroesophageal junction. Cancer Genet Cytogenet 2009; 189: 37 42.*
20. *Li Y, Tan BB, Fan LQ, Zhao Q, Song ZC, Wang D. Proteomic identification and comparison of differentiation related proteins in gastric carcinoma cell lines. Zhonghua Zhong Liu Za Zhi2010; 32: 179 184 (In Chinese).*
21. *Li Y, Tan BB, Zhao Q, Fan LQ, Liu Y, Wang D. Regulatory mechanism of ZNF139 in multi drug resistance of gastric cancer cells. Mol Biol Rep 2014; 41: 3603 3610.*

22. Roy MJ, Vom A, Czabotar PE, Lessene G. Cell death and the mitochondria: Therapeutic targeting of the BCL 2 family driven pathway. Br J Pharmacol 2014; 171: 1973 1987.

23. Rubio N, Garcia Segura LM, Arevalo MA. Survivin prevents apoptosis by binding to caspase 3 in astrocytes infected with the BeAn strain of Theiler's murine encephalomyelitis virus. J Neurovirol 2012; 18: 354 363.