

RESEARCH ARTICLE

Expression and Underlying Roles of IGFBP-3 in Paclitaxel-Treated Gastric Cancer Sgc-7901 Cells

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Abstract

Purpose: To study the expression of insulin-like growth factor binding proteins (IGFBPs) in paclitaxel-treated gastric cancer SGC-7901 cells, and to further investigate underlying mechanisms. **Materials and Methods:** Real time PCR and Western blot assays were applied to detect the mRNA and protein expression of IGFBP-2, -3 and -5 after paclitaxel (10 nM) treatment of SGC-7901 cells. In addition IGFBP-3 expression was silenced by RNA interference to determine effects. Cell viability was determined by MTT assay. Cell cycling and apoptosis were assessed by flow cytometry. **Results:** Compared to the control group, only IGFBP-3 expression was elevated significantly after paclitaxel (10 nM) treatment ($p < 0.05$). Paclitaxel treatment caused cell cycle arrest and apoptosis via downregulating Bcl-2 expression. However, the effect could be abrogated by IGFBP-3 silencing. **Conclusions:** IGFBP-3 exhibits anti-apoptotic effects on paclitaxel-treated SGC-7901 cells via elevating Bcl-2 expression.

Keywords: IGFBPs - paclitaxel - gastric cancer - apoptosis

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Introduction

Surgery remains the first option for the treatment of gastric cancer. However, chemotherapy is nevertheless indispensable, especially for advanced gastric cancer. Paclitaxel, which arrests tumor cells in G2/M phase and induces apoptosis, is one of the most effective drugs for malignant tumor (Vallon et al., 2012; Hossein G et al., 2013). Several signaling pathways were involved in the anti-tumor effect of paclitaxel, including cyclins and cyclin-dependent kinases (CDKs). Although as first line chemotherapeutic agent for gastric cancer, paclitaxel remains restricted for its drug resistance and side effects during clinical application. A previous study showed that RhoA signaling pathway was associated with paclitaxel resistance by regulating Cdk2 and PCNA expression (Kang et al., 2005). Furthermore, the tumor cell sensitivity had an inverse relationship with Ki-67 expression (Wang et al., 2014). Therefore, it is warranted to further investigate the underlying mechanisms of paclitaxel resistance in gastric cancer cells.

Recent studies showed that insulin-like growth factor binding proteins (IGFBPs), which specifically bind to insulin-like growth factor (IGF), were closely related to cell cycle and apoptosis. Among the six members of IGFBPs, IGFBP-2, -3 and -5 stood at most studied. In both breast cancer and colon cancer, the expression of IGFBP-2 was elevated while the expression of IGFBP-3 was down-regulated (Soubry et al., 2012; Sunderic et al., 2012; Foulstone et al., 2013; Duggan et al., 2013;

Dang et al., 2014) Moreover, the overexpression of IGFBP-5 indicated a poor prognosis for bladder cancer patients (Liang et al., 2013). Since IGFBPs were closely related to cell proliferation and apoptosis, their roles on chemoresistance have also been investigated. In prostate cancer cells, IGFBP2 played a pivotal role in the hyperglycemia-induced chemoresistance (Biernacka et al., 2013). While in non-small cell lung cancer, the deficiency of IGFBP-3 mediated the chemoresistance to cisplatin (Cortes-Sempere et al., 2013). Bcl-2, one of important anti-apoptotic factors, overexpressed in various tumor cells was also considered to be possibly related to chemoresistance (Eichhorn et al., 2014). Suppression of Bcl-2 expression by HDAC inhibitor could attenuate the chemoresistance to concanamycin A1 in human oral squamous cells (Kiyoshima et al., 2013). Herein, we aimed to investigate whether IGFBPs were involved in the chemoresistance of paclitaxel in SGC-7901 cells, and its underlying mechanisms

Materials and Methods

Cell culture

SGC-7901 cell, purchased from Shanghai Institute for Biological Sciences (Shanghai, China), was maintained in RPMI1640 medium (HyClone, Logan, UT) supplemented with 10% FBS (Tianhang, Hangzhou, China) and was incubated in 5% CO₂ at 37 °C. When reaching 90% confluent, cells were passaged by using 0.25% trypsin.

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Real time polymerase chain reaction (real time PCR) assay

Total RNA was extracted by using RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The purity and concentration of extracted RNA was determined by a spectrophotometer and further reverse-transcribed to cDNA by using ImProm-II Reverse Transcription System (Promega, Beijing, China). Real time PCR with GO Taq qPCR Master Mix (Promega) was performed with primers for targeted genes as follows: IGFBP-2 (forward: 5'-CAA GCA TGG CCT GTA CAA CCT C-3'; reverse: 5'-GGG TTC ACA CAC CAG CAC TC-3'); IGFBP-3 (forward: 5'-CAG CCA GCG CTA CAA AGT TGA CTA-3'; reverse: 5'-CTG GGA CTC AGC ACA TTG AGG A-3'); IGFBP-5 (forward: 5'-GCT CAA CTT CGA AAA TGG CA-3'; reverse: 5'-TTC CTC CCC ACA TCG ACT CT-3'); Bcl-2 (forward: 5'-TGG GAT GCC TTT GTG GAA CTA T-3'; reverse: 5'-AGA GAC AGC CAG GAG AAA TCA AAC-3'). The expression of target genes were analyzed by $\Delta\Delta$ cycle threshold ($2^{-\Delta\Delta Ct}$) method with GAPDH as reference gene (forward: 5'-TGG CAC CCA GCA CAA TGAA-3'; reverse: 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3').

Western blot analysis

Proteins were extracted with RIPA lysis buffer (Applygen, Beijing, China) mixed with 1% proteinase inhibitors cocktail and the concentration were determined by BCA commercial kit (Applygen, Beijing, China). Samples (30 μ g) were loaded on each well and further separated on 12% SDS-PAGE. Protein bands were then transferred to PVDF membranes and were blocked by 5% non-fat milk for 1 h at room temperature. After binding with specific primary antibodies (mouse anti-human IGFBP-2, 1:1000; mouse anti-human IGFBP-3, 1:1500; mouse anti-human IGFBP-5, 1:500; mouse anti-human Bcl-2, 1:1000 and mouse anti-human β -actin, 1:1000; all from Abcam, Cambridge, MA), the protein bands were incubated with DyLight 800 (KPL, Gaithersburg, ND) conjugated secondary antibody for 2 h and further visualized using Odyssey infrared imaging system.

Gene-silencing by small interfering RNA (siRNA)

Anti-sense siRNA against human IGFBP-3 was designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). The transfection of siRNA was performed using EntransterTM-R (Engreen Biosystem, Beijing, China) at a final concentration of 100 nM according to the manufacturer's instruction. Forty-eight hours after transfection, the gene-silencing effect was determined in both mRNA and protein level.

Cell viability assay

The viability of SGC-7901 cells was detected by MTT assay. Normal and IGFBP-3-silenced SGC-7901 cells seeded in 96-well plates were incubated with paclitaxel (10 nM) for 48 h. Four hours before the end of the incubation, aliquots (15 μ L) of MTT were added into each well. The medium were aspirated and 100 μ L DMSO were added to dissolve the formazan crystals. The absorbance at 490 nm presents cell viability.

Cell cycle analysis

After indicated treatment for 48 h, cells were harvested and washed by ice cold PBS. The cells were further fixed in pre-cold 70% ethanol for 12 h. After that, 500 μ L propidium iodide (PI, 50 μ g/mL) was added and incubated away from light for 30 min. The cell cycle was determined by flow cytometry immediately.

Cell apoptosis analysis

Cell apoptosis was analyzed by using Annexin V-FITC/PI method. Cells were treated as indicated for 48 h and were further stained with Annexin V-FITC and PI according to the manufacturer's protocol (Sigma, St. Louis, MO).

Statistical analysis

All data were expressed as mean \pm standard deviation (SD). Differences between control and experimental groups were analyzed by one-way analysis of variance with Dunnett's test post hoc, and $p < 0.05$ was considered as statistically significant. All calculations were performed using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA).

Results**IGFBPs expression after paclitaxel treatment**

After paclitaxel (10 nM) treatment, the expression of IGFBP-2 and -5 in mRNA level were similar to control group (0.94 \pm 0.11 and 1.08 \pm 0.15 fold, respectively, both $p > 0.05$). However, the mRNA level of IGFBP-3 was significantly elevated (1.82 \pm 0.10 fold compared to control group, $p < 0.05$). Results from Western blot analysis were consistent with real time PCR assay (Figure 1).

Effect of IGFBP-3 silencing on cell viability of paclitaxel-treated SGC-7901 cells

As shown in (Figure 2), paclitaxel (10 nM) treatment could notably decrease the viability of SGC-7901 cells (61.2 \pm 8.4%). The silencing of IGFBP-3 by RNA

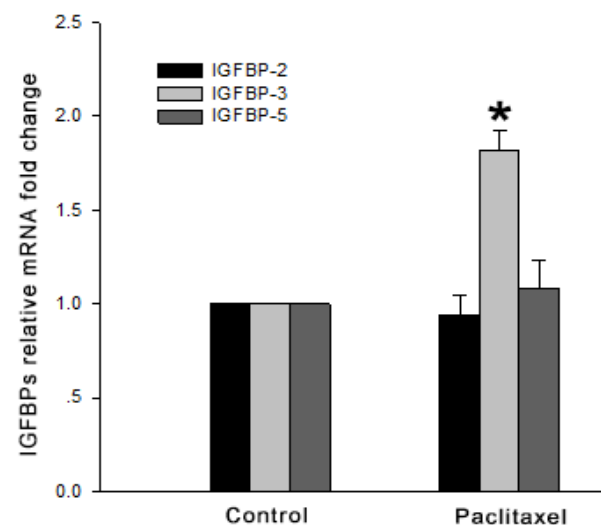


Figure 1. IGFBPs mRNA Expression After Paclitaxel Treatment. Relative fold change was determined by $2^{-\Delta\Delta Ct}$ method with GAPDH as reference gene. * $p < 0.05$, compared with control group

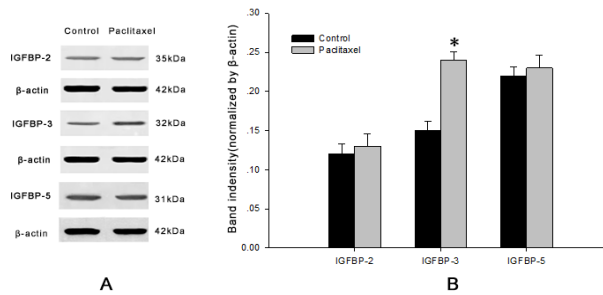


Figure 2. IGFBPs Protein Expression After Paclitaxel Treatment. (A) Western blots of IGFBP-2, -3, -5 and β -actin; (B) relative optical density normalized by β -actin; * p <0.05, compared with control group.

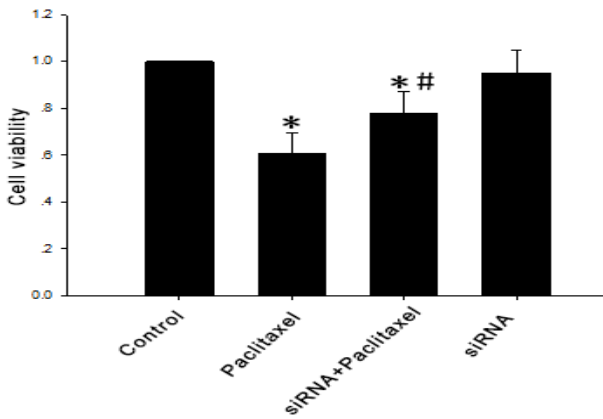


Figure 3. Effect of IGFBP-3 Silencing on Cell Viability of Paclitaxel-Treated SGC-7901 Cells. * p <0.05, compared with control group; # p <0.05, compared with the paclitaxel-treated group

Table 1. Values of Cell Cycle Analysis and Apoptotic Proportion by Flow Cytometry

Group	Proportion of G1 phase (%)	Proportion of G2/M phase (%)	Apoptotic proportion (%)
Control	55.2±7.5	7.6±2.2	5.1±1.9
Paclitaxel	45.5±6.6*	19.6±4.2*	32.1±7.2*
siRNA+Paclitaxel	55.9±6.4*#	12.5±3.6*#	18.4±6.5*#
siRNA	57.3±4.9	7.1±1.8	4.4±1.6

*compared with Control p <0.05. #compared with the group only treated with Paclitaxel p <0.05.

interference could significantly attenuate the inhibitory effect of paclitaxel treatment (78.6±9.1%, p <0.05).

Effect of IGFBP-3 silencing on cell cycle arrest of paclitaxel-treated SGC-7901 cells

As shown in (Figure 3) and (Table 1), paclitaxel (10 nM) treatment could arrest SGC-7901 cells to G2/M phase (from 7.6±2.2% to 19.2±4.2% before and after paclitaxel incubation). Single silencing of IGFBP-3 did not affect cell cycle without paclitaxel treatment. However, there was a decline of cells in G2/M phase after IGFBP-3 silencing when cells are treated with paclitaxel (12.5±3.6%, p <0.05).

Effect of IGFBP-3 silencing on apoptosis of paclitaxel-treated SGC-7901 cells

As shown in (Figure 4) and (Table 1), paclitaxel incubation could significantly induce apoptosis in SGC-

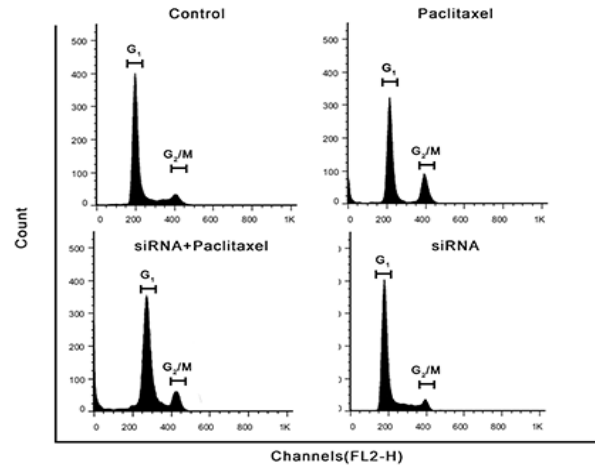


Figure 4. Effect of IGFBP-3 Silencing on Cell Cycle Arrest of Paclitaxel-Treated SGC-7901 Cells

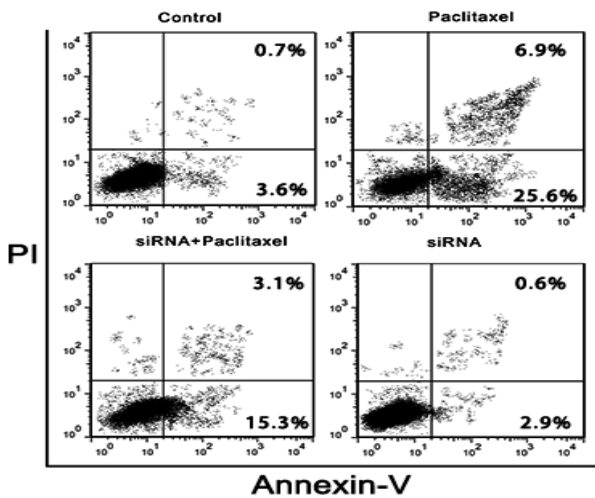


Figure 5. Effect of IGFBP-3 Silencing on Apoptosis of Paclitaxel-Treated SGC-7901 Cells

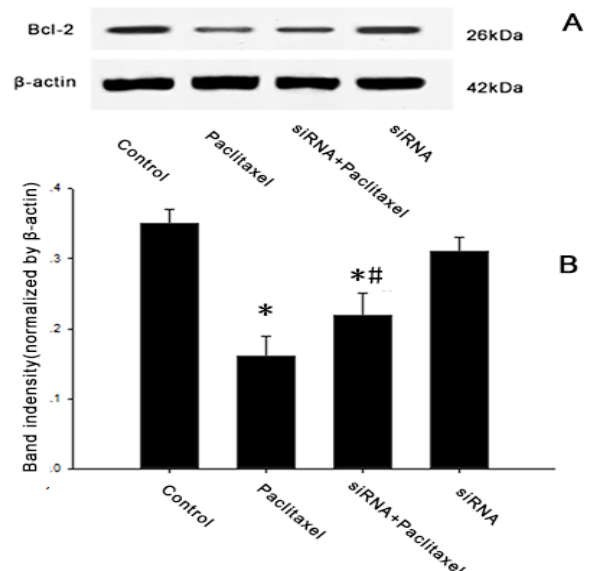


Figure 6. Bcl-2 Protein Expression After Paclitaxel and/or Igfbp-3 Silencing. (A) Western blots of Bcl-2 and β -actin; (B) relative optical density normalized by β -actin. * p <0.05, compared with control group; # p <0.05, compared with paclitaxel-treated group

7901 cells (32.1±7.2% vs 5.7±1.9%, $p<0.05$). However, this effect could be partially abrogated by IGFBP-3 silencing (24.4±6.5%).

Bcl-2 expression after paclitaxel and/or IGFBP-3 silencing

Compared to control group, the mRNA level of Bcl-2 decreased to 0.65±0.06 fold after paclitaxel (10 nM) incubation. While the expression of Bcl-2 increased to a level of 0.86±0.07 fold in IGFBP-3 knockdown SGC-7901 cells after paclitaxel treatment. Results from Western blot analysis of Bcl-2 expression in protein level exhibited a similar trend (Figure 5).

Discussion

Most of the currently available chemotherapeutic agents exhibit their anti-tumor effect by inducing cell cycle arrest and apoptosis. IGFBPs, closely related to cell cycle regulation and apoptosis, have been reported to modulate cancer cell chemoresistance to various anti-tumor agents. Biernacka et al demonstrated that the overexpression of IGFBP-2 could attenuate docetaxel-induced prostate cancer cell apoptosis (Biernacka et al., 2013). Further, IGFBP-3 expression could enhance the anti-tumorigenic effect of irinotecan possibly via inhibiting of Akt phosphorylation (Alami et al., 2008). Moreover, a study from Zeng and colleagues showed elevated IGFBP-3 was found in 5-Aza-2'-deoxycytidine (AZA) treated in breast cancer cells. Inhibition of IGFBP-3 could palliate the anticancer effect of AZA (Zeng et al., 2013).

In our current study, paclitaxel (10nM) treatment could significantly up-regulate IGFBP-3 expression in SGC-7901 gastric cancer cells, while having no effect on either IGFBP-2 or IGFBP-5 expression. Similar to a previous study (Zeng et al., 2013), silencing of IGFBP-3 by RNA interference could attenuate the effect of paclitaxel on cell viability, cell cycle arrest and apoptosis. Our result indicated that up-regulation of IGFBP-3 might enhance gastric cancer cell sensitivity to paclitaxel. More investigations on various chemotherapeutic agents and their relationship to IGFBP-3 in different tumor cell lines are warranted.

However, there are nevertheless some disputes concerning IGFBP-3 expression and chemoresistance. A study from Holdaway's group suggested overexpression of IGFBP-3 would lead to chemoresistance in breast cancer cells (Holdaway et al., 2003). While in our study, we tended to interpret IGFBP-3 overexpression as an adjuvant to paclitaxel in inducing cell apoptosis. The apoptotic effect of IGFBP-3 has been extensively studied, which could be categorized into IGF-dependent and IGF-independent. For the former one, IGFBP-3 could specifically bind to IGF-1, resulting reduced free IGF-1 level, and thus induce cell apoptosis (Kalluri et al., 2011). On the other hand, IGFBP-3 itself could directly or indirectly induce apoptosis via NF- κ B, p38 MAPK and Akt signaling pathway (Peters et al., 2006; Williams et al., 2007; Koyama et al., 2010). Further, Bcl-2 is a classical anti-apoptotic factor, which exerts its anti-apoptotic effect via NF- κ B signaling pathways (Adams, 1998; Zhang et

al., 2013). Our observation of Bcl-2 level also indicated IGFBP-3 could induce cell apoptosis via down-regulating Bcl-2 expression. The underlying mechanisms should be elucidated in future studies.

In conclusion, IGFBP-3 expression is closely related to chemoresistance of paclitaxel in SGC-7901 gastric cancer cells. Clinical monitoring of IGFBP-3 level in patient might be beneficial when applying paclitaxel.

Acknowledgements

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References

- Adams JM, S Cory (1998). The Bcl-2 protein family: arbiters of cell survival. *Science*, **281**, 1322-6.
- Alami N, V Page, Q Yu, et al (2008). Recombinant human insulin-like growth factor-binding protein 3 inhibits tumor growth and targets the Akt pathway in lung and colon cancer models. *Growth Horm IGF Res*, **18**, 487-96.
- Biernacka KM, CC Uzoh, LZeng, et al (2013). Hyperglycaemia-induced chemoresistance of prostate cancer cells due to IGFBP2. *Endocr Relat Cancer*, **20**, 741-51.
- Cortes-Sempere M, MP de Miguel, O Pernia, et al (2013). IGFBP-3 methylation-derived deficiency mediates the resistance to cisplatin through the activation of the IGFBP3/Akt pathway in non-small cell lung cancer. *Oncogene*, **32**, 1274-83.
- Dang YM, Huang G, Chen YR, et al (2014). Sulforaphane inhibits the proliferation of the BIU87 bladder cancer cell line via IGFBP-3 elevation. *Asian Pac J Cancer Prev*. **15**, 1517-20.
- Duggan C, CY Wang, ML Neuhaus, et al (2013). Associations of insulin-like growth factor and insulin-like growth factor binding protein-3 with mortality in women with breast cancer. *Int J Cancer*, **132**, 1191-200.
- Eichhorn JM, SE Alford, N Sakurikar, et al (2014). Molecular analysis of functional redundancy among anti-apoptotic Bcl-2 proteins and its role in cancer cell survival. *Exp Cell Res*. **322**, 415-24.
- Foulstone EJ, LZeng, CM Perks, et al (2013). Insulin-like growth factor binding protein 2 (IGFBP-2) promotes growth and survival of breast epithelial cells: novel regulation of the estrogen receptor. *Endocrinology*, **154**, 1780-93.
- Holdaway IM, BH Mason, AE Lethaby, et al (2003). Serum insulin-like growth factor-I and insulin-like growth factor binding protein-3 following chemotherapy for advanced breast cancer. *ANZ J Surg*, **73**, 905-8.
- Hossein G, Keshavarz M, Ahmadi S, et al (2012). Synergistic effects of PectaSol-C modified citrus pectin an inhibitor of galectin-3 and paclitaxel on apoptosis of human SKOV-3 ovarian cancer cells. *Asian Pac J Cancer Prev*, **14**, 7561-8.
- Liang PI, YH Wang, TF Wu, et al (2013). IGFBP-5 overexpression as a poor prognostic factor in patients with urothelial carcinomas of upper urinary tracts and urinary bladder. *J Clin Pathol*, **66**, 573-82.
- Kiyoshima T, H Yoshida, H Wada, et al (2013). Chemoresistance to concanamycin A1 in human oral squamous cell carcinoma is attenuated by an HDAC inhibitor partly via suppression of Bcl-2 expression. *PLoS One*, **8**, 80998.
- Koyama S, LJ Cobb, HH Mehta, et al (2010). Pomegranate extract induces apoptosis in human prostate cancer cells by modulation of the IGF-IGFBP axis. *Growth Horm IGF Res*, **20**, 55-62.

- Kalluri HS, RJ Dempsey (2011). IGFBP-3 inhibits the proliferation of neural progenitor cells. *Neurochem Res*, **36**, 406-11.
- Williams AC, H Smartt, HZ AM, et al (2007). Insulin-like growth factor binding protein 3 (IGFBP-3) potentiates TRAIL-induced apoptosis of human colorectal carcinoma cells through inhibition of NF-kappaB. *Cell Death Differ*, **14**, 137-45.
- Peters I, I Tossidou, J Achenbach, et al (2006). IGF-binding protein-3 modulates TGF-beta/BMP-signaling in glomerular podocytes. *J Am Soc Nephrol*, **17**, 1644-56.
- Soubry A, D Il'yasova, R Sedjo, et al (2012). Increase in circulating levels of IGF-1 and IGF-1/IGFBP-3 molar ratio over a decade is associated with colorectal adenomatous polyps. *Int J Cancer*, **131**, 512-7.
- Sunderic M, B Dukanovic, V Malenkovic, et al (2014). Molecular forms of the insulin-like growth factor-binding protein-2 in patients with colorectal cancer. *Exp Mol Pathol*, **96**, 48-53.
- Vallon M, C Seidl, B Blechert, et al (2012). Enhanced efficacy of combined ²¹³Bi-DTPA-F3 and paclitaxel therapy of peritoneal carcinomatosis is mediated by enhanced induction of apoptosis and G2/M phase arrest. *Eur J Nucl Med Mol Imaging*, **39**, 1886-97.
- Kang WK, I Lee, U Ko, et al (2005). Differential effects of RhoA signaling on anticancer agent-induced cell death. *Oncol Rep*, **13**, 299-304.
- Wang X, JJ Beitler, H Wang, et al (2014). Honokiol enhances paclitaxel efficacy in multi-drug resistant human cancer model through the induction of apoptosis. *PLoS One*, **9**, 86369.
- Zhang GJ, Z Zhang (2013). Effect of Bcl-2 on apoptosis and transcription factor NF-kappaB activation induced by adriamycin in bladder carcinoma BIU87 cells. *Asian Pac J Cancer Prev*, **14**, 2387-91.
- Zeng L, C Jarrett, K Brown, et al (2013). Insulin-like growth factor binding protein-3 (IGFBP-3) plays a role in the anti-tumorigenic effects of 5-Aza-2'-deoxycytidine (AZA) in breast cancer cells. *Exp Cell Res*, **319**, 2282-95.