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Inhibitory effect of oleanolic acid on cisplatin-resistant non-small cell lung carcinoma

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Abstract: **Present studies have been investigted the inhibiting effect of oleanolic acid on cisplatin-resistant non-small cell lung carcinoma (NSCLC) cell growth and epithelial-mesenchymal transition (EMT). The MTT assay was employed to determine the effect of oleanolic acid (OA) on the viability of A549/CDDP cell line. Assays related to cell migration and invasion was performed to detect the migratory and invasive characteristics of A549/CDDP after treated with OA. The expression of ERCC1 protein, P-gp protein, E-cadherin protein and Vimentin protein were determined by immunocytochemistry and western blotting. These results suggested that OA could significangtly inhibit drug resistance. OA can not only inhibit cell proliferation of A549/CDDP, but also inhibit its EMT phenotype. This research will broaden the clinical application fields of oleanolic acid in the treatment of cancer.**

Keywords: NSCLC; Cisplatin Resistance; Oleanolic Acid; EMT

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1. Introduction

Currently, lung cancer has become a popular malignant tumor with highest incidence in the world. According to the result of statistics from big data, there are about more than 60 million people every year die of lung cancer. Lung cancer is the leading cause of death among people with cancer comparing with other kinds of cancer [1]. About 85% of lung cancers are non-small cell lung cancer (NSCLC), the left 15% are small cell lung cancer [2]. Despite efforts to improve efficacy of therapy, the 5-year survival rate of NSCLC over all stages is still only 17% [3].

Cisplatin, cis-Diamminedichloro-platinum (II) (CDDP) is one of the most commonly-used chemotherapeutic agents in the treatment of lung cancer, especially in NSCLC [4]. The mechanism of action (MOA) of cisplatin is its interaction with DNA will result in the formation of DNA adducts, which in turn activate several signal transduction pathways and culminate relevant to apoptosis [5]. However, current investigation on clinical application of cisplatin is not optimistic, drug resistance is more and more common and severe, regardless of inherent or acquired non-response.

Oleanolic acid (OA) is a pentacyclic triterpenoid exists in considerable plants and medical herbs [6,7], it is relatively nontoxic and hepatoprotective. Moreover, it is of great antiviral and antitumor properties. OA has been shown to inhibit cell proliferation of lung carcinoma cells via the miR-122/cyclin G1/MEF2D axis pathway and suppress hepatocellular carcinoma via ERK-p53-mediated cell cycle arrest and mitochondrial-dependent apoptosis. Besides, OA suppresses cell proliferation and invasiveness of Kras-transformed cells via autophagy [8-13]. Therefore, we hypothesized that OA can increase the sensitivity of cisplatin on cisplatin-resistant NSCLC. To verify our hypothesis, a series of experiments will carried out to detect the effect of OA about reversing drug resistance and inhibiting migration on cisplatin-resistant NSCLC.

2. Materials and Methods

2.1. Reagents and chemicals

OA was purchased from Sigma-Aldrich (St Louis, MO, USA). For the in vitro studies, OA was stored at -20 ℃ after dissolved in dimethyl sulfoxide (DMSO) to create a stock solution (0.1M). The stock solution was further diluted with culture medium for working solutions. The secondary antibodies (goat antirabbit and goat antimouse) were purchased from ABGENT (San Diego, USA). 3-[4,5-dimethylthiazol-2-yl]-2,5-di phenyl-tetrazolium bromide (MTT), was purchased from Solarbio (Beijing, China).

2.2. Cell viability assay

The MTT assay was used to measure cell viability. A549 and A549/CDDP cells (5×10^3/well) were seeded into 96-well plates, cultured for 24h, and then treated with OA at final concentrations of 0, 30, 50, 70 and 90 μmol/L for 24, 48, and 72h. After treatment, 20μL of MTT solution (5mg/mL) was added to each well and the plates were incubated at 37 ℃ for about 4 hours. Then replaced the culture medium with 150μL of DMSO. A microplate reader (Bio-Tek, Winooski, VT, USA) would be used to measured the absorbance of the solution at 490nm.

2.3. Wound-healing assay

Cell migration was assessed using wound-healing assays. Cells were grown in a confluent monolayer in a

6-well plate. A wound was inflicted in the cell layer by scratching the plate with a sterile pipette tip. Plates were rinsed gently with phosphate saline buffer (PBS) twice to remove non-adherent cells before incubating. The medium was changed to serum-free RPMI-1640 with 60μmol/L OA and the cells were continuously cultured for 12h and 24h. Digital images of the wound were obtained at times 0, 12 and 24h at 200×magnification.

2.4. Transwell-matrigel invasion assays

Cells were serum starved for 12h prior to use. Media with 15% FBS was added to the wells of a 24-well plate. The polycarbonate filters (8μm poresize, Corning) were precoated with Matrigel Matrix (BD Biosciences, Sparks, MD, USA). 2×10^3 cells in serum-free media with 60μmol/L OA were added to the interior of each upper chamber. Plates were incubated for 48 h at 37℃ in 5% CO2, and media removed from the upper chamber, which was then washed with PBS. The upper chamber membranes were fixed with cold methanol for 20mins, stained with 0.1% Crystal Violet in 25% methanol for 30 mins and rinsed with PBS. The number of cells was counted by a phase contrast microscope (five fields perchamber were collected).

2.5. Immunocytochemistry assays

A459 cells (10^5/well) were seeded on cover slips in 24-well plates, then treated with 0 and 60 μmol/L OA. After 24 h incubation, the cells were washed twice with PBS, and fixed with a 4% buffered paraformaldehyde solution for 40 mins. After three washes in PBS, the cover slips were incubated for 10 mins in 60 μl endogenous peroxide, and then washed again in PBS (3×), remove PBS liquid, add 60 μl antibodies, incubation overnight. The next step was to add the secondary antibody selected for that trial: biotinylated anti-rabbit IgG or anti-mouse IgG. After that step, the coverslips were washed (3×) with PBS. Next, the coverslips were stained with 60 μl DAB for 3-5 mins. Following two more washes in distilled water, then used 60 μl hematoxylin stained cells to light blue. After washing, the cover slips were observed by microscopy (Eclipse E-800, Nikon, Japan).

2.6. Western blot analysis

A549/CDDP were treated with OA (0, 60 and 90μmol/L) for 48h, A549 were treated without OA relatively. After which both adherent and floating cells were harvested, washed the cells twice in ice-cold PBS, and lysed in RIPA buffer (Beyotime Biotechnology, Shanghai, China) and protease inhibitor (Solarbio, Beijing, China) at 4℃ for 5mins. After centrifugation at 14,000g for 25mins, extracted the supernatant. A BCA Protein Kit (Beyotime Biotechnology, Shanghai, China) were used to quantify the protein concentrations. Cell lysates (40μg/lane) were separated by 10% SDS-PAGE, then electrophoretically transferred to polyvinyl diflouride (PVDF) membranes (Millipore, Billerica, MA, USA) and blocked with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) and 5% skimmed milk powder. The membranes were incubated with anti-E-cadherin, anti-Vimentin (Santa Cruz Biotechnology, China) and anti-ERCC1, anti-P-gp (ABCAM, Cambridge, MA, USA) antibodies (1:1000) at 4℃ overnight, washed three times with TBST and then incubated with the appropriate HRP-conjugated secondary antibodies (1:5000) for 1h at room temperature. The protein bands were visualized using Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). Protein levels were normalized to GAPDH (1:2000, Santa Cruz Biotechnology, China).

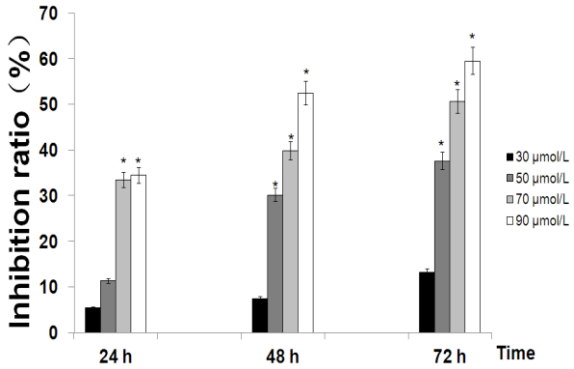

Figure 1. MTT assay detected that OA inhibited cisplatin resistance cancer NSCLC cells proliferation (*P<0.05).

2.7. Statistical analysis

The experimental results are expressed as x±s. The Student's t-test was used to compare the difference between treated groups and their controls. Data were considered to be statistically significant when P<0.05.

3. Results

3.1. OA inhibited cisplatin resistance cancer NSCLC cells proliferation

As shown in Figure 1, the results indicated that OA significantly inhibited the proliferation of A549/CDDP, and presented as time and concentration-dependence. OA inhibits cancer cells viability begining in 24h and achieved 50% inhibition rate at 90μmol/L after treatment for 48h.

3.2. OA effectively inhibite the movement of A549/CDDP

We performed wound healing assay and transwell-matrigel invasion assays to determine the migration and invasion ability of A549/CDDP. After the treatment of 60μmol/L OA for 48h, A549/CDDP cells appearing a reduction in their migration and invasion capabilities compared A549/CDDP cells without treatment of OA (Figure 2A, B).

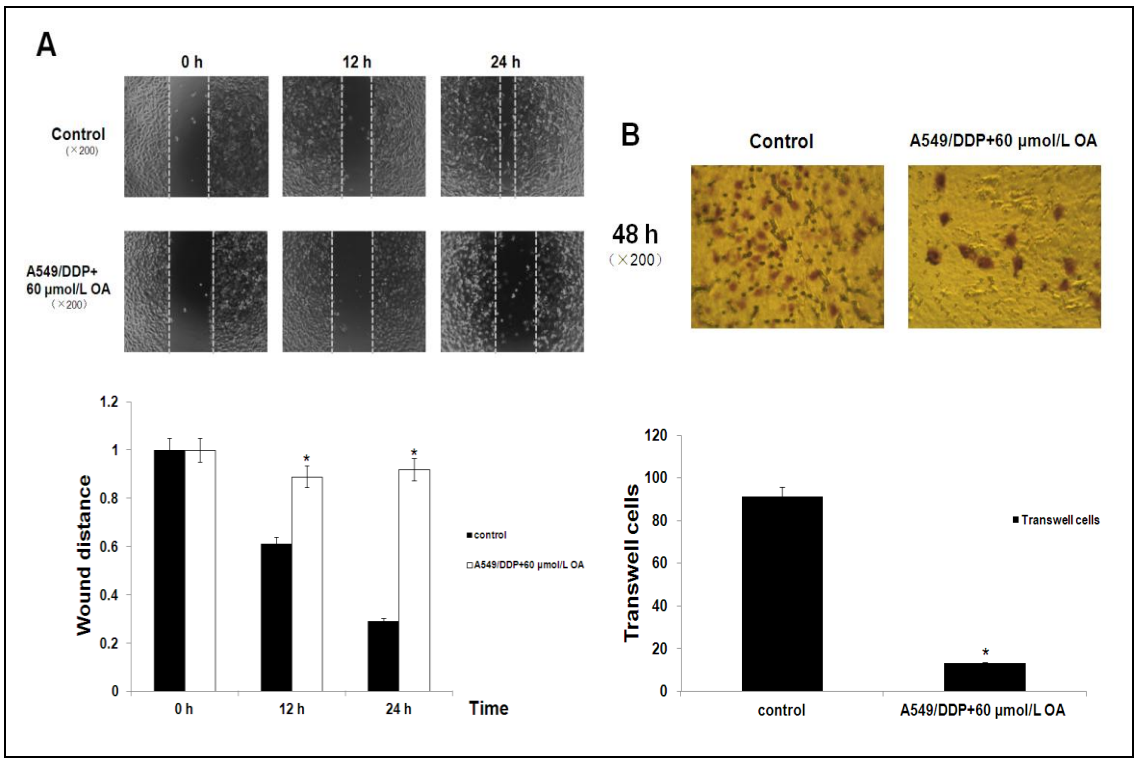

Figure 2. Wound healing assay and transwell-matrigel invasion assays (*P<0.05).

3.3. OA caused the reversal of cisplatin resistance and EMT phenotype cisplatin resistance in A549/CDDP

Western blot and immunocytochemistry assay displayed that, after the treatment of OA, resistance-associated proteins ERCC1, P-gp and the EMT markers E-cadherin, Vimentin in A549/CDDP cells both reversed expression compared with A549 cells (Figure 3A, B).

4. Discussion

The results of our study suggested that OA can effectively inhibit cisplatin-resistant NSCLC cells' proliferation, drug resistance and EMT. In MTT assay (Figure 1), the results showed that OA with high concentrations (50, 70 and 90μmol/L) in the processing time could significantly inhibit A549/CDDP cell proliferation (P<0.05). The treatment of low concentrations (30μmol/L) of OA for 72h could inhibit the proliferation of A549/CDDP cells (P<0.05) and this concentration of OA in the treatment of 24 and 48h on A549/CDDP cells proliferation had no significant inhibition (P>0.05). After the treatment of 60μmol/L OA, the wound distance of A549/CDDP was no significant change and compared to the control group which without treatment of OA, the invasion cells was few (Figure 2A, B). The Immunocytochemistry assays and Western blotting analysis (Figure 3A, B) showed that after the treatment of 60 or 90μmol/L OA for 48h, the expressions of P-gp, ERCC1 was decreased, which suggested that OA could reduce drug resistance inducing by cisplatin. Furthermore, the expression of E-cadherin protein and Vimentin protein has increased and decreased, respectively, their changes verifid that OA could reverse the phenotype of EMT of A549/CDDP. That was a futher evidence of superior anticancer efficacy of OA treated on cisplatin-resistant NSCLC. The chemotherapy based on Cisplatin, one of the main drugs used to treat lung cancer, seemed to have reached a dead-end regarding malignant lung cancer, leading clinical researchers to evaluate the validity of continuing its use in advanced lung cancer patients [14]. Drug resistance is a severe limitation of chemotherapy on the therapy of various malignant carcinomas. Advanced lung cancer is a complicated disease that urges treatments targeting on multiple signaling pathways [15-17]. Researchers are of great interest for lung cancer therapy about new drugs available to overcome the chemoresisitance of cancer and surpress the progress of the metastasis resulted from this disease [18]. As an anticancer agent, the importance of research on OA is apparent. However, the MOA of OA on cisplatin-resistant NSCLC remains unknown. In this study, we found that OA could inhibit cell proliferation, reverse the EMT phenotype, and downregulate the expression of drugresisitance associated proteins. The cell viability assay showed that OA could inhibit proliferation of cisplatin resistance NSCLC cells. Interestingly, an in vivo study found that OA is of low toxicity that it seldomly do harm to the xenagraft mouse [18]. All in all, OA has the potential to become a candidate for treatment of cisplatin-resistant human lung carcinoma.

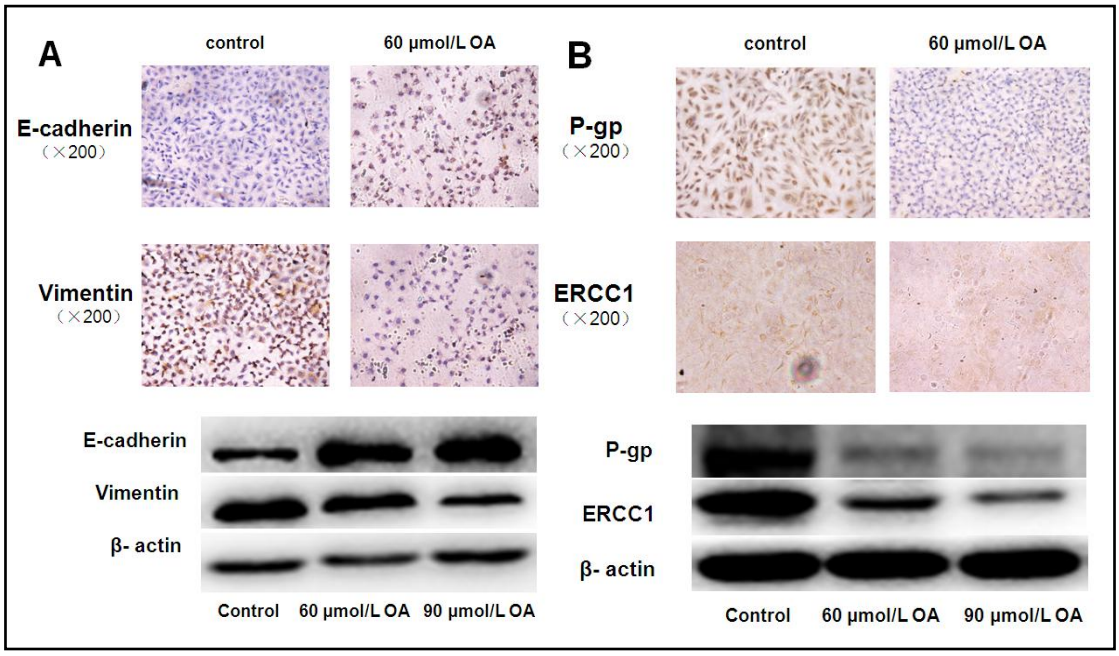

Figure 3. OA caused the reversal of cisplatin resistance and EMT phenotype cisplatin resistance in A549/CDDP.

Data presented in this paper demonstrated that after the treatment of OA, the expression of proteins related to MDR (multidrug resistance), such as P-glycoprotein (P-gp)/ABCB1 (efflux pumps that are good at removing a variety of drugs out the cell, and thus, preventing cell death [19]) and ERCC1 (involved in the nucleotide excision and repair (NER) pathway. Patients with low levels of ERCC1 expression have been reported to have an improved response and a longer OS in lung tumors treated with cisplatin [20-23]) were decreased, which suggests that OA may enhance drug-sensitivety of the NSCLC cells through decreasing the expression of resistance-associated proteins.

In multiple studies, poor prognosis has been shown to be correlated with the altered expression of various proteins relevant to EMT development, including vimentin [24], certain epithelial cytokeratins [25] and E-cadherin [26]. Additionally, EMT can be induced in breast cancers after treated with standard chemotherapies [27] and hormonal therapies [28], suggesting a potential role for EMT in chemoresistance. In our study, we showed that OA weakened the EMT of A549/CDDP. OA not only inhibited the matasis and invasion of A549/CDDP, but also upregulate the expression of Vimentin protein and downregulate the expression of E-cadherin protein. The study had just examined that the inhibitory effect of OA on cisplatin-resistant NSCLC, but the MOA of OA sensitizing the effect of cisplatin on cisplatin-resistant cancer cells is not clear.

In conclusion, the results presented in this paper indicate that OA can be a chemotherapeutic agent with great antitumor activity and a co-adjuvant for the treatment of chemoresistant lung cancer. Taken all together, this study demonstrates the promising clinical application of OA on the treatment of drug resistant cancer.

References

[1] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics. CA Cancer J. Clin., 55 2005 74-108.

[2] Yin J, Huang Q, Zhao Y, Zhou H, Liu Z. Meta-analysis on pharmacogenetics of platinum-based chemotherapy in non small cell lung cancer (NSCLC) patients. PLoS One, 7 2012 38150.

[3] Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics. CA Cancer. J. Clin., 64 2014 9-29.

[4] Prestayko AW, D'Aoust JC, Issell BF, Crooke ST. Cisplatin (cisdiamminedichloroplatinumII). Cancer Treat Rev. 6 1979 17-39.

[5] Kelland LR. Preclinical perspectives on platinum resistance. Drugs, 59(4) 2000 37-38.

[6] Sultana N and Ata A. Oleanolic acid and related derivatives as medicinally important compounds. J. Enzyme Inhib Med Chem, 23 2008 739-756.

[7] Ovesná Z, Vachálková A, Horváthová K and Tóthová D. Pentacyclic triterpenoic acids: new chemoprotective compounds. Minireview. Neoplasma, 51 2004 327-333.

[8] Feng L, Au-Yeung W, Xu Y, Wang S, Zhu Q, Xiang P. Oleanolic acid from Prunella Vulgaris L. induces SPC-A-1 cell line apoptosis via regulation of Bax, Bad and Bcl-2 expression. Asian Pac. J Cancer Prev., 12 2011 403-408.

[9] Liu J, Zheng L, Ma L, Wang B, Zhao Y, Wu N,

Liu G, Lin X. Oleanolic acid inhibits proliferation and invasiveness of Kras-transformed cells via autophagy. J. Nutr. Biochem., 25 2014 1154-1160.
[10] Pratheeshkumar P, Kuttan G. Oleanolic acid induces apoptosis by modulating p53, Bax, Bcl-2 and caspase-3 gene expression and regulates the activation of transcription factors and cytokine profile in B16F. J. Environ. Pathol. Toxicol. Oncol., 30: 2011 21-31.
[11] Wang X, Bai H, Zhang X, Liu, J, Cao P, Liao N, Zhang W, Wang Z, Hai C. Inhibitory effect of oleanolic acid on hepatocellular carcinoma via ERK-p53-mediated cell cycle arrest and mitochondrial-dependent apoptosis. Carcinogenesis, 34 2013 1323-1330.
[12] Wei J, Liu M, Liu H, Wang H, Wang F, Zhang Y, Han L, Lin X. Oleanolic acid arrests cell cycle and induces apoptosis via ROS-mediated mitochondrial depolarization and lysosomal membrane permeabilization in human pancreatic cancer cells. J. Appl. Toxicol., 33 2013 756-765.
[13] Zhao X, Liu M, Li D. Oleanolic acid suppresses the proliferation of lung carcinoma cells by miR-122/cyclin G1/MEF2D axis. Mol. Cell Biochem., 400 2015 1-7.
[14] Kelly Araújo L, Rocha GDG, Leonardo Campos MR, Fernandes J, Maeda C, Gattass CR. Oleanolic acid initiates apoptosis in non-small cell lung cancer cell lines and reduces metastasis of a B16F10 melanoma model in vivo. Plos One, 6(12) 2011 65-65.
[15] Fernandes J, Gattass CR. Topological Polar Surface Area Defines Substrate Transport by Multidrug Resistance Associated Protein 1 (MRP1/ABCC1). J. Med. Chem., 52 2009 1214-1218.
[16] Ikuta K, Takemura K, Kihara M, Naito S, Lee E, Shimizu E, Yamauchi A. Defects in apoptotic signal transduction in cisplatin-resistant non-small cell lung cancer cells. Oncol. Rep., 13 2005 1229-1234.
[17] Krysan K, Dalwadi H, Sharma S, Dubinett SS. Cyclooxygenase2-Dependent Expression of Survivin Is Critical for Apoptosis Resistance in Non-Small Cell Lung Cancer. Cancer Res., 64 2004 6359-6362.
[18] Li H, Wang X, Xiang S, Hu Y, Jiang L, Shu Y, Li M, Wu X, Zhang F, Ye Y, Weng H, Bao R, Cao Y, Lu W, Dong Q, Liu Y. Oleanolic acid induces mitochondrial-dependent apoptosis and G0/G1 phase arrest in gallbladder cancer cells. Drug Design Development & Therapy, 9 2015 3017-3030.
[19] Sharon FJ. ABC Multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics, 9 2008 106-127.
[20] Shirota Y, Stoehlmacher J, Brabender J, Xiong YP, Uetake H, Danenberg KD, Groshen S, Tsao-Wei DD, Danenberg PV, Lenz HJ. ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. Journal of clinical oncology, 19(23) 2001 4298-4304.
[21] Joshi MB, Shirota Y, Danenberg KD, Conlon DH, Salonga DS, Herndon JE 2nd, Danenberg PV, Harpole DH Jr. High gene expression of TS1, GSTP1, and ERCC1 are risk factors for survival in patients treated with trimodality therapy for esophageal cancer. Clinical cancer research, 11(6) 2005 2215-21.
[22] Kwon HC, Roh MS, Oh SY, Kim SH, Kim MC, Kim JS, Kim HJ. Prognostic value of expression of ERCC1, thymidylate synthase, and glutathione S-transferase P1 for 5-fluorouracil/oxaliplatin chemotherapy in advanced gastric cancer. Annals of oncology, 18(3) 2007 504-509.
[23] Metzger R, Leichman CG, Danenberg KD, Danenberg PV, Lenz HJ, Hayashi K. ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. Journal of clinical oncology, 16(1) 1998 309-316.
[24] Fuchs IB, Lichtenegger W, Buehler H, Henrich W, Stein H, Kleine Tebbe A, Schaller G. The prognostic significance of epithelial-mesenchymal transition in breast cancer. Anticancer Res., 22 2002 3415-3419.
[25] Willipinski-Stapelfeldt B, Riethdorf S, Assmann V, Woelfle U, Rau T, Sauter G, Heukeshoven J, Pantel K. Changes in cytoskeletal protein composition indicative of an epithelial-mesenchymal transition in human micrometastatic and primary breast carcinoma cells. Clin. Cancer Res., 11 2005 8006-80014.
[26] Kashiwagi S, Yashiro M, Takashima T, Nomura S, Noda S, Kawajiri H, Ishikawa T, Wakasa K, Hirakawa K. Significance of E-cadherin expression in triple-negative breast cancer. Br. J. Cancer, 103 2010 249-55.
[27] Qu C, Zhang W, Zheng G, Zhang Z, Yin J, He Z. Metformin reverses multidrug resistance and epithelial-mesenchymal transition (EMT) via activating AMP-activated protein kinase (AMPK) in human breast cancer cells. Mol. Cell Biochem., 386 2014 63-71.
[28] Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, Rimm DL, Wong H, Rodriguez A, Herschkowitz JI, Fan C, Zhang X, He X, Pavlick N, Gutierrez MC, Renshaw L, Larionov AA, Faratian D, Hilsenbeck SG,. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc. Natl. Acad. Sci. USA, 106 2009 13820-13825.