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ORIGINAL ARTICLE

Expression of miR-335 in triple-negative breast cancer and its effect on chemosensitivity

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Summary

Purpose: *To investigate the expression of miR-335 in triple-negative breast cancer (TNBC) and its effect on chemosensitivity.*

Methods: *The expression of miR-335 in cancer tissues and adjacent tissues of 42 patients with TNBC who underwent mastectomy in our hospital was detected by qRT-PCR. Liposome was used to transfect miR-335 mimics (miR-335-mimic) and empty vectors (miR-NC) into cells of TNBC cell line MDA-MB-231, and untransfected cells were used as blank control cells (NC). Three groups of cells were cultured in culture Levbeit's medium supplemented with 2 μmol/L paclitaxel, 5 μmol/L cisplatin and 4 μmol/L doxorubicin. Proliferation rate and apoptosis rate of tumor cells were measured by MTT assay and TUNEL assay 48 h after transfection.*

Results: *The relative expression level of miR-335 in cancer tissues was significantly lower than that in adjacent tissues of TNBC patients ($p<0.05$). After paclitaxel, cisplatin and doxorubicin treatment, the proliferation and apoptosis rates of the three groups were statistically different ($p<0.05$). There was no significant difference in cell proliferation rate and apoptosis rate between NC group and miR-NC group ($p>0.05$), but the proliferation rate of cells was higher and apoptosis rate was lower in the NC group and miR-NC group than that in miR-335-mimic group ($p<0.05$).*

Conclusion: *The expression level of miR-335 in cancer tissues of TNBC patients is lower than that in adjacent tissues. Overexpression of miR-335 can increase the sensitivity of tumor cells to paclitaxel, cisplatin and doxorubicin, and improve the effect of chemotherapy.*

Key words: *triple negative breast cancer, MDA-MB-231 cells, miR-335, chemotherapy, proliferation, apoptosis*

Introduction

Triple-negative breast cancer (TNBC) refers to breast cancer with negative immunohistochemical results of estrogen receptor, progesterone receptor and proto-oncogene HER-2. TNBC is a subtype of breast cancer. Overall incidence rate of breast cancer ranks first among all cancers in females [1,2]. Compared with luminal type A breast cancer, luminal type B breast cancer, and HER-2 positive type breast cancer, TNBC has a higher grade of malignancy and worse prognosis. Because of lack of special treatment of TNBC, this disease subtype has become a major clinical problem [3,4]. Chemotherapy is an important treatment for TNBC. The main chemotherapeutic drugs used in breast cancer are paclitaxel, cisplatin, and doxorubicin. However, with the prolongation of treatment duration, some patients show decreased efficacy or even develop drug resistance [5]. So, how to improve the therapeutic effect of chemotherapeutic drugs has become a hot spot for researchers.

MicroRNAs are small, non-coding, endogenous RNAs that are widely expressed in eukaryotic cell organisms and have pleiotropic effects on gene expression [6,7]. Abnormal changes in biosynthesis

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of microRNAs are involved in a variety of pathophysiological processes, and microRNAs have been shown to regulate chemosensitivity in some diseases [8,9]. miR-335 can also regulate the sensitivity of tumor cells to anticancer drugs. It has been reported that miR-335 is downregulated in human small cell lung cancer (SCLC) multidrug resistant cell lines, and overexpression of miR-335 can increase the chemosensitivity of SCLC cells by reducing PARP-1/NF-κB, which is an important regulator of chemoresistance in this kind of tumor and a new potential therapeutic target [10]. However, studies on the role of miR-335 in TNBC are rare.

Therefore, this study analyzed the expression level of miR-335 in tumor tissues of TNBC patients and investigated its effect on chemosensitivity. Our study provided a new therapeutic target for clinical treatment of TNBC.

Methods

Research subjects

Tumor tissues and corresponding adjacent healthy tissues were obtained from 42 patients with TNBC who were subjected to mastectomy from March 2011 to June 2013 in our hospital.

Inclusion criteria

All patients were diagnosed with TNBC by pathological examination after surgical resection in our hospital and their medical records were complete.

Exclusion criteria

HER-2 was positive but not detected by fluorescence *in situ* hybridization, tumor metastasis occurred before surgery, patients with advanced or recurrent breast cancer, patients with other malignant tumors.

The study was approved by Ethics Committee of our hospital, and patients and their families signed informed consent.

Materials

MDA-MB-231 breast cancer cell line (Cat. XB-0006) were purchased from Shanghai Aolu Biotechnology Co., Ltd. Cells were cultured in 89% Levbeit's medium + 10% fetal bovine serum (FBS) + 1% double antibiotics (penicillin and streptomycin) at 37°C in a 5% CO_2 incubator. Levbeit's medium (Cat. CD-02591-ML) was purchased from Wuhan Chundu Biotechnology Co., Ltd. FBS (Cat. 11011-8611) was purchased from Zhejiang Tianhang Biotechnology Co., Ltd. Penicillin/streptomycin (Cat. M31411) were purchased from Shanghai Meiruil Chemical Technology, Ltd.

Cell subculture

MDA-MB-231 cells were cultured to reach 90% confluence, followed by digestion with 0.25% trypsin and then were observed under a microscope. Then, the cells were cultured and placed in a 37°C and 5% CO_2 incubator. Cells were collected at passage 3 for the following experiments.

Constructions of miR-335 expression vector and transfection

MiR-335 mimic (miR-335-mimic group) and empty vector (miR-NC group) were designed and synthesized by Thermo Fisher Scientific (Waltham, MA, USA). MDA-MB-231 cells were trypsinized 24 h before transfection, and the expression vector was transfected when cells reached 80% confluence. All operations were performed in strict accordance with manufacturer's instructions. Cells were cultured in a 37°C and 5% CO_2 incubator for 48 h and cell culture Levbeit's medium was replaced every 6 h. Transfection was confirmed by qRT-PCR. LipofectamineTM 2000 transfection kit (Cat.11668027) was purchased from Shanghai Hengfei Biotechnology Co., Ltd.

Chemotherapy protocol

After transfection, 2 μmol/L paclitaxel, 5 μmol/L cisplatin and 4 μmol/L of adriamycin were added to untreated cells of NC group, miR-NC group, and miR-335-mimic group. Treatment was administered for 48 h. Paclitaxel (Cat.D0177) was purchased from Shanghai Baoman Biotechnology Co., Ltd. Cisplatin (Cat.156202) was purchased from Jiaxing Nanjian Biomaterial Co., Ltd. Doxorubicin (Cat. T1456) was purchased from Nanjing Sairui Biotechnology Co., Ltd.

MTT in vitro proliferation experiment

Chemotherapy group: MDA-MB-231 cells of the NC group, miR-NC group and miR-335-mimic group were treated for 48 h. Following this, cells were collected to prepare single cell suspensions with a cell density of 4×10^6 cells/ml. Cells were transferred to a 96-well plate and were cultured under normal conditions for 48 h. After that, 20 μL MTT (5 mg/mL) were added into each well and culture was continued at 37°C for an additional 4 h. Supernatant containing impurities was removed and dimethyl sulfoxide preparation was added. After shaking for 10 min, a CLARIOstar microplate reader (Hong Kong Boqi Technology Co., Ltd.) was used to measure the absorbance at 570 nm. *Non-chemotherapy group:* the same actions were performed on cells without drug treatment. Blank control group: cell culture medium with cells. Cell proliferation rate = (chemotherapy group A570 - blank control group A570) / (non-chemotherapy group A570 - blank control group A570). MTT test kit (Cat. C-8294) was purchased from Shanghai Caiyou Industrial Co., Ltd.

TUNEL cell apoptosis assay

After chemotherapy for 48 h MDA-MB-231 cells were collected and single cell suspensions were prepared with a cell density of 5×10^7/ml. Cells were fixed with 4% neutral formaldehyde for 10 min at room temperature and washed twice with PBS. Then, cells were treated with 2% hydrogen peroxide in PBS for 5 min at room temperature, followed by washing twice with PBS, 5 min per time. Cell staining was performed according to the instructions of the Tunel kit (Xiamen Huijia Biotechnology Co., Ltd.). Tunel positive cells in 5 fields

were counted using image analysis software (Image-proPlus5.0). The proportion of cumulative Tunel positive cells to the total number of cells in the 5 visual fields was the apoptotic rate. This experiment was repeated 3 times. Tunel kit (AP-11772457001) was purchased from Shanghai Yuda Industrial Co., Ltd.

qRT-PCR

After 100 mg of TNBC tissue or adjacent tissues were ground and pulverized, 1 ml of TRIzol lysate was added to extract total RNA from the tissues.

The concentration of MDA-MB-231 cell suspension was adjusted to 1×10^7/ml, and TRIzol lysate was added in a ratio of 3:1 for total RNA extraction. After RNA extraction, the integrity of the RNA was analyzed by 1.5% agarose gel electrophoresis, and the purity of the extracted RNA was detected using a micro nucleic acid analyzer.

RNA samples with a A260/A280 value between 1.8 and 2.1 were used. Reverse transcription reaction system was 1.0 μl of oligo dt-adaptor primer (500 μg/mL), 5.0 μl of dNTP mixture (2.5 mM), 0.5 μl of PrimerScript RT Enzyme Mix, total RNA 2 μg, and Rnase Free ddH2O was added to make a total volume of 12 μl. Reverse transcription reaction conditions were 65°C for 5 min and 85°C for 5 s. PCR amplification system included 2 μl of cDNA template, 10 μl of SYBR Green Real-time PCR Master Mix, 1 μl of upstream primer and downstream primer, and double distilled water was added to make a final volume of 20 μl. PCR reaction conditions were: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 45 s. Melt curve analysis was performed after the end of the experiment. GAPDH was used as endogenous control. Three replicate wells were set for each sample and data were analyzed by $2^{-\Delta Ct}$ method. TRIzolTM Reagent (Cat. 15596026,) was purchased from Chengdu Dongsheng Kechuang Technology Co., Ltd. SYBER GREEN real-time fluorescent quantitative PCR kit (Cat.4110251) was purchased from Nanjing Kehao Biotechnology Co., Ltd. Primers were designed and synthesized by Herzen (Shanghai) Biotechnology. Co., Ltd. (Table 1).

Follow-up and prognosis

Forty-two patients with TNBC were followed up by telephone. Follow-up data of all patients were complete, and the 5-year overall survival rate of the patients was calculated.

Statistics

Statistical analyses were performed using SPSS 19.0 (Asia Analytics Formerly SPSS China). Measurement data were expressed as percents, and ratios were compared using the x^2 test. Count data were expressed as mean ± standard deviation (mean±SD). T-test was used for comparison between the two groups. Comparisons among multiple groups were performed by One-way analysis of variance (ANOVA) and least significant difference (LSD) test. Comparisons of different time points within the group were performed using ANOVA with repeated measures. Kaplan-Meier survival curves were drawn to analyze the prognostic values of miR-335 for TNBC. Differences with $p<0.05$ were statistically significant.

Results

Relative expression level of miR-335 in cancer tissues of TNBC patients

qRT-PCR results showed that the relative expression level of miR-335 in cancer tissues of TNBC patients was 1.024±0.063, and the relative expression level of miR-335 in adjacent tissues was 1.753±0.086. The relative expression level of miR-335 in cancer tissues was significantly lower than that in adjacent tissues (Figure 1, $p<0.05$).

Analysis of MDA-MB-231 cell transfection

The results of qRT-PCR showed that the relative expression level of miR-335 in miR-NC group after transfection was 1.083±0.034, and the relative expression level of miR-335 in miR-335-mimic group was 1.982±0.074, showing a significant in-

Table 1. Primer sequences

	Forward	*Reverse*
miR-335	5'-AGCCGTCAAGAGCAATAA CGAA-3	5'-GTGCAGGGTCCGAGGT-3'
GAPDH	5'-CGGAGTCAACGGATTTGGT CGTAT-3'	5'-AGCCTTCTCCATGGTGGTG AAGAC-3'

Table 2. Analysis of cell proliferation rate after chemotherapy

	NC (n=3) mean±SD	*miR-NC (n=3) mean±SD*	*miR-335-mimic (n=3) mean±SD*	*F*	*p*
Paclitaxel	0.38±0.02	0.37±0.02	0.27±0.01*	37.000	<0.001
Cisplatin	0.42±0.04	0.43±0.03	0.26±0.02*	28.241	0.001
Doxorubicin	0.41±0.03	0.40±0.02	0.32±0.02*	12.882	0.007

*compared with NC and MIR-NC groups, $p<0.05$.

crease of miR-335-mimic group (Figure 2, p<0.05), suggesting successful transfection.

Analysis of cell proliferation ability of MDA-MB-231 cells after chemotherapy

After paclitaxel, cisplatin and doxorubicin treatment, the proliferation rates of the three groups were significantly different (p<0.05). There was no significant difference in cell proliferation rate between NC group and miR-NC group (p>0.05). However, the proliferation rate of cells in miR-335-mimic group was higher than that in the other 2 groups (Table 2, p<0.05).

Analysis of cell apoptosis after chemotherapy

After paclitaxel, cisplatin and doxorubicin treatment, apoptosis rates of the three groups were significantly different (p<0.05). There was no significant difference in the apoptosis rate between NC group and miR-NC group (p>0.05). However, apoptotic rate was lower in the miR-335-mimic group than that in the other two groups (Table 3, p<0.05).

Correlation between miR-335 and survival of TNBC patients

Patients were divided into high (>1.168) and low (≤1.168) expression groups according to the median relative expression level of miR-335 (1.168). Kaplan-Meier survival analysis showed that the 5-year overall survival rate was 76.19% (16 cases) in the high expression group and 66.67% (14 cases) in the low expression group (Figure 3, p>0.05).

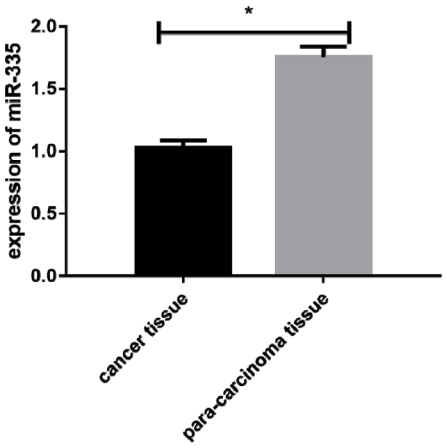

Figure 1. Relative expression levels of miR-335 in cancer and paracancer tissues of triple-negative breast cancer patients. The results of qRT-PCR showed that the relative expression level of miR-335 in cancer tissues of triple-negative negative breast cancer patients was significantly lower than that in adjacent normal tissues. *p<0.05.

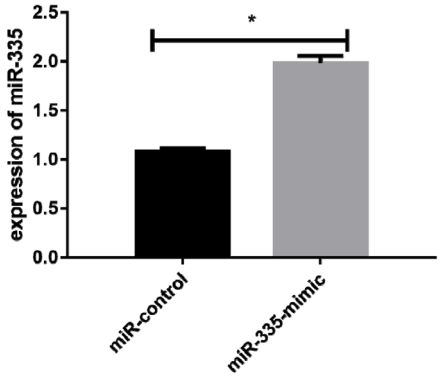

Figure 2. Analysis of MDA-MB-231 cell transfection. The results of qRT-PCR showed that the relative expression level of miR-335 was significantly increased in MDA-MB-231 cells transfected with miR-335 mimic, suggesting successful transfection. *p<0.05.

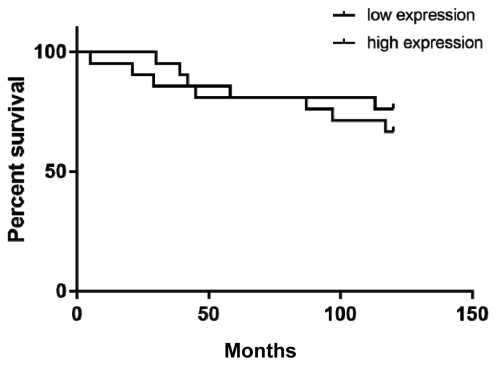

Figure 3. Correlation between miR-335 and survival in triple-negative breast cancer patients. The 5-year survival rate was 76.19% in the high-expression group and 66.67% in the low-expression group. There was no significant difference in 5-year overall survival rate between the two groups. p>0.05.

Table 3. Analysis of apoptotic rate of MDA-MB-231 cells after chemotherapy

	NC (n=3) mean±SD	*miR-NC (n=3) mean±SD*	*miR-335-mimic (n=3) mean±SD*	*F*	*p*
Paclitaxel	9.72±0.65	10.34±1.11	19.63±1.24*	89.935	<0.001
Cisplatin	11.13±1.18	12.04±1.24	20.21±1.28*	49.259	<0.001
Doxorubicin	10.07±0.96	10.59±0.85	16.47±1.32*	33.577	0.001

*compared with NC and MIR-NC groups, p<0.05.

Discussion

With the gradual increase of the incidence of TNBC and the frequent use of chemotherapy, the therapeutic effect of chemotherapy on TNBC patients is gradually reduced. Because TNBC lacks specific molecular targets, the current targeted therapy and endocrine therapy are almost ineffective [11,12]. In addition, development of drug resistance during chemotherapy also negatively affects the treatment. miRNAs may have an impact on chemosensitivity. This study explored the expression of miR-335 in TNBC and analyzed its effect on the sensitivity of commonly used chemotherapeutic drugs in order to improve the efficacy of clinical treatment of this breast cancer subtype.

Studies on the role of miR-335 in TNBC are rare. However, studies have reported that miR-335 is lower in sporadic breast cancer cells than that in normal breast cells, and it is closely related to cell proliferation and apoptosis. Overexpression of miR-335 regulates BRCA 1 and results in decreased cell viability, and increased apoptosis [13]. In our study, the expression level of miR-335 in cancer tissues of TNBC patients was significantly lower than that in adjacent normal tissues. Studies have also reported that miR-335 is down-regulated in breast cancer, and that miR-335 inhibits migration and metastasis of breast cancer cells by targeting the progenitor cell transcription factor SOX4 and the extracellular matrix component tenascin [14], which is consistent with our results. This study did not investigate the effect of miR-335 on the biological behavior of TNBC cells, but some authors [15] have reported that miR-335 may inhibit MDA-MB-231 cell migration by down-regulating the expression of MMP2 and MMP9, while no significant effect of overexpression of miR-335 on the proliferation and apoptosis of MDA-MB-231 cells was observed.

This study mainly analyzed the effect of miR-335 on the sensitivity of TNBC to chemotherapeutic drugs. We used MDA-MB-231 cells as the research object. MDA-MB-231 is an experimental cell line of TNBC. Different subtypes of breast cancer have different chemotherapy regimens. There is still no well-accepted treatment guide for TNBC. Therefore, the treatment is generally carried out according to systemic chemotherapy standards. The standard of chemotherapy is usually based on paclitaxel and platinum drugs, but TNBC often presents drug resistance [16-18]. Therefore, in order to improve the therapeutic effect of basic chemotherapy drugs, it is necessary to conduct in-depth research on factors associated with the sensitivity of these chemotherapeutic drugs in TNBC.

Recent studies have reported the chemotherapeutic sensitization capacity of miR-335. miR-335 is able to target ubiquitin ligase E3 and reverse the resistance of SNU387 and Malme3M tumor cells to paclitaxel and vincristine by increasing the expression of histone deacetylase 3 [19]. miR-335 can also regulate the resistance of SCLC cells to cisplatin, doxorubicin and other chemotherapeutic drugs by inhibiting WW domain binding protein 5 and regulating the Hippo signaling pathway [20]. We constructed a miR-335 overexpression vector transfected into MDA-MB-231 cells. After treatment with paclitaxel or cisplatin or doxorubicin for 48 h, cells were cultured under normal conditions for 48 h. There was no significant difference in cell proliferation rate and apoptosis rate between miR-NC group and NC group, indicating that the vector used in this study did not affect the sensitivity of MDA-MB-231 cells to chemotherapeutic drugs. However, the proliferation rate of miR-NC group was higher than that of miR-335-mimic group, and apoptosis was lower than that of miR-335-mimic group, indicating that overexpression of miR-335 can increase the sensitivity of MDA-MB-231 cells to paclitaxel, cisplatin and doxorubicin. However, this study failed to reveal the mechanism of action of miR-335 to increase the sensitivity of chemotherapeutic drugs. miR-335 may also function through ubiquitin ligase and WW domain binding protein 5, which will be further investigated in our future studies.

We also analyzed the correlations between the relative expression levels of miR-335 and the survival of patients with TNBC, but this study did not find a difference in 5-year overall survival rates between patients with high and low expression of miR-335. It has been reported that gastric and ovarian cancer patients with high expression of miR-335 have a higher overall survival rate than patients with low expression of miR-335, and low expression of miR-335 is an independent prognostic factor for patients with epithelial ovarian cancer [21,22]. This inconsistency may be caused by the small sample size in our study.

In summary, the expression level of miR-335 in cancer tissues of TNBC patients is lower than that in adjacent normal tissues. Overexpression of miR-335 can increase the sensitivity of tumor cells to paclitaxel, cisplatin and doxorubicin.

Conflict of interests

The authors declare no conflict of interests.

References

1. Sahlberg KK, Bottai G, Naume B et al. A serum microRNA signature predicts tumor relapse and survival in triple-negative breast cancer patients. Clin Cancer Res 2015;21:1207-14.
2. Sikov WM, Berry DA, Perou CM et al. Impact of the addition of carboplatin and/or bevacizumab to neoadjuvant once-per-week paclitaxel followed by dose-dense doxorubicin and cyclophosphamide on pathologic complete response rates in stage II to III triple-negative breast cancer: CALGB 40603 (Alliance). J Clin Oncol 2015;33:13-21.
3. Nanda R, Chow LQ, Dees EC et al. Pembrolizumab in patients with advanced triple-negative breast cancer: phase Ib KEYNOTE-012 study. J Clin Oncol 2016;34:2460-7.
4. Burstein MD, Tsimelzon A, Poage GM et al. Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. Clin Cancer Res 2015;21:1688-98.
5. Deng ZJ, Morton SW, Ben-Akiva E et al. Layer-by-layer nanoparticles for systemic codelivery of an anticancer drug and siRNA for potential triple-negative breast cancer treatment. ACS nano 2013;7:9571-84.
6. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Molec Med 2014;20:460-9.
7. Isaacs SR, Wang J, Kim KW et al. MicroRNAs in type 1 diabetes: complex interregulation of the immune system, β cell function and viral infections. Curr Diabetes Rep 2016;16:133.
8. Kong W, He L, Coppola M et al. MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. J Biol Chem 2016;291:22855.
9. Hollis M, Nair K, Vyas A et al. MicroRNAs potential utility in colon cancer: Early detection, prognosis, and chemosensitivity. World J Gastroenterol 2015;21:8284.
10. Luo Y, Tong L, Meng H et al. MiR-335 regulates the chemo-radioresistance of small cell lung cancer cells by targeting PARP-1. Gene 2017;600:9-15.
11. Lehmann BD, Jovanović B, Chen X et al. Refinement of triple-negative breast cancer molecular subtypes: implications for neoadjuvant chemotherapy selection. PLoS One 2016;11:e0157368.
12. Namini SN, Swain M, Al-Rashdan A et al. Predictors of Outcome and Patterns of Failure for High Risk Triple Negative Breast Cancer Patients Treated with Neoadjuvant Chemotherapy, Surgery and Radiation. Int J Radiat Oncol Biol Physics 2017;99:E37-8.
13. Heyn H, Engelmann M, Schreek S et al. MicroRNA miR-335 is crucial for the BRCA1 regulatory cascade in breast cancer development. Int J Cancer 2011;129:2797-806.
14. Tavazoie SF, Alarcón C, Oskarsson T et al. Endogenous human microRNAs that suppress breast cancer metastasis. Nature 2008;451:147.
15. Wu J, Gao Y, Zeng F et al. Effect of miR-335 on biological behavior of breast cancer MDA-MB-231 cells. J Third Military Med University 2015;37:1406-11.
16. Li Z, Dong J, Zou T et al. Dexamethasone induces docetaxel and cisplatin resistance partially through up-regulating Krüppel-like factor 5 in triple-negative breast cancer. Oncotarget 2017;8:11555.
17. Wen J, Yeo S, Wang C et al. Autophagy inhibition re-sensitizes pulse stimulation-selected paclitaxel-resistant triple negative breast cancer cells to chemotherapy-induced apoptosis. Breast Cancer Res Treat 2015;149:619-29.
18. Cheng CC, Shi LH, Wang XJ et al. Stat3/Oct-4/c-Myc signal circuit for regulating stemness-mediated doxorubicin resistance of triple-negative breast cancer cells and inhibitory effects of WP1066. Int J Oncol 2018;53:339-48.
19. Kim Y, Kim H, Park D et al. miR-335 targets SIAH2 and confers sensitivity to anti-cancer drugs by increasing the expression of HDAC3. Molecules Cells 2015;38:562.
20. Tang R, Lei Y, Hu B et al. WW domain binding protein 5 induces multidrug resistance of small cell lung cancer under the regulation of miR-335 through the Hippo pathway. Br J Cancer 2016;115:243.
21. Yang B, Huang J, Liu H et al. miR-335 directly, while miR-34a indirectly modulate survivin expression and regulate growth, apoptosis, and invasion of gastric cancer cells. Tumor Biol 2016;37:1771-9.
22. Cao J, Cai J, Huang D et al. miR-335 represents an independent prognostic marker in epithelial ovarian cancer. Am J Clin Pathol 2014;141:437-42.