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Correlations of the MiR-330 expression with the pathogenesis and prognosis of breast cancer

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Abstract. – OBJECTIVE: To investigate the expression of micro ribonucleic acid-330 (miR-330) in breast cancer tissues and cancer-adjacent tissues as well as the correlations of the miR-330 expression with clinicopathological features and the prognosis of breast cancer patients.

PATIENTS AND METHODS: The expression levels of miR-330 in cancer tissues and cancer-adjacent tissues of 120 breast cancer patients with complete follow-up data were detected via the reverse transcription-polymerase chain reaction (RT-PCR). Meanwhile, the expression level of miR-330 in serum of breast cancer patients was measured using enzyme-linked immunosorbent assay (ELISA). The correlations of the expression level of miR-330 with clinicopathological data and the prognosis of breast cancer patients were explored.

RESULTS: The expression level of miR-330 in breast cancer tissues was remarkably higher than that in cancer-adjacent tissues, and it was also higher in serum of breast cancer patients than that of healthy controls. In breast cancer patients with axillary lymph node metastasis, the proportion of patients with the high expression of miR-330 [30/54 (55.6%)] was markedly larger than that of patients with the low expression of miR-330 [6/30 (20%)] (p=0.003). In terms of tumor-node-metastasis (TNM) stage, the proportion of patients with the high expression of miR-330 in stage II or above was evidently larger than that of patients with the low expression of miR-330, in which the proportion was 46/60 (77.2%) in stage III and 11/15 (73.3%) in stage IV (p=0.002). Additionally, the tumor size, the histological grade, the expression of human epidermal growth factor receptor 2 (HER2), the expression of hormone receptors and the tissue type, were not related to the expression level of miR-330 (p>0.05). It was manifested in the survival curve that the median survival time was 84.8 months in the miR-330 high expression group and 96.8 months in the miR-330 low expression group, displaying a statistical difference (p=0.01).

CONCLUSIONS: MiR-330 is highly expressed in cancer tissues and serum of patients with breast cancer, and it can promote the axillary lymph node metastasis, which is an important factor affecting the prognosis of breast cancer patients. However, no obvious correlations of the expression level of miR-330 with the tumor size, the histological grade, the HER2 expression and the expression of estrogen receptors are found.

Key Words:
Breast Cancer, miR-330, Clinical features, Prognosis.

Introduction

Breast cancer is one of the most common and deadly malignant tumors in women around the world, and more than 90% of breast cancer patients eventually die from the tumor metastasis and recurrence[1]. In the meantime, a younger trend also appears in the occurrence and development of breast cancer, which is the predominant cause of death in female tumor patients aged over 45 years old[2,3]. Currently, the etiology, pathogenesis and diagnostic and therapeutic markers for breast cancer have not yet been fully clarified, bringing difficulties to the early prevention and standardized treatment of breast cancer in clinical practice[4]. Clarifying the occurrence and development mechanisms of breast cancer, therefore, is of great significance for the future clinical diagnosis and treatment of breast cancer. Micro ribonucleic acids (miRNAs) are a group of single-stranded, non-coding RNAs that are 20-24 nt in length and exist in eukaryotes[5]. By binding to specific genes, miRNAs are capable of regulating the expression of various genes, thus exerting crucial effects on such physiological activities as cell proliferation,

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differentiation and apoptosis[6]. Currently, it has been manifested that various miRNAs exert a vital effect on the pathogenesis of breast cancer. For example, the miR-21 expression is significantly increased in cancer tissues of patients with breast cancer, which has close correlations with the clinical stage, lymph node metastasis and poor prognosis[7]. *In vitro* experiments have shown that miR-1204 can target vitamin D receptor (VDR) to promote the endothelial-mesenchymal transition and metastasis of breast cancer cells, thus playing its cancer-promoting role[8]. The long-chain non-coding RNA nuclear enriched abundant transcript 1 (NEAT1) stimulates the occurrence and development of breast cancer through regulating the expressions of miR-448 and zinc finger E-box binding homeobox 1 (ZEB1)[9]. However, there is no report on the expression of miR-330 in breast cancer and its correlations with pathological features and prognosis of breast cancer currently. In this study, the specimens of clinical breast cancer patients were collected, the expression levels of miR-330 in cancer tissues and cancer-adjacent tissues were measured, respectively, and the correlations of the miR-330 expression level with clinicopathological features of patients were analyzed. Finally, the correlation between the miR-330 expression level and the prognosis of patients was analyzed by the analysis of the follow-up data of patients.

Patients and Methods

Patients

A total of 120 breast cancer patients aged (59.6±12.4) years old who underwent treatment in our hospital from December 2014 to December 2017 with complete follow-up data were enrolled. All the patients were definitely diagnosed with breast cancer by pathology, and the surgical method was the modified radical surgery. They received adriamycin, cyclophosphamide, paclitaxel (AC-T) or 5-fluorouracil, doxorubicin, cyclophosphamide (FAC) regimen after operation, and those with positive estrogen receptors were given tamoxifen endocrine therapy. Breast cancer patients with tumor size > 5 cm or metastases of more than 4 axillary lymph nodes were treated with supraclavicular region + chest wall radiotherapy. Cases excluded were those with the lung or bone metastasis alone in stage IV or and neoadjuvant chemotherapy cases.

Main Instruments and Reagents

Total RNA separation reagent TRIzol was purchased from Invitrogen (Carlsbad, CA, USA), SYBR Green Real-Time Polymerase Chain Reaction (PCR) Master Mix kit from Toyobo (Osaka, Japan), reverse transcriptases and protein kinase K from Promega (Madison, WI, USA), Real-time PCR instrument from StrateGene (San Diego, CA, USA) and the DU-600 microplate reader from Beckman Coulter (Brea, CA, USA) and primers were designed by BGI (Shenzhen, China).

MiR-330 Expression in Breast Cancer Tissues Detected via Reverse Transcription PCR (RT-PCR)

(1) The total RNA of breast cancer tissues was extracted using the TRIzol assay, and then the concentration and purity of the extracted RNA were detected via an ultraviolet spectrophotometer. When the absorbance A_{260}/A_{280}=1.8-2.0, the RNA could be used. (2) Messenger RNAs (mRNAs) were synthesized into complementary deoxyribonucleic acids (cDNAs) through RT and stored in a refrigerator at -80°C. (3) RT-PCR system: 2.5 μL 10×Buffer, 2 μL cDNAs, 0.25 μL forward primers (20 μmol/L), 0.25 μL reverse primers (20 μmol/L), 0.5 μL deoxy-ribonucleotide triphosphates (10 mmol/L), 0.5 μL Taq enzymes (2×10^6 U/L) and 19 μL double distilled water. The amplification systems for RT-PCR were identical. (4) CT value calculation: the number of cycles when the fluorescence signal in each well plate reached the set threshold was recorded. The relative quantitative method was adopted to calculate the expression level of miR-330 in cancer tissues and cancer-adjacent tissues.

Detection of the Expression Level of miR-330 in Serum via THE Enzyme-Linked Immunosorbent Assay (ELISA)

(1) 3 mL blood samples were collected from the patients. (2) Standards were prepared according to the kit instructions. (3) The standards and samples were added into each reaction well. (4) Streptavidin-horseradish peroxidase (HRP) was added for incubation. (5) The plate was washed and the color was developed. (6) After the addition of the stop buffer, the absorbance was measured via an ultraviolet spectrophotometer.

Statistical Analysis

All data were analyzed using Statistical Product and Service Solutions 22.0 (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). Count data were expressed as frequency and per-

centage, and measurement data were expressed as mean ± standard deviation. Multiple groups were compared using the one-way analysis of variance. Fisher's exact test was applied to assess the relationship between the miR-330 expression and clinicopathological features. The chi-square test was used for multiple comparisons of count data, and the *t*-test and analysis of variance (ANOVA) were applied for measurement data. The Cox regression analysis and Kaplan-Meier method were used to analyze the relationship between the miR-330 expression and the prognosis of breast cancer. $p<0.05$ represented that the difference was statistically significant.

Results

General Data

A total of 120 patients aged 24-89 years old, with an average age of (59.6±12.4) years old were included in this study. The maximum diameter of the tumor was 12.4 cm, with an average value of (4.42±2.39) cm. The specific data are shown in Table I.

Identification of the Expression of miR-330 in Breast Cancer Tissues

According to RT-PCR results, the expression level of miR-330 in breast cancer tissues was evidently higher than that in cancer-adjacent tissues ($p<0.05$), and the expression level of miR-330 in metastatic cancer tissues was also remarkably higher than that in non-metastatic cancer tissues ($p<0.05$) (Figure 1).

Determination of the Level of miR-330 in Serum of Breast Cancer Patients

Furthermore, healthy controls were tested. Figure 2 shows the expression levels of miR-330 in serum of patients with non-metastatic breast cancer and metastatic breast cancer. The results, consistent with those in the test of tissues, revealed that the expression of miR-330 in serum of patients with metastatic cancer was significantly higher than that of patients with non-metastatic breast cancer, followed by that of healthy controls.

Correlation Analyses of Clinicopathological Indicators with the miR-330 Expression

In 120 cases of breast cancer patients, there were 40 patients (33.3%) with the low expression of miR-330 and 80 patients (66.7%) with the high expression of miR-330. In breast cancer-adjacent tissues, there were 100 patients (83.3%) with the low expression of miR-330 and 20 patients (16.7%) with the high expression of miR-330. In the TNM stage, the proportion of patients with the high expression of miR-330 in stage II or above was evi-

Table I. Clinicopathological features of 120 patients with breast cancer.

Item		Case (n)	%
Age (years old)	< 30	8	6.7
	31-40	22	18.3
	41-50	30	25.0
	51-60	32	26.7
	61-70	11	9.2
	> 70	17	14.2
Tumor size (cm)	≤ 2	26	21.7
	2-5	53	44.2
	≥ 5	41	34.1
Histological grade	Grade I	41	34.1
	Grade II	30	25.0
	Grade III	49	40.9
Tumor-node-metastasis (TNM) stage	Stage I	21	17.5
	Stage II	24	20.0
	Stage III	60	50.0
	Stage IV	15	12.5
Metastatic number (n)	≤ 3	84	70.0
	> 3	36	30.0
Estrogen receptor	(-)	39	32.5
	(+)	81	67.5
Human epidermal growth factor receptor 2 (HER2)	(-)	45	37.5
	(+)	75	62.5

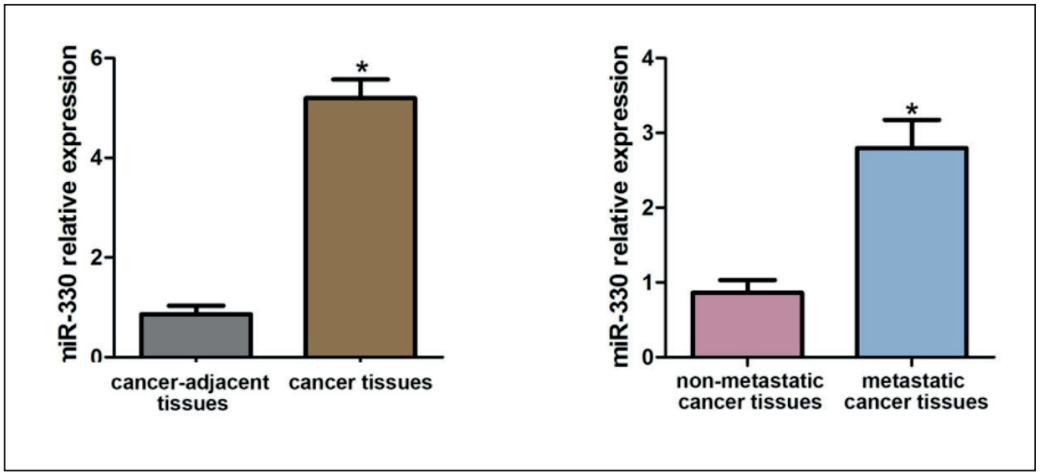

Figure 1. MiR-330 expression levels in breast cancer patients. *Represents a statistical difference compared with the previous group shown in the Figure (p<0.05).

dently larger than that of patients with the low expression of miR-330, in which the proportion was 46/60 (77.2%) in stage III and 11/15 (73.3%) in stage IV (χ^2=0.891, p=0.002). Additionally, in patients with metastasis in more than 3 lymph nodes, the proportion of the miR-330 low expression was significantly higher than that of the miR-330 high expression (χ^2=0.627, p=0.003). However, the tumor size, the histological grade, the expression of human epidermal growth factor receptor 2 (HER2), the expression of hormone receptors and the tissue type were not significantly related to the expression level of miR-330 (p>0.05) (Table II).

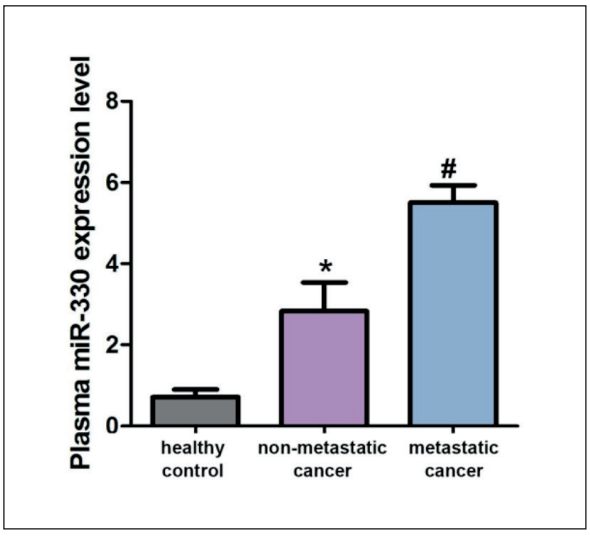

Figure 2. MiR-330 expression level in serum of breast cancer patients. *Indicates a statistical difference compared with healthy control group, and # displays a statistical difference compared with non-metastatic cancer group (p<0.05).

Correlation Analysis of the miR-330 Expression with the Prognosis of Breast Cancer Patients

The median follow-up time of 120 patients in this study was 92 months. The Kaplan-Meier analysis showed that the median survival time was 84.8 months in the miR-330 high expression group and 96.8 months in the miR-330 low expression patients, displaying a statistical difference. Log-rank analysis results manifested χ^2=0.219 and p=0.001 (Figure 3). Therefore, it was considered that the expression level of miR-330 had a notable association with the prognosis of breast cancer patients.

Discussion

In recent years, as the molecular biology technique rapidly develops and innovates, people have become more aware of the pathogenesis of tumors, and their attention gradually turns from oncogenes initially to non-coding RNAs, including miRNAs and lnRNAs. miRNA is an endogenous and highly conservative RNA with approximately 19-22 nt in length and does not encode a protein[10-13]. miRNA can incompletely or completely bind to the three prime untranslated regions of the messenger RNA (mRNA), so as to inhibit the translation of the mRNA or directly degrade[14]. At present, about 600 kinds of miRNAs have been discovered, and nearly half of them have been confirmed to be closely associated with the occurrence and development of tumors. The mechanism by which

Table I. Correlations of clinicopathological features with the expression of miR-330 in 120 patients with breast cancer.

Clinicopathological indicator		Case (n)	MiR-330		χ^2	*p*
			Low expression group	High expression group		
Total		120	40	80		
Histological grade	Grade I	41	20	21	0.231	0.942
	Grade II	30	12	18		
	Grade III	49	30	19		
TNM stage	Stage I	21	8	13	0.891	0.002
	Stage II	24	4	20		
	Stage III	60	14	46		
	Stage IV	15	4	11		
Metastatic number (n)	≤ 3	84	30	54	0.627	0.003
	> 3	36	6	30		
Tumor size (cm)	≤ 2	26	12	14	0.382	0.771
	2-5	53	24	29		
	≥ 5	41	18	23		
Estrogen receptor	(-)	39	19	20	0.229	0.612
	(+)	81	38	43		
HER2	(-)	45	15	30	0.394	0.559
	(+)	75	25	50		

miRNAs affect tumors is mainly related to the regulation of genes involved in cell proliferation, differentiation, angiogenesis and invasion, and miRNAs affect the biological behavior of tumors by activating or inhibiting these genes[15]. Due to the increased morbidity and mortality rates of breast cancer worldwide in recent years, the roles of miRNAs in the invasion and metastasis of breast cancer have also received more and more attention. For example[16], miRNA-183-5p inhibits the apoptosis and promotes the proliferation of breast cancer cells by means of the targeted inhibition of programmed cell death protein 4. MiR-26b inhibits [17] the apoptosis of breast cancer cells by inhibiting prostaglandin-endoperoxide synthase 2. Suzuki et al[18] revealed that miR-9, miR-148 and miR-34b/c can inhibit the endothelial-mesenchymal transition and metastasis of breast cancer through the targeted regulation on E2F transcription factor 3, C-myc, TGIF2 and cyclin-dependent kinase 6. In this study, the expressions of miR-330 in cancer tissues and cancer-adjacent tissues of breast cancer patients were examined, revealing that the expression level of miR-330 in cancer tissues was significantly higher than that in cancer-adjacent tissues. This finding was also confirmed by Mesci et al[19]. Besides, the correlations of the expression level of miR-330 with the clinicopathological features of breast cancer were further analyzed, demonstrating that the proportion of the high expression of miR-330 was higher in breast cancer patients with axillary lymph node metastasis and in those in stage III-IV in TNM stage. However, the ex-

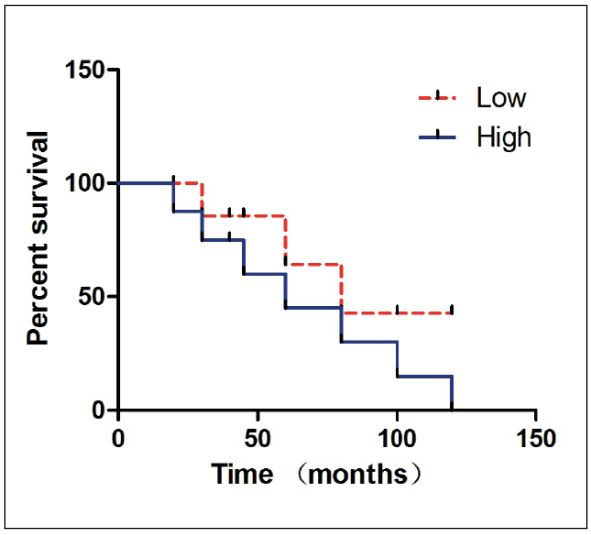

Figure 3. Correlation between the expression level of mwiR-330 and the survival rate of patients with breast cancer. Low: low expression group. High: high expression group.

pression level of miR-330 had no correlations with the expressions of estrogen receptors and HER2, histological grade and tumor size. Results of the survival analysis denoted that the median survival time of patients with the high expression of miR-330 was significantly lower than that of patients with the low expression of miR-330 (84.8 *vs.* 96.8 months), but there is no report on the specific pathogenesis of miR-330 in breast cancer. In colon cancer, miR-330 is able to inhibit the proliferation of cancer cells via the targeted inhibition of cell division control protein 42 homolog[20,21] and it also inhibits the progression of gastric cancer through the targeted inhibition of the expression of MIS1[22]. These results indicate that miR-330 may exert a bidirectional regulatory effect in the occurrence and development of tumors, so the regulation of miR-330 on breast cancer needs to be further investigated using experiments. In spite of this, certain limitations still exist in this study: 1) the clinical sample size was relatively small; 2) only malignant breast cancer patients were included in this study, and no benign lesions were considered.

Conclusions

To sum up, this study identifies for the first time that miR-330 is highly expressed in cancer tissues of patients with breast cancer, and it has a certain correlation with the tumor metastasis and the clinical stage. In the meantime, the highly expressed miR-330 is negatively related to the prognosis of breast cancer patients; however, the specific mechanism still needs to be explored in depth via experiments.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Zhou L, Zhao L C, Jiang N, Wang XL, Zhou XN, Luo XL, Ren J. MicroRNA miR-590-5p inhibits breast cancer cell stemness and metastasis by targeting SOX2. Eur Rev Med Pharmacol Sci 2017; 21: 87-94.
2) Ding Q, Wang Y, Zuo Z, Gong Y, Krishnamurthy S, Li CW, Lai YJ, Wei W, Wang J, Manyam GC, Diao L, Zhang X, Lin F, Symmans WF, Sun L, Liu CG, Liu X, Debeb BG, Ueno NT, Harano K, Alvarez RH, Wu Y, Cristofanilli M, Huo L. Decreased expression of microRNA-26b in locally advanced and inflammatory breast cancer. Hum Pathol 2018; 77: 121-129.
3) Zhao J, Jiang GQ. MiR-4282 inhibits proliferation, invasion and metastasis of human breast cancer by targeting Myc. Eur Rev Med Pharmacol Sci 2018; 22 : 8763-8771..
4) Alkawaa FM, Chaudhary K, Garmire LX. Deep learning accurately predicts estrogen receptor status in breast cancer metabolomics data. J Proteome Res 2018; 17: 337-347.
5) Shishodia G, Verma G, Das BC, Bharti AC. miRNA as viral transcription tuners in HPV-mediated cervical carcinogenesis. Front Biosci (Schol Ed) 2018; 10: 21-47.
6) Axtell MJ, Meyers BC. Revisiting criteria for plant miRNA annotation in the era of big data. Plant Cell 2018; 30: 272-284.
7) Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, Zeng YX, Shao JY. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA 2008; 14: 2348-2360.
8) Liu X, Bi L, Wang Q, Wen M, Li C, Ren Y, Jiao Q, Mao JH, Wang C, Wei G, Wang Y. miR-1204 targets VDR to promotes epithelial-mesenchymal transition and metastasis in breast cancer. Oncogene 2018; 37: 3426-3439.
9) Jiang X, Zhou Y, Sun AJ, Xue JL. NEAT1 contributes to breast cancer progression through modulating miR-448 and ZEB1. J Cell Physiol 2018; 233: 8558-8566.
10) Mirzaei H, Masoudifar A, Sahebkar A, Zare N, Sadri Nahand J, Rashidi B, Mehrabian E, Mohammadi M, Mirzaei HR, Jaafari MR. MicroRNA: a novel target of curcumin in cancer therapy. J Cell Physiol 2018; 233: 3004-3015.
11) Li JH, Sun SS, Fu CJ, Zhang AQ, Wang C, Xu R, Xie SY, Wang PY. Diagnostic and prognostic value of microRNA-628 for cancers. J Cancer 2018; 9: 1623-1634.
12) Tsai HP, Huang SF, Li CF, Chien HT, Chen SC. Differential microRNA expression in breast cancer with different onset age. PLoS One 2018; 13: e0191195.
13) Dufresne S, Rebillard A, Muti P, Friedenreich CM, Brenner DR. A review of physical activity and circulating-miRNA expression: implications in cancer risk and progression. Cancer Epidemiol Biomarkers Prev 2018; 27: 11-24.
14) Wang V, Wu W. MicroRNA-based therapeutics for cancer. Biodrugs 2009; 23: 15-23.
15) Fujii H, Chiou T J, Lin SI, Aung K, Zhu JK. A miRNA involved in phosphate-starvation response in Arabidopsis. Curr Biol 2005; 15: 2038-2043.
16) Cheng Y, Xiang G, Meng Y, Dong R. MiRNA-183-5p promotes cell proliferation and inhibits apoptosis in human breast cancer by targeting the PDCD4. Reprod Biol 2016; 16: 225-233.
17) Jia L, Kong X, Zhang J, Luo Q, Li X, Fang L. MiRNA-26b inhibits proliferation by targeting PTGS2 in breast cancer. Cancer Cell Int 2013; 13: 7.

18) Suzuki M, Yoshino I. Identification of microRNAs caused by DNA methylation that induce metastasis. Future Oncol 2008; 4: 775-777.

19) Mesci A, Huang X, Taeb S, Jahangiri S, Kim Y, Fokas E, Bruce J, Leong HS, Liu SK. Targeting of CCBE1 by miR-330-3p in human breast cancer promotes metastasis. Br J Cancer 2017; 116: 1350-1357.

20) Li Y, Zhu X, Xu W, Wang D, Yan J. miR-330 regulates the proliferation of colorectal cancer cells by targeting Cdc42. Biochem Biophys Res Commun 2013; 431: 560-565.

21) Li HG, Zhao LH, Bao XB, Sun PC, Zhai BP. Meta-analysis of the differentially expressed colorectal cancer-related microRNA expression profiles. Eur Rev Med Pharmacol Sci 2014, 18: 2048-2057.

22) Guan A, Wang H, Li X, Xie H, Wang R, Zhu Y, Li R. MiR-330-3p inhibits gastric cancer progression through targeting MSI1. Am J Transl Res 2016; 8: 4802-4811.