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Original Article

Changes in miR-29 levels and their predictive value of a cure after diamminedichloroplatinum chemotherapy in patients with nasopharyngeal carcinoma

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Abstract: Objective: This study aimed to investigate the serum expressions of miR-29a/b/c before and after diamminedichloroplatinum administration in patients with nasopharyngeal carcinoma and their predictive roles for chemotherapy efficacy. Methods: 86 patients with the clinical stages of II-IVa nasopharyngeal carcinoma were enrolled as the study cohort. An ROC curve was used to analyze the predictive performance of the miR-29a, miR-29b, and miR-29c expression levels before chemotherapy to determine the diamminedichloroplatinum treatment efficacy. A Kaplan-Meier curve was used to analyze the relationship between miR-29a/b/c expressions after chemotherapy and the 5-year survival rate. Results: The AUC, threshold, sensitivity, and specificity of miR-29a in predicting the chemotherapy efficacy were 0.609, 1.947, 80.34%, and 38.67%, respectively. The AUC, threshold, sensitivity, and specificity of miR-29b in predicting the chemotherapy efficacy were 0.641, 3.276, 72.23%, and 62.67%, respectively. The AUC, threshold, sensitivity, and specificity of miR-29c in predicting the chemotherapy efficacy were 0.713, 1.394, 90.91%, and 42.67%, respectively. The AUC, sensitivity, and specificity of combined miR-29a/b/c in predicting the chemotherapy efficacy were 0.775, 72.73% and 74.67%, respectively. A Kaplan-Meier survival analysis showed that the 5-year survival rate of patients with high expressions of miR-29a and miR-29b before and after chemotherapy was lower than the rate of patients with low expressions of miR-29a and miR-29b ($P<0.05$). The 5-year survival rate of patients with high expressions of miR-29c before and after chemotherapy was higher than it was in patients with a low expression of miR-29c ($P<0.05$). Conclusion: After chemotherapy, the expressions of miR-29a/b were decreased, and the expression of miR-29c was increased. The combined miR-29a/b/c expressions have a good predictive value for the chemotherapy effect of diamminedichloroplatinum and prognosis.

Keywords: Nasopharyngeal carcinoma, diamminedichloroplatinum, miR-29, efficacy, prognosis

Introduction

Nasopharyngeal carcinoma originates from nasopharyngeal epithelial tissues. It often occurs in people over 40 years old, predominantly affecting male patients (1:100,000) [1, 2]. The early diagnosis rate of nasopharyngeal carcinoma is low. About 40%-80% of patients are initially diagnosed with lymph node or distant metastasis, and they have a poor prognosis and a 50% survival rate at five years [3, 4]. At present, the pathogenesis of nasopharyngeal carcinoma has not been fully studied. It is mainly considered to be a multi-gene hereditary disease. In addition, it is related to viral infections and chromosomal abnormalities [5].

miRNA is widely expressed in eukaryotic cell organisms. It is a short (18-25 nucleotide) single-stranded RNA molecule. It can regulate a variety of biological activities such as cell proliferation, invasion, apoptosis, and differentiation by binding to the 3'-untranslated region of target mRNAs and controlling gene expression [6, 7]. In recent years, some studies have found that miRNAs can regulate the development and progression of nasopharyngeal carcinoma by controlling specific mRNAs. As a key modifier of the extracellular matrix (ECM) steady state, the miR-29 family is found in various tissues. The upregulation of miR-29 can directly target apoptosis-related factors and extracellular matrix proteins to induce tumor cell apoptosis. Its tu-

Table 1. Primer sequence

	Forward primer	Reverse primer
miR-29a	CCGTCCTCCGTAGCACCATCTGAAAT	CTCAACTGGTGTCGTGGAGTCGGC
miR-29b	CGCTCCTCCGTAGCACCATTTGAAATC	CTCAACTGGTGTCGTGGAGTCGGC
miR-29c	TGACCGATTTCTCCTGGTGTTC	GCGAGCACAGAATTAATACGAC
U6	GCGCGTCGTGAAGCGTTC	GTGCAGGGTCCGAGGT

morigenicity is reduced [8, 9]. Qiu et al. [10] reported that miR-29a/b is lowly expressed in nasopharyngeal carcinoma tissues and serum. Over-expressed miR-29a/b inhibits SPARC and COL3A1, and promotes the migration and invasion of nasopharyngeal carcinoma cells. Liu et al. [11] also reported that the miR-29c ectopic expression for targeting TIAM1 inhibits the migration and invasion of nasopharyngeal carcinoma cells in vitro, and inhibits the lung metastasis of nasopharyngeal carcinoma cells. Xiong et al. [12] reported that miR-29 expression is low in hepatocellular carcinoma tissues. The down-regulation is associated with the poorer disease-free survival rate of patients with liver cancer. What's more, miR-29 can make hepatocellular carcinoma more sensitive to serum starvation, anoxia, and chemotherapy, triggering apoptosis. It plays a potential role in the prognosis and treatment evaluation of liver cancer. Chemotherapy is also the main method for treating nasopharyngeal carcinoma. There are few studies about the influence of miR-29 on the efficacy of chemotherapy for patients with nasopharyngeal carcinoma.

This study analyzed the miR-29 expression after administering diamminedichloroplatinum in patients with nasopharyngeal carcinoma and explored the predictive performance of miR-29 for diamminedichloroplatinum treatment efficacy.

Materials and methods

Research subjects

86 patients with the clinical stages of II-IVa nasopharyngeal carcinoma were enrolled as the study cohort from August 2011 to February 2014. Inclusion criteria: II-IVa nasopharyngeal carcinoma patients diagnosed by pathology and in line with the AJCC/UICC 8th edition staging criteria [13] for nasopharyngeal carcinoma. Patients who did not undergo any chemoradiotherapy. Patients with cisplatin included in their current chemotherapy regimens. Patients who did not have any abnormal bleeding or coagulopathy. All the patients had complete medical records and follow-up data. All the patients and their families signed the informed consents, and the medical staff were required to follow the Helsinki Declaration. The study was approved by the Medical Ethics Committee of our hospital.

Exclusion criteria: Patients with other benign tumors or malignant tumors. malnourished patients. Patients with severe organ dysfunction. Patients with a history of tumors, or pregnant or lactating women.

Treatment methods

Chemotherapy with cisplatin includes cisplatin monotherapy, gemcitabine (1000 mg/m^2 ivgtt, at the first day and the eighth day) + cisplatin (GP regimen), docetaxel (100 mg/m^2 ivgtt, on the first day) + cisplatin (TP regimen), docetaxel (75 mg/m^2 ivgtt, at the first day) + cisplatin + 5-fluorouracil (400 mg/m^2 civ 96 hours, from the first to the fifth days) (TPF regimen), cisplatin + 5-fluorouracil (1000 mg/m^2 civ 96 hours, from the first to the fourth days) (PF regimen). For all the cisplatin regimens, the cisplatin was administered at a dose of 75 mg/m^2, administrated from the first to the fifth days. All the patients received at least 3 cycles of chemotherapy, with each cycle lasting 21 days. Following the end of chemotherapy, docetaxel or pemetrexed was used for the maintenance treatment. The patients were followed up regularly every 3 months for 5 years by telephone or in-person interview to record their health status and their 5-year survival rate.

qRT-PCR

Peripheral blood from all patients was collected before the chemotherapy and at three days after three cycles of chemotherapy, and serum was obtained after centrifugation. The total serum RNA was extracted by TRIzol (Guangzhou Lanji Biotechnology Co., Ltd.). The specific steps were conducted following the kits' instructions. The micro-ultraviolet spectropho-

Table 2. General information

	General information (n = 86)
Sex [n (%)]	
Men	60 (69.77)
Women	26 (30.23)
Age (years)	46.35±10.77
BMI	23.85±2.76
Clinical stages [n (%)]	
II	14 (16.28)
III	44 (51.16)
IV	28 (32.56)
Differentiated degree [n (%)]	
Medium and high differentiation	60 (69.77)
Poor differentiation	26 (30.23)
T stage [n (%)]	
T1 + T2	53 (61.63)
T3 + T4	33 (38.37)
N stage [n (%)]	
N0	21 (21.42)
N1 + N2 + N3	65 (75.58)
Smoking [n (%)]	
Yes	27 (31.40)
No	59 (68.60)
Family history [n (%)]	
Yes	16 (18.60)
No	70 (80.40)
EBV-DNA [n (%)]	
Positive	41 (47.67)
Negative	45 (52.33)
Tumor site [n (%)]	
After the wall	39 (45.35)
Side wall	29 (33.72)
Inferior wall	18 (20.93)
The nasal invasion [n (%)]	
Yes	60 (69.77)
No	26 (30.23)
5 year Relapse [n (%)]	
Yes	46 (53.49)
No	40 (46.51)
5 year Prognosis [n (%)]	
Survival	65 (75.58)
Death	21 (24.42)

tometer DanoProp1000 (Thmorgan Biotechnology Co., Ltd.) was used to analyze the concentration and purity of the extracted RNA. The 3% Sepharose gel electrophoresis (the gel electrophoresis kit was purchased from Shanghai Jingke Chemical Technology Co., Ltd.) was used to analyze the integrity of the RNA. It is qualified when the A260/A280 value was between 1.8 and 2.1. After the RNA extraction, the qRT-PCR reaction was carried out. The reverse transcription reaction system was 5*PrimerScript Buffer 2 μL, PrimerScript RT Enzyme Mix 0.5 μL, Random 6 mers (100 μM) 0.5 μL, Oligo dT Primer (50 μM) 0.5 μL, and total RNA 2 μg. Ribonuclease-free distilled water was added to obtain the final 10 μL solution. The reverse transcription reaction was conducted at 37°C for 15 min; the reverse transcriptase inactivation reaction was conducted at 85°C for 5 s; the reaction was completed at 4°C; after that, the PCR amplification was carried out. The PCR amplification system was cDNA template 4 μL, SYBR Green Mix (2x) 25 μL, upstream primer and downstream primer 1 μL each, reference dye (optional) 1 μL, and double distilled water 50 μL. After predenaturation at 95°C for 3 min, the denaturation was performed at 95°C for 30 s, annealed at 55°C for 30 s, and extended at 72°C for 60 s, for 30 cycles total. The extension was conducted at 72°C for 5 minutes after the completion of the cycle. U6 was used as the reaction internal reference. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer sequence was designed and synthesized by Hepeng (Shanghai) Biotechnology Co., Ltd. (**Table 1**).

Evaluation criteria

The patient received the first review within the first week after chemotherapy. The efficacy was evaluated after the end of the chemotherapy according to the response evaluation criteria in solid tumors (RECIST) 1.1: complete remission (CR): all lesions disappear, which lasts for more than 4 weeks; partial remission (PR) : the sum of the largest single path of the tumor has been decreased by over 30%, which lasts for more than 4 weeks; stable condition (SD): the sum of the largest single path of the tumor has not been decreased by over 30% or increased by over 20%, which lasts for more than 4 weeks; disease progression (PD): the sum of the largest single path of the tumor has been increased by over 20% or

Table 3. Analysis of the curative effect

	n = 86 [n (%)]
CR	33 (38.37)
PR	42 (48.84)
SD	9 (10.46)
PD	2 (2.33)
Effective rate	75 (87.21)
Ineffective rate	11 (12.79)

new lesions appear. Effective rate = CR + PR, ineffective rate = SD + PD.

Outcome measures

The changes in the expression levels of miR-29a, miR-29b, and miR-29c in the peripheral blood were compared before and after the treatment. An ROC curve was used to analyze the predictive values of the miR-29a, miR-29b, and miR-29c expression levels before chemotherapy for the patients treated with diamminedichloroplatinum. A Kaplan-Meier curve was used to analyze the relationship between the expression levels of miR-29a/b/c before and after chemotherapy and the 5-year survival of patients with nasopharyngeal carcinoma.

Statistical analysis

SPSS 19.0 (AsiaAnalytics formerly SPSS China) was used in this study. The enumeration data were expressed as the rate, and the rates were compared using χ^2 tests. The measurement data was expressed as the mean ± sd, and paired t-tests were used before and after the treatment while independent sample t tests were used for the comparisons between groups. A survival curve was drawn using the Kaplan-Meier method, and an ROC curve was used to analyze the predictive value for the efficacy. The logistics regression prediction model was used to detect the predictive value of the combined detection. P<0.05 indicated a statistically significant difference.

Results

General information

Among the 86 patients with nasopharyngeal carcinoma, 60 (69.77%) patients were male, 26 (30.23%) patients were female, the average age was (46.35±10.77) years old, 14 patients (16.28%) were in stage II, 44 patients (51.16%) were in stage III, 28 patients (32.56%) were in stage IV, and additional basic information is shown in **Table 2**.

Analysis of the curative effect

The CR of the 86 patients who received diamminedichloroplatinum for nasopharyngeal carcinoma was 38.37% (33 cases), the PR was 48.84% (42 cases), the SD was 10.46% (9 cases), and the PD was 2.33% (2 cases). The effective rate was 87.21% (75 cases) and the ineffective rate was 12.79% (11 cases) (**Table 3**).

Changes in the expression levels of miR-29 before and after the chemotherapy for nasopharyngeal carcinoma

After chemotherapy, the relative expression levels of miR-29a and miR-29b in the peripheral blood of the nasopharyngeal carcinoma patients were lower than those before chemotherapy (P<0.05), while the relative expression of miR-29c was higher than it was before the chemotherapy (P<0.05) (**Table 4**).

The predictive value of miR-29 expression before chemotherapy for diamminedichloroplatinum treatment efficacy

The patients with an effective outcome were placed into the effective group, and the patients with an ineffective outcome were placed in the ineffective group. The relative expression levels of miR-29a, miR-29b, and miR-29c in the peripheral blood of the two groups are displayed in **Table 5**. The relative expression level of miR-29a in the effective group was lower than it was in the ineffective group (P<0.05), but the miR-29c expression was higher in the effective group than it was in the ineffective group (P<0.05). There was no significant difference in miR-29b expression levels in the two groups (P>0.05). The AUC, threshold, sensitivity, and specificity of miR-29a in predicting the efficacy of diamminedichloroplatinum for nasopharyngeal carcinoma before chemotherapy were 0.609, 1.947, 80.34%, and 38.67%, respectively. Before chemotherapy, the AUC, threshold, sensitivity, and specificity of miR-29b in predicting the efficacy of diamminedi-

Table 4. Changes in the expression levels of miR-29 before and after chemotherapy for nasopharyngeal carcinoma

	Before Chemotherapy (n = 86)	After Chemotherapy (n = 86)	T	P
miR-29a	2.234±0.679	1.822±0.549	4.376	<0.001
miR-29b	3.206±1.095	2.522±1.040	4.200	<0.001
miR-29c	1.189±0.573	1.512±0.738	3.206	0.002

Table 5. miR-29 expression levels before chemotherapy

	Effective Group (n = 75)	Ineffective Group (n = 11)	T	P
miR-29a	2.166±0.651	2.699±0.720	2.493	0.015
miR-29b	3.133±1.128	3.702±0.685	1.625	0.108
miR-29c	1.242±0.561	0.825±0.546	2.310	0.023

Table 6. The predictive value of serum miR-29 expression before chemotherapy for diamminedichloroplatinum treatment efficacy

	miR-29a	miR-29b	miR-29c	Combination
AUC	0.609	0.641	0.713	0.775
95% CI	0.542-0.838	0.555-0.826	0.536-0.851	0.651-0.899
Cut off	1.947	3.276	1.394	
Sensitivity%	80.34	72.23	90.91	72.73
Specificity%	38.67	62.67	42.67	74.67

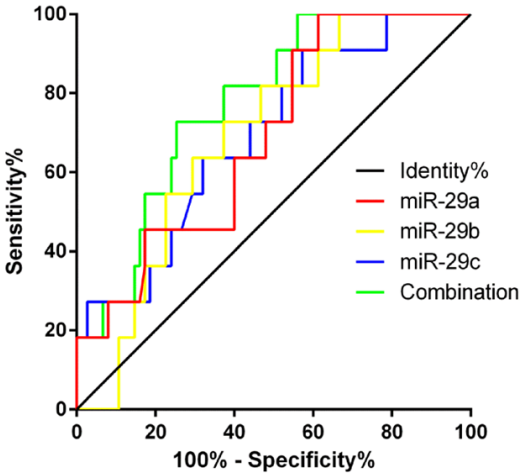

Figure 1. Predictive value of the miR-29 expression level before chemotherapy for the curative effect of nasopharyngeal carcinoma diamminedichloroplatinum. AUC (miR-29a) = 0.609, AUC (miR-29b) = 0.641, AUC (miR-29c) = 0.713, AUC (combination) = 0.775.

chloroplatinum for nasopharyngeal carcinoma were 0.641, 3.276, 72.23%, and 62.67%, respectively. The AUC, threshold, sensitivity, and specificity of miR-29c in predicting the efficacy of diamminedichloroplatinum for nasopharyngeal carcinoma before chemotherapy were 0.713, 1.394, 90.91%, and 42.67%, respectively. The AUC, sensitivity, and specificity of combined miR-29a, miR-29b, and miR-29c in predicting the efficacy of diamminedichloroplatinum for nasopharyngeal carcinoma before chemotherapy were 0.775, 72.73%, 74.67%, respectively (**Table 6**; **Figure 1**).

The relationship between miR-29 expression and the 5-year survival rate before and after chemotherapy

According to the median expression levels of miR-29a/b/c after chemotherapy, the patients were divided into high and low expression groups. A Kaplan-Meier survival analysis showed that the 5-year survival rate of the patients with high expressions of miR-29a and miR-29b before and after chemotherapy was lower than the rate of the patients with low expressions of miR-29a and miR-29b (P<0.05). The 5-year survival rate of patients with high expressions of miR-29c before and after chemotherapy was higher than the survival rate of the patients with a low expression of miR-29c (P<0.05) (**Figure 2**).

Discussion

The incidence and mortality of nasopharyngeal carcinoma have been increasing for years [14]. Chemotherapy is the main method for treating advanced nasopharyngeal carcinoma, but the overall therapeutic effect is still not very satisfactory. How to effectively evaluate a patient's therapeutic effect is of great significance to the implementation of the patient's follow-up treatment plan.

miRNAs play a similar role with oncogenes or tumor suppressor genes. They are important in tumor diagnosis, treatment and prognosis evaluation [15, 16]. The miR-29 family includes miR-29a, miR-29b, and miR-29c. There are only two or three different bases among the three

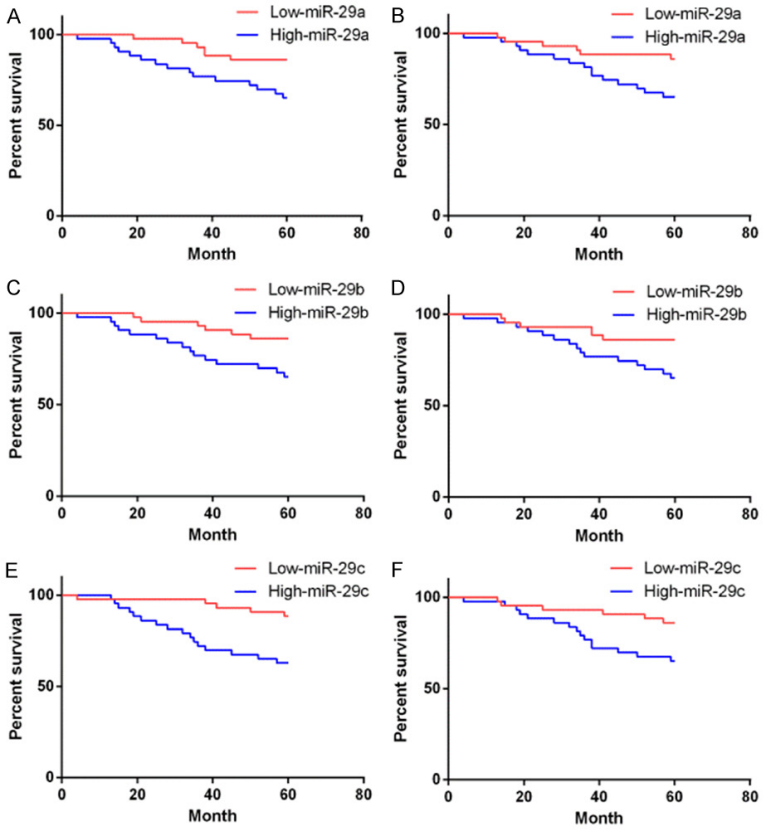

Figure 2. The relationship between miR-29 expressions before and after chemotherapy and the 5-year survival rates of patients with nasopharyngeal carcinoma. A. miR-29a expression before chemotherapy and 5-year survival. B. miR-29a expression after chemotherapy and 5-year survival. C. miR-29b expression before chemotherapy and 5-year survival. D. miR-29b expression after chemotherapy and 5-year survival. E. miR-29c expression before chemotherapy and 5-year survival. F. miR-29c expression after chemotherapy and 5-year survival.

members. In many tumors, miR-29a, miR-29b, and miR-29c can inhibit the expression of the PI3-kinase subunit and CDC42, promote the expression of p53, and induce apoptosis [17, 18]. Over-expressed miR-29a promotes the epithelial mesenchymal conversion and accelerates breast cancer with the oncogenic signaling pathway RAS [19]. miR-29b can inhibit the leukemia 1 protein expression of KMCH cell line myeloid cells. It also can make tumor cells sensitive to the apoptosis-inducing ligands related to the tumor necrosis factor, thereby regulating apoptosis. The myeloid cell leukemia 1 (MCL-1) protein is a multi-domain anti-apoptotic protein [20]. The expression of miR-29c is down-regulated at different degrees in many different tumors, and it can regulate tumor-related genes such as MCL-1 and T cell leukemia 1 (TCL-1) and play a role in the occurrence and development of tumors [21].

In their study of the miR-29 family and nasopharyngeal carcinoma, Luo et al. [22] found that the expressions of miR-29a and miR-29c were down-regulated in metastatic nasopharyngeal carcinoma. However, no change in the miR-29b level was found. The study by Qiu et al. [10] found that the expressions of miR-29a/b in nasopharyngeal carcinoma tissue are lower than they are in normal paracancerous tissues. Over-expressed miR-29b can inhibit the cell cycle progression of nasopharyngeal carcinoma cells at the G1/S phase. In contrast, miR-29a has little effect on the progression of the nasopharyngeal carcinoma cell cycle. A high expression of miR-29a/b can promote the migration and invasion of nasopharyngeal carcinoma cells. We found that the 5-year survival rates of patients with high and low expressions of serum miR-29b were 57.3% and 78.6%. There was no significant difference in the overall survival rate between the high and low expression groups of miR-29a. Zhang et al. [23] showed that the down-regulation of mir-29c in tumors was closely related to poor tumor prognosis. Over-expressed miR-29c can make nasopharyngeal carcinoma cells sensitive to cisplatin and can also promote apoptosis.

This study mainly analyzed the predictive value of the miR-29 family for patients with nasopharyngeal carcinoma treated with diamminedichloroplatinum. The miR-29a/b expression levels after chemotherapy were decreased, and the miR-29c expression level was increased compared with the levels before chemotherapy. The miR-29a/b/c expression levels before chemotherapy were used to predict the curative effect. The results showed that the curative effect AUC of the combined detection of miR-29a/b/c in predicting nasopharyngeal diamminedichloroplatinum was 0.775, and the

specificity was 76.47%. However, the sensitivity was less than 90.91% of the miR-29c single prediction. The lower sensitivity of the combined detection may be related to our experimental method-parallel testing of the combined detection. Other tumor markers were not detected in this study. There are few reports on the predictive efficacy of miR-29a/b/c. In future studies, other tumor markers combined with miR-29a/b/c may be included, which could improve the predictive value of the miR-29a/b/c efficacy. The relationship between miR-29a/b/c and the 5-year survival rate in patients with nasopharyngeal carcinoma was also analyzed. The 5-year survival rate of patients with high expressions of miR-29a/b before and after chemotherapy was lower than it was in the patients with low expressions of miR-29a/b. The 5-year survival rate of patients with high expressions of miR-29c before and after chemotherapy was higher than it was in patients with a low expression of miR-29c. This finding verifies the value of miR-29a/b/c in the prognosis evaluation of patients with nasopharyngeal carcinoma.

There are some shortcomings in this study. Due to the limitation of cases, this study enrolled patients receiving chemotherapy with different cisplatin regimens but did not further explore t whether different cisplatin regimens brought different chemotherapy outcomes or not. Also, the real value of miR-29a/b/c combined detection for predicting the efficacy of cisplatin chemotherapy failed to meet our expectations before the study, which may be related to the statistical method we used - the logistic regression prediction model. We will use more analytical methods in future studies, and will continue to include qualified patients to make up for the case shortage.

In summary, the expressions of miR-29a/b after chemotherapy were lower than they were before chemotherapy, and the expression of miR-29c was higher than it was before chemotherapy. The combined miR-29a/b/c has some predictive performance for diamminedichloroplatinum, and miR-29a/b/c are closely related to the prognosis of patients. The 5-year survival rate of patients with high expressions of miR-29a/b and a low expression of miR-29c is lower.

Disclosure of conflict of interest

None.

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