Original Article
Clinical diagnostic value of combined detection of microRNA-373 and microRNA-204 expression in retinoblastoma

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Abstract: Objective: To study microRNA-373 and microRNA-204 expression in retinoblastoma and its clinical diagnostic value. Methods: The cancer tissues and peripheral blood of 32 patients with Retinoblastoma (RB) who underwent enucleation of the eyeball from March 2017 to August 2018 were selected as the study group. Healthy retinal tissues and peripheral blood of 15 corneal transplantation donors in the same period were selected as the control group. The expression levels of microRNA-373 and microRNA-204 were detected by qRT-PCR. The ROC curve was used to analyze the diagnostic value of microRNA-373 and microRNA-204. The study group were followed up for 5 years by telephone or SMS. The Kaplan-Meier survival curve was used to analyze the relationship between microRNA-373, microRNA-204 and the prognosis of patients. Results: The expression level of microRNA-373 in tumor tissues of the study group was significantly higher than that in the control group (P < 0.05). The expression level of microRNA-204 in tumor tissues of the study group was significantly lower than the control group (P < 0.05). The 5-year survival rate of the low microRNA-373 expression group was higher than that of the high microRNA-373 expression group (P = 0.024). There was no difference in 5-year survival rate between the low microRNA-204 expression group and the high microRNA-204 expression group (P = 0.133). The expression level of microRNA-373 in the study group was significantly lower than the control group (P < 0.05). Conclusion: The diagnostic value of combined detection of microRNA-373 and microRNA-204 was higher than single factor alone. The 5-year survival rate of patients with high microRNA-373 expression was lower.

Keywords: MicroRNA-373, microRNA-204, retinoblastoma (RB)

Introduction

Retinoblastoma (RB) is a kind of ophthalmic cancer caused by mutation of the retina which has been a hot topic of tumor study in recent years [1]. RB is more likely to occur in children under 5 years old, and the incidence of RB is about 7%-45%. About 70% of the patients suffer from unilateral intraocular tumors, which is the first tumor found with genetic basis. About 40% of RB is hereditary [2, 3]. Although the survival rate of patients with RB is very high, the mortality rate can’t be ignored because RB is prone to other secondary malignant tumors. The prognosis of RB patients found in an early stage is better, and the outcome rate of RB patients can reach 95% survival [4].

Pathological examination is still the golden standard for diagnosis of tumors. Due to traumatic examination, it is difficult to popularize in clinic. With the development of CT and nuclear magnetic resonance technology, less invasive methods provide for the diagnosis of RB. However, there are still some patients who can’t be diagnosed in this manner because of the similar clinical manifestations to metastatic endophthalmitis and Coats [5]. The sensitivity and specificity of existing molecular markers such as carcinoembryonic antigen (CEA) for RB diagnosis are also not ideal [6]. Therefore, it is necessary to find a sensitive and specific molecular marker to improve the early diagnosis rate and prognosis of children. MicroRNA is a small, non-coding endogenous RNA, which is widely expressed in eukaryotic cells. Gene expression is regulated by the degradation or translation inhibition of mRNA after microRNA and protein form a RNA-induced silencing complex [7].
More and more evidence have shown that microRNAs play an important role in the occurrence and development of tumors [8, 9]. In the screening of biomarkers in RB tumors, it was found that the expression of microRNA-373 in tumor tissues was 4 times higher than that in adjacent tissues [10]. It has also been shown that microRNA-204 was down-regulated in RB and participates in the proliferation and invasion of RB tumor cells. Up-regulation of microRNA-204 expression can inhibit the proliferation and invasion of RB tumor cells [10]. However, the diagnostic value of both microRNAs in RB has not been reported.

This study explored the combined detection of microRNA-373 and microRNA-204 expression in RB and its clinical diagnostic value.

Material and methods

Study subjects

The cancer tissues and peripheral blood of 32 patients with RB who underwent enucleation of the eyeball in our hospital from March 2017 to August 2018 from were selected as the study group. Normal retinal tissues and the corresponding peripheral blood of 15 corneal transplantation donors in the same period were selected as the control group. Inclusion criteria: All patients were diagnosed with RB by pathological examination after resection of tumors in our hospital. No cryocoagulation, radiotherapy (RT) and chemotherapy (CT) were performed before the operation, no organ dysfunction was found, and the medical records were complete. Exclusion criteria: Patients with preoperative metastasis of tumors or advanced tumors; patients with other malignant tumors, congenital heart disease and other congenital diseases; patients with jaundice; patients with a history of cancer; or patients with abnormal bleeding or coagulation dysfunction. This study was approved by the Hospital Ethics Association, and informed consent was given by patients or their relatives.

Outcome measures

The expression levels of microRNA-373 and microRNA-204 in tissues and plasma of the two groups were detected by qRT-PCR. The ROC curve was used to analyze the diagnostic value of single and combined detection of microRNA-373 and microRNA-204 in RB. The patients in the study group were followed for 5 years by telephone or SMS. The Kaplan-Meier survival curve was used to analyze the relationship between microRNA-373 and microRNA-204 and the prognosis of patients.

qRT-PCR

After the grinding and comminution of the cancer tissues, 1 ml TRIzol lysate was added to extract the total RNA from the tissues. The total RNA was extracted from the plasma by 3:1 ratio of TRIzol lysate. After the extraction, the integrity of RNA was analyzed by 1.5% agarose gel electrophoresis, and the purity of RNA was detected by the micro nucleic acid analyzer. The value of A260/A280 in 1.8-2.1 was considered to meet the experimental requirements. After RNA extraction, the reverse transcription reaction was carried out. The first cDNA strand was synthesized and amplified by PCR. The PCR amplification system consisted of 2 μL cDNA template, 1 μL cDNA dilution, 5 μL 2*SYBR Green mixture, 1 μL upstream primer, 1 μL downstream primer, and 10 μL distilled water. After denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds over 40 cycles, the dissolution curve was analyzed. GAPDH was used as the internal reference of the reaction. All samples were repeated in 3 wells, and the results were analyzed by 2-ΔΔCt method. TRIzol™ Reagent was purchased from Chengdu East Creative Technology Co., Ltd. with the article number 1559026. SYBER GREEN Real-time Fluorescence Quantitative PCR Kit was purchased from Nanjing Cobioer Biotechnology Co., Ltd. with the article number 430151. The primer sequence was designed and synthesized by Hepeng (Shanghai) Biotechnology Co., Ltd. (Table 1).

Statistical methods

SPSS 19.0 (Asia Analytics Formerly SPSS China) was used to analyze the data. The measurement data were expressed as % and χ² test was used for the comparison of rate. The enumeration data were expressed as mean ± standard deviation (mean ± sd), and the independent sample t test was used for the comparison between the two groups. The ROC curve was used to analyze the diagnostic value of microRNA-373 and microRNA-204 in RB. The Kaplan-Meier survival curve was used to analyze the relationship between microRNA-373 and micro-
Combined detection of MicroRNA-373 and MicroRNA-204 expression

Table 1. Primer sequence

<table>
<thead>
<tr>
<th></th>
<th>Upstream</th>
<th>Downstream</th>
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<tbody>
<tr>
<td>miR-373</td>
<td>5'-GACGGCTCGAGGACCAAGG-GGCTGTATGCAC-3'</td>
<td>5'-GCCAGAAGCT-TCTGCTGTTCATCTGCAGG-3'</td>
</tr>
<tr>
<td>miR-204</td>
<td>5'-GGGCGCAAAGAATTCTCCT-3'</td>
<td>5'-GTGCAGGGTCGGAGGT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CGGAGTCAACGGATTTGTGTGTAT-3'</td>
<td>5'-AGCCTTCTCCATGGTGTGAAGAC-3'</td>
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</table>

Table 2. General data

<table>
<thead>
<tr>
<th></th>
<th>Study group (n = 32)</th>
<th>Control group (n = 15)</th>
<th>χ²/t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender [n (%)]</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>18 (56.25)</td>
<td>8 (53.33)</td>
<td>0.006</td>
<td>0.078</td>
</tr>
<tr>
<td>Female</td>
<td>14 (43.75)</td>
<td>7 (46.67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>3.8 ± 1.4</td>
<td>4.6 ± 2.1</td>
<td>1.550</td>
<td>0.128</td>
</tr>
</tbody>
</table>

Table 3. Expression levels of microRNA-373 and microRNA-204 in tissues

<table>
<thead>
<tr>
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<th>Control group (n = 15)</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MicroRNA-373</td>
<td>3.518 ± 1.721</td>
<td>2.306 ± 1.606</td>
<td>2.279</td>
<td>0.026</td>
</tr>
<tr>
<td>MicroRNA-204</td>
<td>1.598 ± 0.766</td>
<td>2.881 ± 1.713</td>
<td>3.573</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 4. Diagnostic value of microRNA-373 and microRNA-204 in tissues in RB

<table>
<thead>
<tr>
<th></th>
<th>miR-373</th>
<th>miR-204</th>
<th>miR-373 and miR-204</th>
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</thead>
<tbody>
<tr>
<td>AUC</td>
<td>0.728</td>
<td>0.692</td>
<td>0.792</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.573 to 0.883</td>
<td>0.511 to 0.873</td>
<td>0.635 to 0.948</td>
</tr>
<tr>
<td>Critical value</td>
<td>2.860</td>
<td>3.295</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>80.00%</td>
<td>46.67%</td>
<td>73.33%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>71.88%</td>
<td>100.00%</td>
<td>84.38%</td>
</tr>
</tbody>
</table>

Figure 1. Diagnostic value of microRNA-373 and microRNA-204 in tissues in RB. AUC (microRNA-373) = 0.728, AUC (microRNA-204) = 0.692, AUC (microRNA-373 and microRNA-204) = 0.792.

Results

General data

There were 32 patients in the study group, including 18 males (56.25%), 14 females (43.75%) and 15 patients in the control group, including 8 males (53.33%) and 7 females (46.67%). There was no significant difference in sex ratio and age between the two groups (P > 0.05) (Table 2).

Expression levels of microRNA-373 and microRNA-204 in tissues

The expression level of microRNA-373 in tumor tissues of the study group was (3.518 ± 1.721), the expression level of microRNA-373 in normal retina tissues of the control group was (2.306 ± 1.606), and the study group was higher than the control group (P < 0.05). The expression level of microRNA-204 in tumor tissues of the study group was (1.598 ± 0.766), the expression level of microRNA-204 in normal retina tissues of the control group was (2.881 ± 1.713), and the study group was lower than the control group (P < 0.05) (Table 3).

Diagnostic value of microRNA-373 and microRNA-204 in tissues in RB

The AUC, specificity, sensitivity and critical value in the tissues were 0.728, 80.00%, 71.88% and 2.860 for single detection of microRNA-373 in RB. The AUC, specificity, sensitivity and critical value in the tissues were 0.692, 46.67%, 100.00% and 3.295 for single detection of microRNA-204 in RB. The AUC, specificity and sensitivity in the tissues were 0.792, 73.33% and 84.38% for combined detection of microRNA-373 and microRNA-204 in RB (Table 4 and Figure 1).

RNA-204 and the prognosis of patients. P < 0.05 represented that there was statistical significance.
Combined detection of MicroRNA-373 and MicroRNA-204 expression

Figure 2. Relationship between the expression of microRNA-373 and microRNA-204 in tissues and the prognosis of patients. A. The 5-year survival rate of the low microRNA-373 expression group was higher than that of the high microRNA-373 expression group (P = 0.024). B. There was no difference in 5-year survival rate between the low microRNA-204 expression group and the high microRNA-204 expression group (P = 0.133).

Table 5. Expression levels of microRNA-373 and microRNA-204 in peripheral blood

<table>
<thead>
<tr>
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<th>Control group (n = 15)</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MicroRNA-373</td>
<td>2.755 ± 0.960</td>
<td>1.194 ± 0.755</td>
<td>5.535</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MicroRNA-204</td>
<td>0.269 ± 0.121</td>
<td>1.046 ± 0.643</td>
<td>6.667</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 6. Diagnostic value of microRNA-373 and microRNA-204 in peripheral blood in RB

<table>
<thead>
<tr>
<th></th>
<th>miR-373</th>
<th>miR-204</th>
<th>miR-373 and miR-204</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>0.900</td>
<td>0.912</td>
<td>0.963</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.8104 to 0.9896</td>
<td>0.7901 to 1.033</td>
<td>0.9145 to 1.011</td>
</tr>
<tr>
<td>Critical value</td>
<td>1.679</td>
<td>0.494</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>73.33</td>
<td>80.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>93.75</td>
<td>96.88</td>
<td>78.13</td>
</tr>
</tbody>
</table>

Relationship between the expression of microRNA-373 and microRNA-204 in tissues and the prognosis of patients

According to the relative expression levels of microRNA-373 and microRNA-204 in tumor tissues, the median expression level of microRNA-373 was 3.537. With the median as the critical value, the patients were divided into the high microRNA-373 expression group (> 3.537) and low microRNA-373 expression group (< 3.537). The median expression level of microRNA-204 was 1.369. With the median as the critical value, the patients were divided into the high microRNA-204 expression group (> 1.369) and low microRNA-204 expression group (< 1.369). Kaplan-Meier survival analysis showed that the 5-year survival rate was 87.50% (14 cases) in the low microRNA-373 expression group and 50.00% (8 cases) in the high microRNA-373 expression group. The 5-year survival rate of the low microRNA-373 expression group was higher than that of the high microRNA-373 expression group (P = 0.024). The 5-year survival rate was 56.25% (9 cases) in the low microRNA-204 expression group and 81.25% (13 cases) in the high microRNA-204 expression group. There was no difference in 5-year survival rate between the low microRNA-204 expression group and the high microRNA-204 expression group (P = 0.133) (Figure 2). The expression level of microRNA-373 in peripheral blood of the study group was (2.755 ± 0.960), and that of the control group was (1.194 ± 0.755). The study group was higher than the control group (P < 0.05). The expression level of microRNA-204 in peripheral blood of the study group was (0.269 ± 0.121), and that of the control group was (1.046 ± 0.643). The study group was lower than the control group (P < 0.05) (Table 5).

Diagnostic value of microRNA-373 and microRNA-204 in peripheral blood

The AUC, specificity, sensitivity and critical value in peripheral blood were 0.728, 80.00%, 71.88% and 2.860 for single detection of microRNA-373 in RB. The AUC, specificity, sensitivity and critical value in peripheral blood were 0.692, 46.67%, 100.00% and 3.295 for single detection of microRNA-204 in RB. The AUC, specificity and sensitivity in peripheral blood were 0.792, 73.33% and 84.38% for combined detection of microRNA-373 and microRNA-204 in RB (Table 6 and Figure 3).
Combined detection of MicroRNA-373 and MicroRNA-204 expression

![Graph showing sensitivity and specificity percentages for different expression levels of microRNAs.](image)

**Figure 3.** Diagnostic value of microRNA-373 and microRNA-204 in peripheral blood in RB. AUC (microRNA-373) = 0.900, AUC (microRNA-204) = 0.912, AUC (microRNA-373 and microRNA-204) = 0.963.

**Relationship between the expression of microRNA-373 and microRNA-204 in peripheral blood and the prognosis of patients**

According to the relative expression levels of microRNA-373 and microRNA-204 in tumor tissues, the median expression level of microRNA-373 was 2.762. With the median as the critical value, the patients were divided into the high microRNA-373 expression group (> 2.762) and low microRNA-373 expression group (< 2.762). The median expression level of microRNA-204 was 0.306. With the median as the critical value, the patients were divided into the high microRNA-204 expression group (> 0.306) and low microRNA-204 expression group (< 0.306). Kaplan-Meier survival analysis showed that the 5-year survival rate was 87.50% (13 cases) in the low microRNA-373 expression group and 56.25% (9 cases) in the high microRNA-373 expression group. There was no difference in 5-year survival rate between the low microRNA-373 expression group and the high microRNA-373 expression group (P = 0.085). The 5-year survival rate was 56.25% (9 cases) in the low microRNA-204 expression group and 81.25% (13 cases) in the high microRNA-204 expression group. There was no difference in 5-year survival rate between the low microRNA-204 expression group and the high microRNA-204 expression group (P = 0.133) (Figure 4).

**Discussion**

RB is a very common primary malignant tumor among minors in the ophthalmic department [11]. With the prolongation of the course of disease, the risk of bone tumor, soft tissue sarcoma and melanoma also increased [12]. The occurrence and development of cancer is a multi-step process of multi-genetic diseases. The abnormal expression of microRNAs is associated with the occurrence and development of tumor cells. Detection of related microRNAs as a marker of early diagnosis of malignant tumors is a very hot topic in tumor study in the world [13, 14].

MicroRNA-373 and microRNA-204 are two microRNAs closely related to tumors. MicroRNA-373 has been identified as a potential oncogene of testicular germ cell tumors [15]. In recent years, the role of microRNA-373 in promoting the metastasis of breast cancer cells has also been verified [16]. Reis et al. [17] found that the expression level of microRNA-373 in RB was higher than that in normal retina tissues by RB-related microRNAs screening, suggesting that microRNAs play a role in tumorigenesis and in occurrence. However, there are few reports about the relationship between microRNA-373 and RB since then. In a report on microRNA-204, Ding et al. found that microRNA-204 may be involved in the occurrence and development of RB [18], and was closely related to the differentiation of tumor tissues, nerve invasion and lymph node metastasis. MicroRNA-204 may inhibit proliferation and promote apoptosis of RB cells by down-regulating the expression of Bcl-2 and Sirt-1 in RB, which may become new biological indicators related to RB. MicroRNA-204 was also regulated by non-coding RNA paranuclear set transcription factor 1 (NEAT 1). The down-regulation of NEAT 1 promoted the expression of microRNA-204, significantly reduced the proliferation and metastasis of RB cells, and promoted the apoptosis of RB cells. However, the diagnostic value of both microRNAs in RB has not been reported, and this study will analyze the diagnostic value of the two microRNAs.

The results of this study showed that compared with normal fresh retinal tissue and donor peripheral blood, the expression of microRNA-373 in RB tumor tissues and peripheral blood of RB patients increased, while the expression of microRNA-204 decreased. Then, the diagnostic value of microRNA-373 and microRNA-204 in RB was analyzed. The results showed that the AUC for single detection of...
Combined detection of MicroRNA-373 and MicroRNA-204 expression

The relationship between the expression of microRNA-373 and microRNA-204 and the prognosis of patients with RB was also analyzed. The results showed that the 5-year survival rate of high microRNA-373 expression group in tissues was lower than that of low microRNA-373 expression group. In this study, the relationship between the expression of microRNA-373 in peripheral blood and the prognosis of patients with RB was not found, nor was the relationship between the expression of microRNA-204 in tissues and peripheral blood and the prognosis of patients with RB. Müller et al. [23] found that the elevated level of serum microRNA-373 was significantly related with the advanced stage of clinical cancer in HER-2 positive breast cancer patients. In the study of Tu et al. [24], it was reported that overexpression of microRNA-373 was associated with lymph node metastasis, lymphatic vessel invasion and poor survival. Multivariate analysis showed that high-expression of microRNA-373 was an independent predictor of poor survival in patients with oral squamous cell carcinoma. In a study of breast cancer, Li et al. [25] reported that the expression of microRNA-204 was closely related to TNM staging and metastasis, and the total survival and disease-free survival of low microRNA-204 expression group were lower than those of high microRNA-204 expression group. Ye et al. [26] reported that glioma patients with low microRNA-204 expression had shorter progression-free survival and overall survival than those with high microR-
Combined detection of MicroRNA-373 and MicroRNA-204 expression. In addition, the single factor and multi-factor analysis showed that the expression of microRNA-204 was an independent prognostic factor of progression-free survival. These reports confirmed the relationship between the expression of microRNA-373 and the prognosis of RB patients to some extent. miR-373 orchestrates its functions either by pairing to the 3’ untranslated regions (UTR) of specific mRNAs to post-transcriptionally down-regulate gene expression, or by binding to the promoters of target DNAs to up-regulate gene expression [27]. However, this study failed to verify the specific mechanism of miR-373 and miR-204 in RB, which we will demonstrate in future studies. Also more data is needed to prove the relationship between the expression of microRNA-204 and the prognosis of RB patients.

In conclusion, microRNA-373 was highly expressed in RB, and microRNA-204 was less expressed in RB. MicroRNA-373 and microRNA-204 had better diagnostic value in RB, and the diagnostic value of combined detection of microRNA-373 and microRNA-204 was higher. MicroRNA-373 was also related to prognosis of patients, and the 5-year survival rate of patients with high microRNA-373 expression was lower.

Disclosure of conflict of interest

None.

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References


