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Expression and significance of microRNA-32 in multiple myeloma*

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Abstract *Objective:* This study was aimed to explore the expression of microRNA-32 (miR-32) in multiple myeloma (MM), and study its association with β2-microglobulin and staging of MM by Durie-Salmon classification. *Methods:* The expression level of miR-32 in bone marrow mono-nuclear cells of MM were examined by real-time polymerase chain reaction (real-time PCR), and the correlations between the expression level of miR-32 and related clinic pathologic features β2-microglobulin, staging of MM by Durie-Salmon classification were further analyzed. *Results:* The expression of miR-32 in MM patients was obviously higher than that in normal control ($P < 0.05$). The expression of miR-32 in relapsed/refractory MM patients was obviously higher than that in newly diagnosed MM patients. The expression of miR-32 in MM patients decreased after chemical therapy than that before treatment, especially in effective group ($P < 0.05$). There was no statistically significant change in ineffective/progress group after chemical therapy ($P > 0.05$) .The expression of miR-32 was associated to staging of MM and β2-microglobulin level. *Conclusion:* Expression level of miR-32 in MM patients is significantly higher than that in normal bone marrow, these data indicated that miR-32 may play an important role in the development of MM. High-regulated expression of miR-32 was associated with β2-microglobulin and staging of MM by Durie-Salmon classification.

Key words microRNA-32 (miR-32); multiple myeloma (MM); real-time polymerase chain reaction (real-time PCR)

MicroRNAs (miRNAs) are a relatively recently identified class of regulatory non-coding RNAs, typically 20–25 nucleotides in length, that function primarily by targeting specific messenger RNAs (mRNAs) or degradation or inhibition of translation through base pairing to partially or fully complementary sites [1]. MiRNAs are involved in critical biological processes of cancer, including cell differentiation, apoptosis, and proliferation [2–5]. Multiple myeloma (MM) is a B-cell neoplasm characterized by the accumulation of clonal malignant plasma cells in the bone marrow. Its incidence rate has exceeded that of leukemia, and become the second common malignant tumor of hematological system next to lymphoma. Little is known, however, about miRNA expression in MM. A recent study has shown that, microRNA-32 (miR-32), whose expression was significantly increased in MM, plays an important role in the pathogenesis of MM [6]. However, so far, there have no study on correlation between miR-32 and MM in China. Here we collected 43 patients suffered from MM and 20 normal controls, detected miR-32 expression level by real-time fluorescence quantitative PCR method, analyzed the correlation between miR-32 and MM, and explore the possible pathogenesis in the development of MM.

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Materials and methods

Patients

All the procedures were carried in accordance with the local and national regulatory guidelines. Forty-three bone marrow samples in experimental group were included in the study. The bone marrow samples were obtained from MM patients who were admitted to hospitals from 2006–2011 after proper informed consent in Qingdao Municipal Hospital, China. Males were predominant, with a total of 25 against 18 females. The average age of the patients was 55 years, ranged from 38 to 78 years. Respectively, the patients were treated with MP regimen (melphalan and prednisone), VAD regimen (vincristine, doxorubicin and dexamethasone), TD (velcade and dexamethasone) or thalidomide. The 43 patients in experimental group were

divided into different subgroups, including newly diagnosed subgroup (25 cases), refractory/relapsed subgroup (18 cases), stage I subgroup (2 cases) by Durie-Salmon classification, stage II subgroup (16 cases), stage III subgroup (25 cases). The 43 patients in experimental group included 23 cases with β2-microglobulin level ≥ 4.0 mg/L, and 20 cases with β2-microglobulin level < 4.0 mg/L. The total response rate (CR + nCR + VGPR + PR + MR) was 72% (31 cases), and the others (28%, 12 cases) were NC and PD after 3 cycles of chemotherapy. The 20 patients in control group included 11 males and 9 females. The average age of control group was 51 years, ranged from 39 to 64 years. All the bone marrow samples from control group were normal. The samples were included only if the patients' vital parameters were in the normal physiological range and they did not have any other abnormalities in their hematological system such as an associated autoimmune disease or malignancy.

Bone marrow aspiration and cell isolation

The 5 mL of bone marrow aspirated from the ileac crest in all the patients was transported in an EDTA anticoagulant solution. The samples were subjected to Ficoll gradient centrifugation procedure and the bone marrow mono-nuclear cells (BMMNCs) were collected by removing the buffy coat. The viability of the cells was checked using Trypan blue and the quantity of BMMNC per mL was calculated. The BMMNCs were cultured in suspension in RPMI-1640 medium (Gibco BRL Inc., USA), supplemented with 15% bovine calf serum (Hyclone Inc., USA), 100 U/mL penicillin and 100 U/mL streptomycin, at a final concentration of 2×10^5/mL, in a humidified atmosphere containing 5% CO_2 at 37 ℃.

RNA isolation and quantitative real-time PCR

RNA was extracted from BMMNCs, using TRIzol reagent (Invitrogen Inc., USA). The time of precipitation step in isopropanol was prolonged more than 2 hours at –20 ℃ in order to obtain more RNA. The concentration of RNA solution was tested, with the ratio of OD260/OD280 > 1.8. For reverse transcription reaction, (Oligo)dT was used as primer. The reverse transcription reactions were performed with M-MLV Reverse Transcriptase (Invitrogen Inc., USA). The reactions were subjected to 16 ℃ 30 min, 37 ℃ 30 min, 70 ℃ 10 min, and 4 °C 10 min.

For quantitative real-time PCR (qPCR), U6 snRNA was used as an endogenous control for miRNA detection. PCR primers were 5'-gcagcatgttgtctcctgaa-3' (forward) and 5'-taatcccagcaagcaagagc-3' (reverse) for miR-32. PCR reactions were subjected to hot start at 95 ℃ for 30 s, followed by denaturation at 95 ℃ for 5 s, annealing at 60 °C for 5 s, and extension at 66 °C for 15 s using the Real-Time PCR Detection System. The expression of miR-32 was quantified by measuring cycle threshold (Ct) values and normalized using the $2^{-\Delta\Delta Ct}$ method relative to U6 snRNA. ΔΔCt = (Ct, target genes – Ct, housekeeping gene) experimental group – (Ct, target genes – Ct, housekeeping gene) control group [7].

Statistical analysis

All statistical analyses were performed by using the SPSS 13.0 statistical software package. Differences between variables were assessed by the *t* test. A *P* value less than 0.05 was considered to be statistically significant.

Results

miR-32 was up-regulated in MM (Fig. 1)

The expression level of miR-32 was remarkably up-regulated in MM patients (5.29 ± 0.31) compared with control group, with statistically significant difference ($P < 0.05$). We also observed that the expression level of miR-32 in relapsed/refractory MM patients was remarkably higher than that in newly diagnosed MM patients (6.86 ± 0.24 vs. 4.15 ± 0.29; $P < 0.05$).

The expression of miR-32 in MM patients decreased after chemical therapy compared with that before treat-

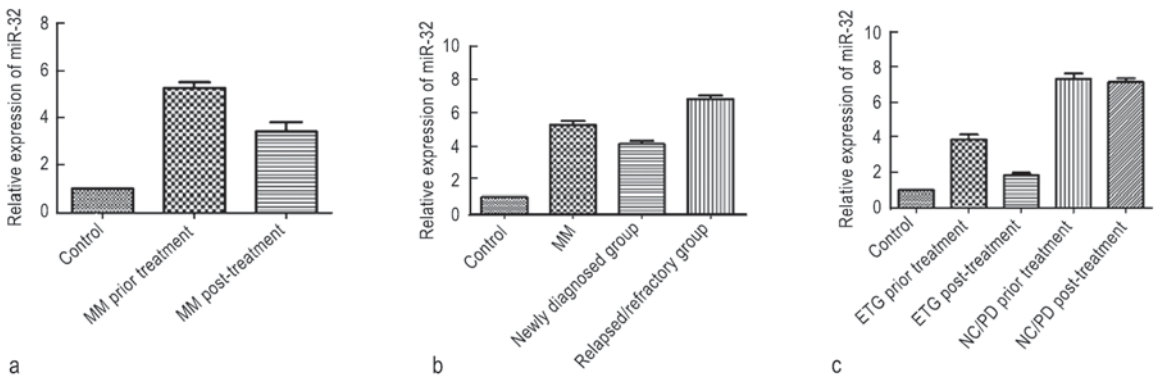

Fig. 1 miR-32 was up-regulated in MM. ETG: effective treatment group; NC/PD: no change/progression of disease

ment (5.29 ± 0.31 vs. 3.43 ± 0.45), especially in effective group (4.57 ± 0.41 vs. 1.92 ± 0.15; $P < 0.05$). There was no statistically significant change in NC/PD group after chemical therapy (7.14 ± 0.39 vs. 7.33 ± 0.47; $P > 0.05$). In addition, we also found that the expression level of miR-32 in NC/PD MM patients was remarkably higher than that in effective treatment group patients (7.14 ± 0.39 vs. 4.57 ± 0.41; $P < 0.05$).

Over-expression of miR-32 was associated with β2-microglobulin and staging of MM (Fig. 2)

We observed that the expression level of miR-32 was remarkably up-regulated in patients of MM stage III compared with stage II (6.17 ± 0.43 vs. 4.13 ± 0.33). Statistical analyses of stage I were failed to performed because of only 2 samples. And the expression of miR-32 in patients with β2-microglobulin level ≥ 4.0 mg/L was remarkably higher than that in patients with β2-microglobulin level < 4.0 mg/L, with statistically significant difference (6.32 ± 0.51 vs. 4.10 ± 0.44; $P < 0.05$).

Discussion

MM is a B-lymphocyte neoplasm, derived from the immune system. With the aging of the population, the incidence of MM increased year by year. The overall incidence rate of MM is 4/100 000 every year, accounting for 2 percent of cancer mortality with several fetal complications including infections, skeletal destruction, cytopenia, renal dysfunction and amyloidosis [8]. However, the pathogenesis of MM is not clear, and the effectiveness of medical therapy has not been satisfactory. Already existing prognostic index cannot reflect the prognosis of MM properly. Here what we need do is to study on the pathogenesis of MM, and find new potential prognosis factors or drug targets for MM.

MicroRNA-32 located on chromosome 9q31.2. Dysregulated expression of miR-32, reported in a number of cancers including MM [6, 9], were directly involved in human cancer processes, such as tumorigenesis, migration and metastasis. Studies found that up-regulated miR-32 in MM targeted p300-CBP-associated factor (PCAF), a gene involved in p53 regulation, decreased the expression of p53, and promoted the occurrence and progress of tumor [6]. Gocek's study found that miR-32 lead to Bim targeting and inhibition of apoptosis. Thus, agents which can inhibit miR-32 expression may offer clinical utility by enhancing therapeutic efficacy [10]. There were also other studies found that transcription factor E2F1, p21, and BTG2 were probable targets of miR-32 [9, 11]. So miR-32 may effect the differentiation, proliferation and apoptosis of cells by adjusting a variety of target genes. MiR-32 and its probable mechanisms of action were deduced to play an important role in the pathogenesis of MM in these studies. But the change of miR-32 expression and its significance have not yet been studied in MM patients during treatment.

Our study found that the expression of miR-32 was up-regulated in MM patients, which is consistent with Pichiorri's findings. We also found that the expression of miR-32 in relapsed/refractory MM patients was higher than that in newly diagnosed MM patients. And the expression of miR-32 in MM patients decreased after chemical therapy. These results suggest that miR-32 may play an important regulatory role in the pathogenesis of MM. In addition, the expression of miR-32 decreased remarkably in effective treatment group after chemical therapy, but not in NC/PD group. And the expression level of miR-32 in NC/PD MM patients was higher than that in effective group before chemical treatment. The result suggested that high level of miR-32 was associated with lower therapeutic response and worse prognosis. The correlation analysis showed that high-level expression of miR-32 was significantly associated with β2-microglobulin level and staging of MM. This result confirmed that the expression of miR-32 was associated with cell proliferation, migration and prognosis of MM. High expression of miR-32 portend a poor prognosis. Isolation of bone marrow mononuclear cell is a accepted method of MM research, but if we have purified plasma cells instead of bone marrow mononuclear cells, the results would be more convincing and perfect.

Our study have confirmed the high expression of miR-32 was closely associated with the occurrence and prognosis of MM. And miR-32 was a promising prognosis

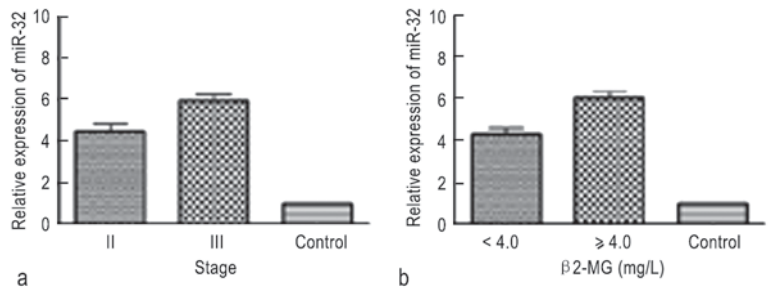

Fig. 2 Over-expression of miR-32 was associated with β2-microglobulin and staging of MM

factor of MM. However, the exact mechanism for microRNA is still unclear. Further study on the mechanism of the action of miR-32 in MM will be carried on in our research. We believe that miR-32 have a good future to be a diagnosis index and new target for treatment of MM, with the successful application of antisense nucleic acid technology.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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