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Expression and significance of miR-21 in multiple myeloma patients

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ABSTRACT. The aim of the present study is to examine the expression level of peripheral mir-21 in multiple myeloma (MM) patients and to determine its clinical significance. MM patients (30), monoclonal gammopathy of undetermined significance (MGUS) patients (14), and normal controls (20) were recruited to determine the serum level of β2-MG, IgA and IgM, IgG, λ, κ, TP, ALB, Hb, LDH, and Ca^{2+}. Gene expression of mir-21 was quantified by SYBR green real-time fluorescent quantitative PCR. We found that the expression level of serum mir-21 in the MM group was significantly higher than the MGUS group and the NC group ($P < 0.01$). According to the ISS installment, the level of mir-21, IgG, κ, and ALB in the MM group in stage I differed from that in stages II and III. The level of IgA, β2-MG in stage III was higher as compared with stage I and II ($P < 0.05$ and $P < 0.01$).The levels of mir-21, κ, (κ+λ), IgG, (IgG + IgA + IgM), and β2-MG in MM patients were positively correlated with ALB ($P < 0.01$). Based on the results, miR-21 plays an important role as an oncogene. Mir-21 may be important in the occurrence, development, and disease prognosis of MM.

Key words: Multiple myeloma; Mir-21; PCR; Correlation

INTRODUCTION

Multiple myeloma (MM) is a common hematologic malignancy, accounting for 10% of all hematologic malignancies (Hatzimichael et al., 2010), and its incidence is rising. The occurrence of MM is considered a gradual progression: many patients first experience monoclonal gammopathy of undetermined significance (MGUS) during early stages, which then progresses to MM, and some patients eventually develop extramedullary myeloma (plasma cell leukemia). During this process, mutations appear on multiple genes in the bone marrow plasma cells of (Garzon et al., 2006; Pichiorri et al., 2008; Schmittgen and Livak, 2008). In recent years, micro RNAs (miRNA) have been shown to play important regulatory roles in cell proliferation, metabolism, apoptosis, and differentiation. In addition, they can also function as oncogenes and cancer suppressor genes during tumorigenesis (Sayed and Abdellatif, 2011). MiR-21 is highly expressed in a variety of tumor cells. Some studies have shown that miR-21 expression in MM bone marrow plasma cells was significantly higher compared to normal plasma cells (Xu et al., 2011). However, the expression of miR-21 in peripheral blood is not clear. The present aims to determine the expression of miR-21 in MM patients, and to generate correlation analysis on related clinical parameters by exploring the expression peripheral mir-21 in multiple myeloma patients and its clinical significance.

MATERIAL AND METHODS

Materials

MM patients from our hospital were recruited between July 2010 and October 2013. MM diagnostic criteria refer to the International Myeloma Working Group guidelines (Siegel and Naishadham, 2012) in 2013. MM group: 60 patients (34 males and 26 females) aged 34-86 years, with an average age of 61.7 ± 12.1 years; MGUS group: 30 patients (18 males and 12 females) aged 35-85 years, with an average age of 60.7 ± 13.2 years. The control group consisted of 30 patients (16 males and 14 females) aged 35-82 years, with an average age of 59.8 ± 10.6 years. None of the participants had autoimmune diseases, cancer, and other diseases that may affect the expression of miR-21 (Table 1).

Table 1. Physical data of 60 MM patients, 30 MGUS patients, and the control subjects.

Items	MM	MGUS	Control
Gender			
Male	34 (56.7)	18 (60.0)	16 (53.3)
Female	26 (43. 3)	12 (40.0)	14 (46.7)
Immmune type			
κ	32 (53.3)	0 (0)	0 (0)
λ	24 (40.0)	0 (0)	0 (0)
No secretion	4 (6.7)	0 (0)	0 (0)
Durie-Salmon(D-S)			
I	10 (16.7)	0 (0)	0 (0)
II	16 (26.7)	0 (0)	0 (0)
III	34 (56.7)	0 (0)	0 (0)
β_2-MG (mg/L)			
<4.0	26 (43.3)	25 (83.3)	28 (93.3)
≥4.0	34 (56.7)	5 (16.7)	2 (6.7)

Apparatus and reagents

The following instruments were used: Spife4000 type electrophoresis apparatus (Helena Laboratories, Beaumout, TX, USA); IMMAGE Immunochemistry Analyzer (Beckman Coulter, Inc., Shanghai, China); 7600-110 automatic biochemical analyzer (Hitachi Instruments Ltd.); Sysmex XE-2100 automated hematology analyzer and their ancillary reagents; ABI7500 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA); NanoPhotometer nucleic acid and protein UV detector (Implen, USA); and 3K18 type refrigerated high-speed centrifuge (Sigma, Germany). The kits used were miRNeasy Mini Kit serum total RNA extraction kit [QIAGEN (Suzhou) Translational Medicine Co., Ltd., Suzhou, China]; mir-21qPCR primer kit (TaKaRa); and TRIzol M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Methods

Detection of clinical specimens

Venous blood (5 mL) was drawn from all fasted subjects, and centrifuged at 3000 rpm for 10 min. A 7600-210 automatic biochemical analyzer was used to detect TP, ALB, LDH, and Ca^{2+}. IMMAGE Immunochemistry Analyzer was used to detect β2-MG, IgA, IgM, IgG, lambda (λ), and kappa (κ) light chain. The Spife4000 type electrophoresis apparatus and immunofixation electrophoresis were used to detect serum protein. Hemoglobin (Hb) level was tested on the Sysmex XE-2100 automated hematology analyzer.

Detection of miR-21 expression

RNA was extracted by using miRNeasy Mini Kit serum total RNA extraction kit, as described by Chen et al. (2005). Briefly, RNA was extracted from 200 μL serum according to QIAGEN's miRNeasy Mini Kit instructions, and was stored at -80°C. Reverse transcription was performed with the reverse transcription kit. Cycling parameters were as follows: 95°C for 10 min, 95°C for 15 s, 55°C for 15 s, and 72°C for 20 s (40 cycles). Comparison threshold was set, Ct values were obtained, and the expression miR-21 was determined using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct$ = experimental group ($Ct_{\text{target gene}}$ -$Ct_{\text{housekeeping gene}}$) - control group ($Ct_{\text{target gene}}$ - $Ct_{\text{housekeeping gene}}$). Each experiment was performed in triplicates, and three individual experiments were carried out.

Treatment and follow-up

MM group: 60 patients used MP (melphalan, prednisone), VAD (vincristine, adriamycin, dexamethasone), TD (Velcade, dexamethasone), and thalidomide for chemotherapy for at least 3 cycles. Therapeutic effect was evaluated based on the PECIST solid tumor effect standard, which were divided into complete response (CR), partial response (PR), stable disease, and progressive disease, with the formula of effective (RR) = CR + PR. Following termination of chemotherapy, telephone follow-up appointment was scheduled every 2-3 months. Survival time was defined as from the date of hospital admission to the date of death or the date of the last follow-up. Follow-up time was 5-24 months, with a median follow-up time of 7.5 months. During the course of the study, five patients passed away (8.3%), and one patient was missing (1.7%). Of the 38 patients that

received initial treatment, 22 patients (36.7%) relapsed. Treatment efficiency was found to be 72% (31/60), and 28% (12/60) of the patients experienced no change following treatment.

Statistical analysis

Data were analyzed by the software SPSS 13.0 (IBM Research, Shanghai, China). Data are reported as means ± SEM, as assessed using the Student *t*-test. Count data was evaluated by the c^2 test, and correlation analysis was tested by linear correlation analysis. $P < 0.05$ was considered to be statistically significant.

RESULTS

Expression levels of miR-21 in each group

Expression levels of miR-21 in the MM group were significantly higher than the that of the MGUS group and the control group ($P < 0.01$). The expression level of miR-21 between the MGUS group and the control group were not statistically different ($P > 0.05$, Table 2).

Table 2. Expression cycle of miR-21 in all groups.

Group	N	miR-21
MM	60	2.38 ± 0.32**
MGUS	30	0.77 ± 0.20
Control	30	0.43 ± 0.13

Comparison made between the MGUS group and the control group. **P < 0.01.

Correlative clinical indexes in each MM stage

According to D-S staging and common prognostic indicators (expression levels of β2 -MG), we found that in the MM group, expression levels of IgA, λ, and β2-MG in stage III were significantly higher as compared to stages I and II ($P < 0.01$). In addition, as shown in Table 3, the levels of ALB in stage I were significantly higher than that in stages II and III, and the levels of IgG and κ in stage I were significantly lower than that in stages II and III ($P < 0.05$). The level of IgA, b2-MG in stage III was higher as compared with stages I and II ($P < 0.05$ and $P < 0.01$).

Table 3. Clinical indicators of MM samples in ISS stages.

Item	Stage		
	I (N = 10)	II (N = 16)	III (N = 34)
IgG (g/L)	7.64 ± 1.78*	19.53 ± 6.81	20.24 ± 13.57
IgA (g/L)	0.49 ± 0.21	0.53 ± 0.22	10.46 ± 7.4**
IgM (g/L)	0.41 ± 0.14	0.36 ± 0.13	0.45 ± 0.17
b2-MG (mg/L)	2.27 ± 0.18	3.10 ± 0.63	11.35 ± 2.88**
κ (mg/L)	612.33 ± 214.36*	1985.80 ± 854.95	1969.57 ± 923
λ (mg/L)	377.00 ± 96.89	249.40 ± 118.09	996.29 ± 653.5**
ALB (g/L)	39.76 ± 1.05*	32.75 ± 2.84	29.31 ± 2.94
TP (g/L)	58.84 ± 1.97	63.80 ± 6.15	60.12 ± 6.99
LDH (U/L)	184.33 ± 19.70	186.40 ± 46.51	174.43 ± 21.73
Hb (g/L)	106.89 ± 5.80	86.4 ± 5.77	89.13 ± 8.69
Ca^{2+} (M)	2.01 ± 0.04	2.14 ± 0.42	2.10 ± 0.25

Comparisons were made between II and III, *P < 0.05; comparisons were made between II and III, **P < 0.01.

Correlation between expression level of miR-21 and D-S staging and plasma b2-MG

In the MM group, expression levels of miR-21 in stages II and III were significantly higher than that in stage I ($P < 0.05$). However, expression levels of miR-21 in stages II and III were not significantly different ($P > 0.05$, Figure 1A). Lastly, when b2-MG ≥ 4.0, the expression levels of miR-21 were significantly higher than when b2-MG < 4.0 ($P < 0.05$, Figure 1B).

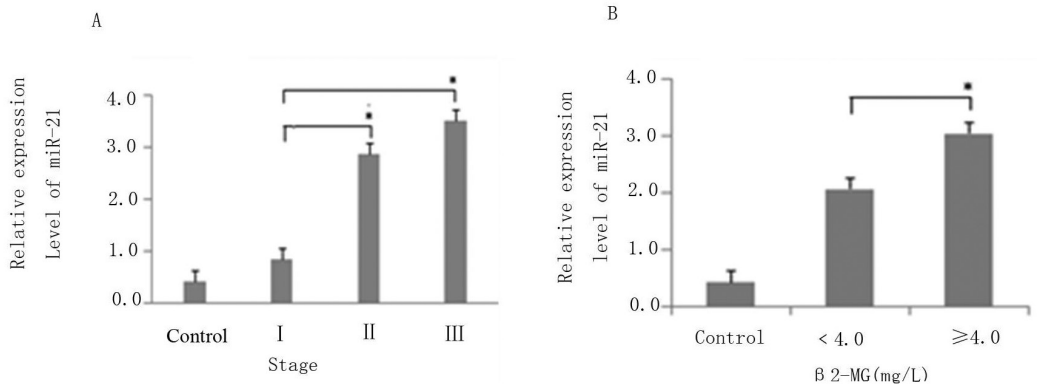

Figure 1. In the MM group, expression levels of miR-21 in stages II and III were significantly higher than that in stage I ($P < 0.05$). However, expression levels of miR-21 in stage II and stage III were not significantly different ($P > 0.05$, Figure 1A). Lastly, when b2-MG ≥ 4.0, the expression levels of miR-21 were significantly higher than when b2-MG < 4.0 ($P < 0.05$, Figure 1B).

Correlation analysis between expression level of miR-21 and clinical parameters

Correlation analysis between expression level of miR-21 and clinical parameters are shown in Table 4. Expression level of miR-21 in MM patients was positively correlated with κ, (κ + λ), IgG, (IgG + IgA + IgM), and b2-MG ($P < 0.01$). miR-21 expression was negatively correlated with ALB ($P < 0.01$). There was no correlation between MiR-21 expression and GLB, LDH, Hb, and Ca^{2+}. ($P > 0.05$).

Table 4. Correlation analysis of miR -21 levels in MM and the clinical index.

Var.	k	κ +l	IgG	IgG+IgA+IgM	b_2-MG	ALB	TP	LDH	Hb	Ca^{2+}
r	0.532	0.614	0.586	0.625	0.490	-0.585	0.358	0.250	-0.285	-0.226
P	0.013	0.003	0.011	0.002	0.024	0.002	0.112	0.280	0.208	0.224

Expression levels of miR-21 in MM patients

Expression levels of miR-21 in the MM group were significantly higher as compared with the control group. Expression levels of miR-21 in the relapsed/refractory were also significantly higher than the newly diagnosed group ($P < 0.05$, Figure 2A). Following chemotherapy, expression levels of miR-21 were significantly lower as compared to that before chemotherapy treatment ($P < 0.05$, Figure 2B). In addition, expression levels of miR-21 in the effective treatment group were significantly lower than that in the invalid/progressive group, both before and after chemotherapy

treatment (P < 0.05). Lastly, the expression level of miR-21 in the invalid/progressive group did not change significantly before and after chemotherapy (Figure 2C).

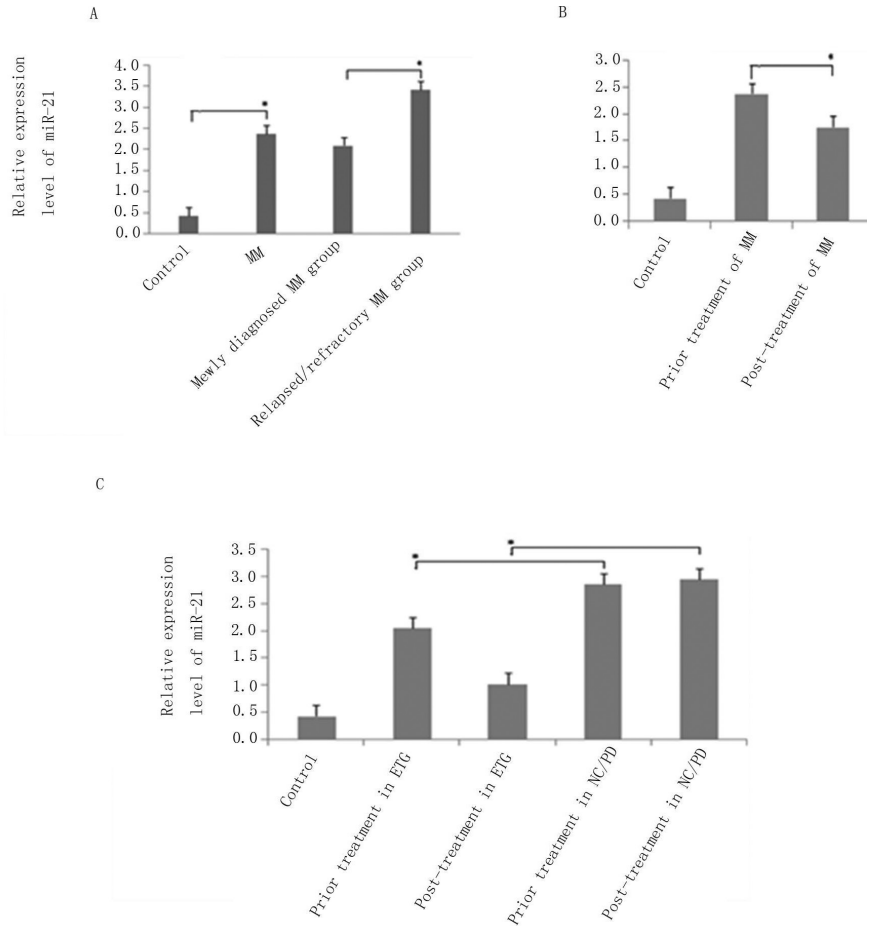

Figure 2. Expression levels of miR-21 in the MM group were significantly higher as compared with the control group. Expression levels of miR-21 in the relapsed/refractory were also significantly higher than the newly diagnosed group (P < 0.05, **A**). Following chemotherapy, expression levels of miR-21 were significantly lower as compared to that before chemotherapy treatment (P < 0.05, **B**). In addition, expression levels of miR-21 in the effective treatment group were significantly lower than that in the invalid/progressive group, both before and after chemotherapy treatment (P < 0.05). Lastly, the expression level of miR-21 in the invalid/progressive group did not change significantly before and after chemotherapy (**C**).

DISCUSSION

MM is a malignant tumor that originates in the immune system lymphocytes. With an aging population, MM incidence increases with each year, and has become the second most common hematologic malignancy (Hatzimichael et al., 2010). Abnormal plasma cells in the bone marrow and monoclonal immunoglobulin hyperplasia lead to severe humoral immune deficiency, bone

damage, and high mortality. The pathogenesis of MM is still unclear at present, and individual difference between treatment effect is large. The existing prognostic indicators do not well reflect the complexity of the disease, and in-depth study of its pathogenesis and prognostic factors may be the key to effectively solving this clinical problem.

A good sampling tumor marker should be non-invasive and easy to detect. miR-21 is a popular miRNA molecule. miR-21 expression is shown to be abnormal in a variety of malignant tumors. It has multiple regulatory molecular targets such as PTEN, RECK, and other tumor suppressor genes and apoptosis-related molecules. When screening for circulating miRNA, researchers found that miR-21 appeared in the peripheral blood of various cancer patients. Lawrie et al. (2008) were the first to report circulating miRNA to be correlated with disease prognosis. They found that expression of miR-21, miR-155, and miR-210 in patients with diffuse large B-cell lymphoma was significantly higher than that in the control group, and the levels of miR-21 were related to disease recurrence rate and patients' survival rate. Recently, Asaga et al. (2011) used real-time PCR to detect circulating miR-21 in 102 cases of breast cancer patients. It was found that the levels of circulating miR-21 in serum were closely related to breast cancer metastasis (Pichiorri et al., 2008; Liu et al., 2010; Asaga et al., 2011). These studies indicate that circulating miR-21 is closely related to tumor progression, and may offer predictive value on disease prognosis.

Our study found that miR-21 is over-expressed in MM patients. Circulating miR-21 in the MM group was significantly higher as compared to the MGUS group and the control group ($P < 0.01$), which is consistent with the literature (Asangani et al., 2008; Xu et al., 2011). Similarly, several studies have also demonstrated that expression of miR-21 in myeloma cells were significantly higher than that in the MGUS groups and normal plasma cell control groups (Wang et al., 2011; Xiong et al., 2012). It is possible that the expression level of circulating miR-21 is positively correlated to tumor cells. High levels of serum circulating miR-21 in MM patients are likely to be secreted by myeloma cells, which can be used to observe and evaluate the various effects on treating MM. Expression level of circulating miR-21 in MGUS group was also found to be slightly higher than that in the control group, albeit non-significant ($P > 0.05$). This may be due to the insufficient sample size used in the study. In the MM Group, expression levels of miR-21, IgG, κ, and ALB in stage I were significantly different compared to stages II and III; expression levels of IgA, b2-MG, and λ in stage III were significantly higher than that in stages I and II ($P < 0.05$ and $P < 0.01$). Our results suggest that miR-21 may be associated with the occurrence and development of MM, and may play an important cancer-causing role in the pathogenesis of MM.

Our correlation analysis showed the following: expression levels of miR-21 in MM patients were positively correlated with κ, ($\kappa + \lambda$), IgG, (IgG + IgA + IgM) and b2-MG ($P < 0.01$); they were negatively correlated with ALB ($P < 0.01$). In the MM group, when b2-MG $\geq$ 4.0 or in stages II and III, the expression of miR-21 was significantly higher than when b2-MG < 4.0 or in stage I. We found that there is a positive correlation between expression level of miR-21 in MM patients and pathogenesis of MM. This suggests that high expression levels of miR-21 may be a prognostic indicator of MM. However, the specific mechanisms need to be further explored.

This study found that in MM patients with over-expressed of miR-21, circulating miR-21 in the relapsed/refractory group was significantly higher than the newly diagnosed group. The miR-21 expressing after chemotherapy was significantly lower, consistent with previous reports (Löffler et al., 2007; Hu et al., 2013), which suggested the relationship between miR-21 pathogenesis of MM. miR-21 expression in the effective treatment group was significantly lower comparing to the invalid/progressive group, suggesting that high expression of miR-21 in MM patients may be prone

to be unresponsive to chemotherapy. These results further confirmed that miR-21 is closely related to tumor cell proliferation, metastasis capacity, and prognosis of MM. Therefore, it can be used as an indicator of poor prognosis. With the development and application of antisense oligonucleotide technology miRNA, miR-21 may become a potential MM diagnostic marker.

In summary, miR-21 regulates cell differentiation, proliferation, apoptosis, and participates in the invasion of vascular invasion and metastasis of tumor cells. Because of the lack of full understanding miRNA target genes, the molecular mechanisms by which miRNA regulates tumor growth is not clearly understood. Future experiments can examine the effect of miR-21 inhibition in combination with anticancer drugs to determine whether miR-21 inhibition can improve drug efficacy.

Conflicts of interest

The authors declare no conflicts of interest.

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