INVESTIGATIVE REPORT

Decreased miR-7 Expression in the Skin and Sera of Patients with Dermatomyositis

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Expression of microRNA (miRNA) in the skin in derma-tomyositis has not previously been studied in detail. In this study, we performed miRNA array analysis using miRNAs purified from dermatomyositis-involved skin and normal skin, and found that several miRNAs were up- or down-regulated in dermatomyositis skin. Among them, we focused on miR-7, one of the most down-regu-lated miRNAs in dermatomyositis skin. Total miRNAs were purified from serum, and hsa-miR-7 levels were measured with quantitative real-time PCR using the specific primer. Serum levels of miR-7 were significantly decreased in patients with dermatomyositis compared with normal subjects or patients with other autoimmune diseases. Thus, serum miR-7 levels might be a possible diagnostic marker for dermatomyositis. Clarifying the up- or down-stream events of down-regulated miR-7 in patients with dermatomyositis may lead to further un-derstanding of the disease and a new therapeutic ap-proach. *Key words: autoimmune diseases; polymerase chain reaction; polymyositis.*

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Polymyositis/dermatomyositis (PM/DM) is an inflam-matory disease that affects multiple organs, including the muscles, skin and lungs. Cancer risk is increased in patients with PM/DM. This disease sometimes overlaps with other autoimmune disorders, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, systemic sclerosis (SSc), Sjögren's syndrome, and mixed connec-tive tissue disease, thus autoimmunity may play a major role in the pathogenesis of PM/DM. However, the exact aetiology of PM/DM is unknown.

microRNAs (miRNAs) are a class of small non-coding RNAs, on average 22 nucleotides long, which usually inhibit the translation of target mRNA by binding to 3' UTR of the target (1). In humans, almost 1,700 miRNAs have been identified, and miRNAs have been implicated in various cellular events, such as immune response as well as cell development, cell differentiation, organogenesis, growth control or apop-tosis. Accordingly, many publications have suggested the possibility that miRNAs are involved in the patho-genesis of various diseases. However, there are few reports about the relationships between miRNAs and PM/DM. Eisenberg et al. (2) reported that expression of several miRNAs are changed in the muscle tissues of PM/DM compared with those of normal control muscle. However, miRNA expression in the skin of DM patients has not been determined. In this study, we performed miRNA array using miRNAs purified from DM skin, and found that several miRNAs were up- or down-regulated in DM skin. Among them, we focused on miR-7, one of the most down-regulated miRNAs in DM skin. We evaluated the possibility that serum levels of miR-7 may be a useful marker for the diagnosis or evaluation of disease activity in DM patients.

MATERIALS AND METHODS

Patient material

Serum samples were obtained from 20 patients with DM. A summary of clinical/laboratory features of DM patients enrolled in this study are shown in Table I. All the patients fulfilled the criteria of Bohan & Peter (3, 4). Ten patients with clinically and histopathologically typical cutaneous lesions, but without myositis were diagnosed as clinically amyopathic DM (CADM) according to the previous criteria (5). Electromyographic examination and muscle biopsies were performed at the time of diagnosis. Lung involvement was diagnosed based on the findings of chest radiography, computed tomography of the chest, and lung function tests. Patients who had received treat-ments including steroids, azathioprine, and methotrexate were excluded. Control serum samples were also collected from 17 healthy volunteers, 5 patients with PM, 10 with SLE, and 10 with SSc. All serum samples were stored at –80ºC prior to use.

Skin specimens were obtained from Gottron's eruption of 8 DM patients. Six SSc skin was used as disease control. Seven normal skin samples were obtained from routinely discarded limb skin of healthy human subjects undergoing skin grafts. Control and patient skin were collected and fixed in formalde-hyde immediately after resections. The study design was appro-ved by the Ethics Review Committee of Kumamoto University (number 177). Written informed consent was obtained before the patients and healthy volunteers were entered into this study according to the Declaration of Helsinki.

Table I. *Correlation of serum miR-7 levels with clinical and laboratory features in patients with dermatomyositis (DM)*

	DM patients ($n=20$)	Serum miR-7		
		Reduced ($n=13$)	Normal ($n=7$)	*p*-value
Clinical features				
Gottron's eruption, %	88.9	100.0	71.4	0.14
Heliotrope coloration, %	58.8	63.6	50.0	0.48
ILD, %	38.9	45.5	28.6	0.42
Internal malignancy , %	25.0	38.5	0.0	0.08
Joint involvement, %	40.0	42.9	33.3	0.67
Dysphagia, %	33.3	36.4	25	0.59
Laboratory features				
ANA, %	45.0	46.2	42.9	0.63
IgG, mg/dl, mean	1,467.8	1,231.3	1,901.3	0.42
CK, IU/l, mean	2,510.8	3,220.8	1,192.1	0.97
Myoglobin, ng/ml, mean	794.8	975.6	484.9	0.93
Aldolase, IU/l, mean	46.0	71.5	10.3	0.32

DM: dermatomyositis; ILD: interstitial lung disease; ANA: antinuclear antibodies; CK: creatinine kinase.

miRNA extraction from tissue and PCR analysis of miRNA expression

Small RNAs were extracted from tissue section using a miR-Neasy FFPE kit (Qiagen, Valencia, CA, USA). For PCR array, RNAs were reverse-transcribed into the first strand cDNA using an RT^2 miRNA First Strand Kit (SABioscience, Frederick, MD, USA). Equal amounts of samples from 3 normal skin or 3 DM skin were pooled and used in a single experiment: the mixed cDNA was added with RT^2 Real-Time SYBR GREEN/ROX PCR Master Mix (SABioscience) and applied into a 96-well RT2 miRNA PCR Array (SABioscience) that includes primer pairs for 88 human miRNAs (6). PCR was performed on a Takara Thermal Cycler Dice (TP800®) following the manufacturer's protocol. Threshold cycle (Ct) for each miRNA was extracted using Thermal Cycler Dice Real Time System ver2.10B. The raw Ct was normalized using the values of small RNA housekeeping genes.

For quantitative real-time PCR, cDNA was synthesized from total miRNA with Mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kit (Takara Bio Inc, Shiga, Japan). Quantitative real-time PCR with a Takara Thermal Cycler Dice (TP800®) used primers and templates mixed with the SYBR Premix. The sequence of miR-7 primer was designed based on miRBase (http://www.mirbase.org):caaagtgcttacagtgcaggtag. The primer set was prevalidated to generate single amplicons. DNA was amplified for 40 cycles of denaturation for 5 s at 95ºC and annealing for 20 s at 60ºC. Transcript level of miR-7 was normalized to that of U6.

miRNA extraction from serum and PCR analysis of miRNA expression

miRNA isolation from serum samples were performed with miRNeasy RNA isolation kit (Qiagen) following the manufacturer's instructions with minor modification (7, 8). Briefly, 100 µl serum were supplemented with 5 µl of 5 fmol/µl synthetic non-human miRNA (C. elegans miR-39, Takara) as a control providing an internal reference for normalization of technical variations between samples. After Qiazol solution (1 ml) was added and mixed well by vortexing, then samples were incubated at room temperature for 5 min. Aqueous and organic phase separation was achieved by the addition of chloroform. The aqueous phase was applied to RNeasy spin column and RNeasy MinElute spin column. miRNA was eluted from the column with nuclease-free water.

cDNA was synthesized from total miRNA with Mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kit. Quantitative real-time PCR with a Takara Thermal Cycler Dice (TP800®) used primers and templates mixed with the SYBR Premix. Transcript level of miR-7 was normalized to that of cel-miR-39.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U* test for comparison of medians and Fisher's exact probability test for the analysis of frequency. *p*-values < 0.05 were considered significant.

RESULTS

miRNA expression profile in dermatomyositis skin

As an initial experiment, to determine which miRNAs were involved in the pathogenesis of DM, we purified total miRNAs from Gottorn's eruption of 3 DM patients and from normal skins from 3 healthy controls. Equal amount of samples from the 3 normal skin or 3 DM skin were pooled and used in a single experiment; various miRNA expression in normal or DM skin *in vivo* was determined using PCR array, consisting of 88 miRNAs involved in human cell differentiation and development. The miRNA expression profile indicated that 5 miRNAs were overexpressed and 27 miRNAs were suppressed in DM skin compared with normal skin (Table SI; available from http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1459). Among them, we focused on miR-7, one of the miRNAs which are expressed in normal skin but not in DM skin, because miR-7 is thought to be increased in SSc skin fibroblasts and may be involved in the pathogenesis of SSc (9, 10). The array analysis was performed as a single experiment, and statistical significance of the data could not be evaluated. Therefore, we confirmed the array results by real-time PCR using specific primer for miR-7 with increased number of samples (8 DM skin, 6 SSc skin and 7 control skin). Consistent with the array data, the miR-7 levels in DM skin were significantly down-regulated compared with those in normal skin, while they tended to be up-regulated in SSc skin (Fig. 1).

Serum concentrations of miR-7 in dermatomyositis patients

We next tried to determine serum concentration of miR-7 by quantitative real-time PCR and evaluated the possibility that serum miR-7 levels could be a disease marker for DM.

There has been no report demonstrating the expression of miR-7 in cell-free body fluid such as serum or urine. To validate that the miRNA in human serum is indeed detectable, miRNA was extracted from sera of healthy individual and quantitative real-time PCR was
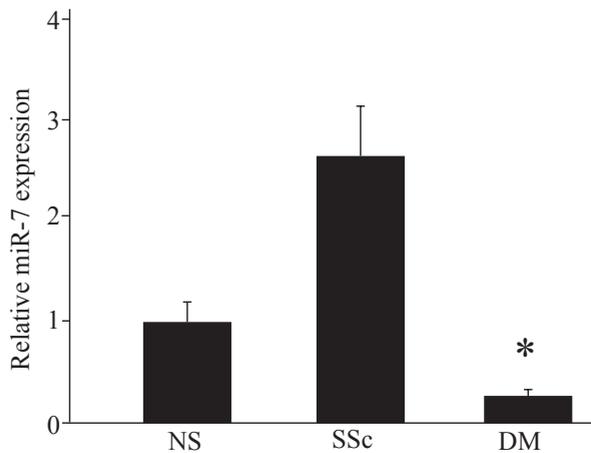
Fig. 1. Mean relative transcript levels of miR-7 in skin tissues of dermatomyositis (DM), systemic sclerosis (SSc) and normal subjects (NS). Error bars represent standard error. *$p < 0.05$ compared with the values in NS (1.0).

performed using primer set specific for miR-7, as described in previous publications (7, 8). The amplification of miR-7 was observed, and Ct values were increased by the serial dilution of the miRNA (data not shown). Thus, miR-7 is likely to be not only detectable, but quantitative, in the serum by our method.

The serum miR-7 levels in patients with DM are shown in Fig. 2. Serum samples were obtained from 20 patients with DM, 5 with CADM, and 10 with PM. Seventeen healthy control subjects, 10 SLE patients and 10 SSc patients were also included in this study.

Mean serum miR-7 levels were lower in DM and CADM patients than in healthy control subjects, and there was statistically significant difference in the values between control subjects and DM patients ($p < 0.00001$) or CADM patients ($p = 0.00036$). In addition, serum
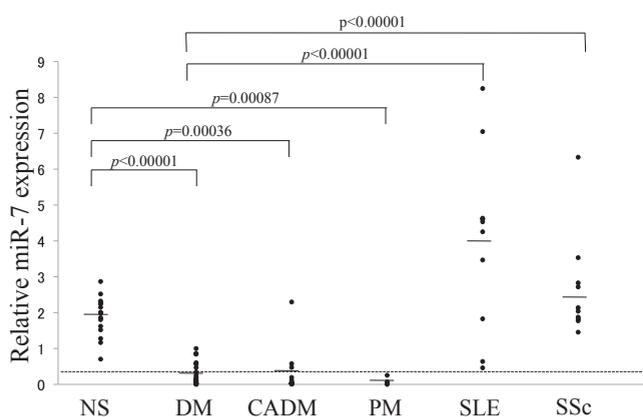

Fig. 2. Serum concentrations of miR-7 in patients with dermatomyositis (DM), clinically amyopathic dermatomyositis (CADM) or polymyositis (PM) and normal subjects (NS). miR-7 levels were measured with real-time PCR, as described in Materials and Methods. The maximum value in patients with DM was set at 1. Serum miR-7 concentrations are shown on the ordinate. Bars show means. The dotted line indicates the cut-off. *p*-values are determined using a Mann–Whitney *U* test. SLE: systemic lupus erythematosus; SSc: systemic sclerosis.

miR-7 levels were also significantly decreased in PM patients compared with healthy controls ($p = 0.00087$). On the other hand, miR-7 levels in patients with SLE or SSc were slightly higher than those in healthy controls, but not statistically significant. We also found significant difference in miR-7 levels between DM patients and patients with SLE ($p < 0.00001$) or SSc ($p < 0.00001$). Taken together, the decrease in serum miR-7 levels was highly specific to the patients with DM, CADM or PM.

When we set the cut-off value at mean –3 SD of serum miR-7 levels in healthy controls subjects, the serum miR-7 levels were decreased in 13 of 20 DM patients (65%), 7 of 10 CADM patients (70%), all of 5 PM patients (100%), and none of 10 SLE and SSc patients. All the values in healthy controls were above the cut-off.

Association of serum miR-7 levels with clinical or laboratory features of dermatomyositis

Finally, we examined the association of serum miR-7 levels with clinical manifestations of DM patients (Table I). Although not statistically significant ($p = 0.14$), the frequency of Gottron's eruption tended to be increased in patients with reduced serum levels of miR-7 than those with normal miR-7 levels, which is consistent with the array result that miR-7 expression is reduced in Gottron's eruption *in vivo* (Table SI). In addition, patients with decreased miR-7 levels tended to have higher prevalence of Heliotrope coloration, lung involvement, internal malignancy, joint involvement or dysphagia, as well as higher levels of CK, myoglobin or aldolase, but we could not find statistical significance.

DISCUSSION

In this study, we first identified several overexpressed or suppressed miRNAs in DM skin compared with normal skin by the miRNA PCR array analysis. Among them, miR-7 expression was down-regulated in DM skin. miR-7 expression is reported to be decreased in various cancer cells including glioblastoma and breast cancer (11, 12). On the other hand, there has been little research on miR-7 expression in the skin. We have shown that miR-7 expression is increased in SSc dermal fibroblasts by array analysis (9, 10). This study also showed that miR-7 expression is up-regulated in SSc skin. Thus, the expression of miR-7 may be differently regulated in each autoimmune disease.

Several studies have indicated that serum miRNAs are stable: miRNAs in serum are thought to be encapsulated in microvesicles shed from cell plasma membrane (13–17). Such microvesicles including miRNAs as well as RNA or DNA can be incorporated into adjacent cells, resulting in the alteration of gene expression in the recipient cell (17). Accordingly, serum miRNA may not merely come from apoptotic cells, but have some

functional roles (18). Although there has been no report determining serum miRNA in DM patients or determining serum miR-7 levels in human, we expected that miR-7 expression may also be decreased in serum of DM patients, and can be correlated with the disease activity of DM. Our results indicate that serum miR-7 levels are specifically decreased in DM patients as well as in patients with PM or CADM, but not decreased in those with other autoimmune diseases including SSc. Thus there is a possibility that serum miR-7 levels can be used as a diagnostic marker for PM/DM. Although we could not find significant association of serum miR-7 levels with clinical and laboratory findings of DM patients, this may be because of the small number of patients. In addition, we could not address the cellular source of miR-7 in the skin, the function of the miRNA, the detailed mechanism(s) by which miR-7 expression is decreased in the skin and sera of DM patients, and the role of decreased miR-7 in the pathogenesis of DM in this study. miR-7 has been reported to be expressed in several cell types including lymphocytes and fibroblasts (9, 19), and may target inflammatory molecules including fibroblast growth factor (FGF) 11 or CC chemokine ligand (CCL) 16 according to TargetScan (http://www.targetscan.org/). Our speculation is that, for example, miR-7 expression is decreased in the infiltrated lymphocyte or fibroblasts of DM skin, which may result in increased production of such inflammatory molecules, leading to the inflammation of skin. In addition, decreased serum miR-7 may indicate systemic down-regulation of miR-7 expression (e.g. in muscles or lung as well as skin). Larger studies are needed to clarify these points in the future.

Nakasa et al. (20) have reported that intravenous injection of miR-146a in collagen-induced arthritis model mice could prevent joint destruction. Clarifying the up- or down-stream events of down-regulated miR-7 in DM patients may lead to further understanding of the disease and a new therapeutic approach.

ACKNOWLEDGEMENTS

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