Original Article

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Expression of collagen-related piRNA is dysregulated in cultured dermal fibroblasts derived from patients with scleroderma

Minako Tanaka[1], Yutaka Inaba[1,*], Azusa Yariyama[1], Yumi Nakatani[1], Kayo Kunimoto[1], Chikako Kaminaka[1], Yuki Yamamoto[1], Katsunari Makino[2], Satoshi Fukushima[2], Masatoshi Jinnin[1]

[1] Department of Dermatology, Wakayama Medical University, Wakayama, Japan;
[2] Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan.

SUMMARY PIWI-interacting RNA (piRNA) is a class of recently discovered small non-coding RNAs. piRNAs derive from an initial transcript encompassing a piRNA cluster via a unique biosynthesis process, interact with PIWI proteins, bind to specific targets, and recruit chromatin modifiers to enable transcriptional repression. Abnormal expression of PIWI proteins and piRNAs has been reported in some human cancers, with participation of some PIWI/piRNAs complexes in tumorigenesis and association with cancer prognosis. Their expression in patients with systemic sclerosis (SSc) has not been widely elucidated. PIWI/piRNAs and their role in the pathogenesis of collagen accumulation in SSc was therefore investigated; no difference was found in the PIWIL1-4 levels between normal and cultured SSc dermal fibroblasts. Among piRNAs predicted to target SSc-related molecules, we first found significant piR-32364 up-regulation in SSc dermal fibroblasts, likely due to intrinsic TGF-β signaling. Forced piR-32364 overexpression in normal fibroblasts significantly reduced COL1A1 expression both at mRNA and protein levels, but not COL1A2. Thus, piR-32364 overexpression in SSc fibroblasts may be the negative feedback against collagen up-regulation, which could suggest the potential of piRNAs as a therapeutic target.

Keywords fibrosis, collagen, piRNA

1. Introduction

Despite the remarkable advances in medical science in recent years, there are still many diseases whose pathogenesis have yet to be unclarified. Among them, systemic sclerosis (SSc) is distinguished by multiple-organ tissue fibrosis, including the skin and lungs. The mechanism of abnormal collagen expression as a cause of the fibrosis is still unknown, and treatments for the disease are sometimes ineffective. Numerous clinical and basic research studies have been conducted, but the disease is heterogeneous, and its manifestation varies in each case.

Genetic and environmental factors could each have involvement in the pathogenesis of collagen diseases, and the latter mainly affecting the heterogeneity. Non-coding RNA has been the focus of our research as an environmental factor. Small non-coding RNAs include microRNAs (miRNAs) and small interfering RNAs (siRNAs) (*1,2*). Mature forms of such RNAs associated with biogenesis pathway proteins include Argonaute protein to guide target gene regulation. Meanwhile, PIWI-interacting RNAs (piRNAs), recently discovered small non-coding RNAs comprising 24–31 nucleotides, have a strong 5′-terminal uridine or tenth position adenosine bias, but lack clear secondary structure motifs (*3,4*). Unlike miRNAs and siRNAs, piRNAs derive from an initial transcript encompassing a piRNA cluster *via* a unique biosynthesis process (*5*), interact with PIWI proteins, bind to specific targets (based on sequence specific complementarity), and recruit chromatin modifiers to enable transcriptional repression (*6*). They were originally believed to control only transposons and development of germinal stem cells, but piRNAs have become known to control epigenetic regulation both at transcriptional and post-transcriptional levels.

Abnormal expression of PIWI proteins and piRNAs has been reported in human cancers, with some PIWI/piRNAs complexes being involved in tumorigenesis and associated with cancer prognosis (*7-9*). Expression in patients with SSc has not been widely elucidated. We therefore investigated the role of PIWI/piRNAs in the

pathogenesis of collagen accumulation in SSc.

2. Materials and Methods

2.1. Cell cultures

This study received approval from the Wakayama Medical University Research Ethics Committee (No. 2446), with written informed consent obtained from all patients. It was conducted in accordance with the Declaration of Helsinki. Human dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearms) of three diffuse cutaneous SSc patients with < 2 years of skin thickening. Control normal dermal fibroblasts were also obtained, as previously described (*10*). Dermal fibroblasts were cultured in Minimum Essential Medium Eagle (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (EQUITECH-BIO, Kerrville, TX) and Antibiotic-Antimycotic (Gibco, Waltham, MA). Monolayer cultures independently isolated from different individuals were maintained at 37°C in 5% CO_2. Cells were serum-starved before all experiments.

2.2. Skin samples

Skin specimens were obtained from the skin of the involved forearms of patients with SSc (n = 5), and normal skin was also collected for use as controls. These skin samples were fixed in formalin and embedded in paraffin immediately after collection.

2.3. Synthetic oligo

Human dermal fibroblasts were transfected with synthetic piR-32368 oligo (sense; GGTGAAAATGGAGCTCCTGGTCAGATG; antisense; CATCTGACCAGGAGCTC CATTTTCACC) as reported previously (*11*). Scrambled and non-targeting RNA was used as a control (Dharmacon, Lafayette, CO).

2.4. RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA from cultured fibroblasts were extracted using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Total RNA from paraffin sections was extracted with RNeasy FFPE Kit (QIAGEN).

cDNA was synthesized from the total RNA with PrimeScript RT Reagent Kit (Takara, Kusatsu, Shiga, Japan). Primers for COL1A1 (forward: GCTTGGTCCACTTGCTTGAAGA, reverse: GAGCATTGCCTTTGATTGCTG), COL1A2 (forward: GAGGGCAACAACAGCAGGTTCACTTA, reverse: TGGGCCAATGTCCACAAAGA), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: AGGGCTGCTTTTAACTCTGGT, reverse: CCCCACTTGATTTTGGAGGGA) were purchased from FASMAC. PIWIL gene primers were designed as previously described (*12*). All of these primers have been pre-verified to produce a single amplicon. Quantitative real-time PCR was performed with TaKaRa Thermal Cycler Dice (TP900) using TB Green Premix Taq II (Tli RNaseH Plus) (Takara). Data from each PCR reaction was analyzed by Thermal Cycler Dice real time system ver. 5. 11 (Takara). The transcript levels of each gene were normalized to those of GAPDH.

2.5. Quantitative real-time PCR for piRNA

Mir-X miRNA First-Strand Synthesis Kit (Takara) was used for cDNA synthesis from piRNAs. The sequences of piRNA primers were designed based on piRNAdb (*https://www.pirnadb.org*). The template was amplified and annealed as previous reported (*13,14*). Transcript levels of all piRNAs were normalized to those of U6 in the same sample.

2.6. Cell lysis and immunoblotting

Fibroblasts were cultured until they were confluent, before removal of the medium from the culture dishes. Cells were washed twice with phosphate buffered saline (PBS) and lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA). Aliquots of the cell lysates were separated by electrophoresis, as previously described (*15*). The primary antibody for type I collagen were purchased from Southern Biotechnology Associates (Birmingham, AL). The immunoreactive bands were visualized using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA).

2.7. Statistical analysis

The bar graphs were the means + standard deviation (SD). Mann-Whitney U test was used for statistical analysis. P values < 0.05 were considered significant.

3. Results

3.1. PIWIL gene expression in cultured SSc dermal fibroblasts

Expression of PIWIL genes were first compared using real-time PCR with RNA extracted from cultured dermal fibroblasts of three SSc patients and three normal subjects. Only PIWIL4 was detected by our protocol (Figure 1A). When the transcription level of PIWIL4 was corrected with that of GAPDH in each sample, PIWIL4 expression in SSc fibroblasts were similar to the normal control cells, with no significant differences.

3.2. piRNA expression in cultured SSc dermal fibroblasts

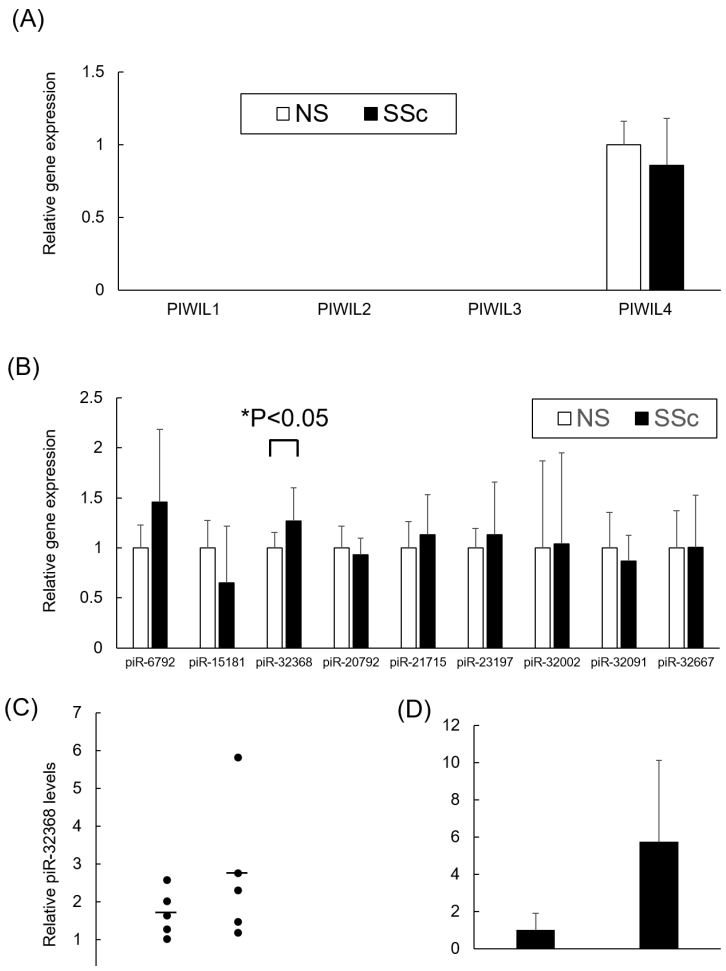

Figure 1. Expression of piR-32368 in the skin fibroblasts and tissues of patients with SSc. (A) Relative PIWI gene expression in cultured dermal fibroblasts derived from SSc patients and normal subjects (NS). Total RNA was extracted, and levels of indicated genes were analyzed by real-time PCR. The transcript level of each gene was normalized to that of GAPDH. The bars represent the mean values, and the T bars indicate standard deviations. The mean value of NS cells was set at 1. **(B)** Relative piRNA expression in cultured dermal fibroblasts derived from SSc patients and normal subjects (NS). RNA was extracted, and indicated piRNA levels were analyzed by real-time PCR. The transcript level of each gene was normalized to that of U6. The bars represent the mean values, and the T bars indicate standard deviations. The mean value of NS cells was set at 1. *$P < 0.05$ ($n = 3$). **(C)** Relative piRNA expression in skin tissues of five SSc patients and of control subjects (cont). RNA was extracted, and mRNA levels of piR-32368 were analyzed by real-time PCR. The lowest value in control tissues was set at 1. **(D)** piR-32368 levels in normal fibroblasts in the presence or absence of TGF-β1 (5 ng/mL) for 3 hours. *$P < 0.05$ compared to untreated normal fibroblasts (1.0).

Next, we compared piRNA expression of normal and SSc dermal fibroblasts. As the key molecules in the pathogenesis of SSc, we focused on COL1A1, COL1A2, COL3A1, KLF5, and Fli1. According to piRNA target gene predictions using piRNAdb (https://www.pirnadb.org), nine piRNAs were selected: has-piR-6792 for COL3A1, piR-15181 for COL1A1 and Fli1, piR-32368 for COL1A1, piR-20792 for Fli1, piR-23197 for Fli1 and KLF5, piR-32002 for Fli1, piR-21715 for Fli1, piR-32091 for COL3A1, and piR-32667 for COL1A2. Quantitative real-time PCR analysis was performed using the specific primer for each piRNA. As a result, among the nine piRNAs, only piR-32368 level was significantly up-regulated in SSc dermal fibroblasts (Figure 1B). Expression of piR-32368 in RNA extracted from five SSc whole skin tissues and normal skin tissues was also investigated: It was slightly increased in SSc skin, but without statistical significance (Figure 1C). piR-32368 up-regulation may therefore be highly specific to fibroblasts, but not in other cell types. To examine the possibility that the up-regulation of piR-32368 in SSc fibroblasts is due to the stimulation of intrinsic transforming growth factor (TGF)-β activation seen in these cell types as described previously (*16*), normal fibroblasts were stimulated with exogenous TGF-β1. TGF-β1 induced piR-32368 expression significantly (Figure 1D), suggesting that up-regulation of piR-32368 is consequence of activation of TGF-β signaling in SSc fibroblasts, at least partly.

3.3. Collagen expression in cultured dermal fibroblasts with transfected piR-32368

To better understand the role of piR-32368 up-regulation in SSc dermal fibroblasts, total RNA was isolated from cultured normal dermal fibroblasts transfected with or without piR-32368 synthetic oligo. Expression analysis showed that the oligo significantly induced the expression of piR-32368, and that piR-32368 overexpression was achieved with transfection of the oligo (Figure 2A). Furthermore, transfection of dermal fibroblasts with the piRNA oligo significantly reduced the expression of COL1A1 both at the mRNA levels (Figure 2B) and protein levels (Figure 2C). However, COL1A2 expression was not affected. These results indicate that COL1A1 is the specific target of piR-32368 in dermal fibroblasts.

3.4. Expression of piR-32368 in the sera of SSc patients

Lastly, we aimed to determine serum piR-32368 levels in patients with SSc and in control subjects using the same

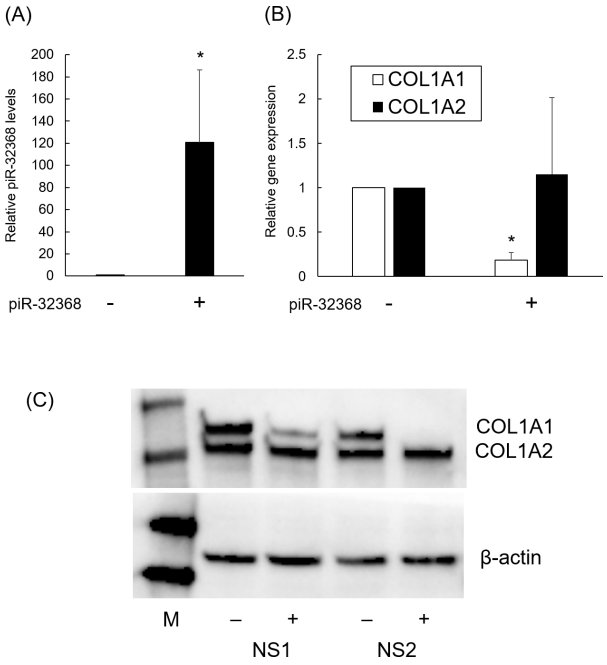

Figure 2. The function of piR-32368 in dermal fibroblasts. (A) Relative gene expression in cultured normal dermal fibroblasts transfected with or without piR-32368 synthetic oligo. RNA was extracted, and mRNA levels of piR-32368 were analyzed by real-time PCR. The mean value of cells transfected with control oligo was set at 1. *$P < 0.05$ ($n = 3$). **(B)** Relative gene expression in cultured normal dermal fibroblasts transfected with or without piR-32368 synthetic oligo. RNA was extracted, and mRNA levels of COL1A1 and COL1A2 were analyzed by real-time PCR. The mean value of cells transfected with control oligo was set at 1. *$P < 0.05$ ($n = 3$). **(C)** Lysates from two different normal dermal fibroblasts treated with or without synthetic oligo for 48 hours were subjected to immunoblotting with antibody against type I collagen or β-actin. M; molecular marker.

method as used in the studies of miRNAs and yRNAs (*14,15*), but amplification of the piRNA in the serum using real-time PCR could not be detected (data not shown).

4. Discussion

piRNAs have been implicated in the pathogenesis of several cancers (*2,4*). In patients with SSc, however, the expression and role of PIWI/piRNA has not been understood. In the current study, PIWIL1-3 was not detected in normal dermal fibroblasts. PIWIL1 and PIWIL2 have been reported to be found in the testes, while PIWIL3 could not be detected in human adult testes but is expressed in fetal testes and ovaries (*17*). The tissue specificity of PIWIL4 seems to be low, and the present study may be the first to demonstrate the expression of PIWIL4 in dermal fibroblasts according to PubMed search using keywords "fibroblast" and "PIWIL4". However, the PIWIL4 levels in normal and cultured SSc dermal fibroblasts were similar.

On the other hand, we focused on COL1A1, COL1A2, COL3A1, KLF5, and Fli1 as the SSc-related molecules, because they were are all reported to be affected by epigenetic regulation in patients with SSc (*18-22*). As far as we are aware base on search by PubMed using keywords "systemic sclerosis", "fibroblast" and "piRNA", we first found significant piR-32368 up-regulation in SSc dermal fibroblasts. There are no known reports on piR-32368, and its role and regulatory mechanism in human diseases remains unknown. Excess collagen production by dermal fibroblasts is thought, at least in part, to be caused by intrinsic activation of TGF-β signaling in SSc (*16*). Our study also indicated that the up-regulation of piR-32368 in SSc fibroblasts may also result from the activated endogenous TGF-β signaling.

SSc is an autoimmune disease in which fibroblasts produce collagen fibers more aggressively than normal. We therefore hypothesized that up-regulation of piR-32368 in SSc dermal fibroblasts stimulates collagen expression. Contrary to the expectation, however, forced piR-32368 overexpression in normal fibroblasts significantly reduced the expression of COL1A1 both at the mRNA levels and protein levels, but not COL1A2. COL1A1 is therefore thought to be the specific target of piR-32368. The vast majority of collagens accumulated in lesional skin of SSc are type I collagen: Type I collagen molecules consists of two α1(I) chains and one α2(I) chain, and COL1A1 and COL1A2 genes are located on chromosomes 17 and 7, respectively. COL1A1 and COL1A2 may therefore be regulated by different mechanisms. COL1A1 expression is, at least in part, regulated by piRNAs, while COL1A2 is unlikely to be targeted by piRNAs because the expression of COL1A2-associated piRNA was not altered in SSc dermal fibroblasts. piRNAs may be a clue to the distinct regulation of COL1A1 and COL1A2. Taken together, piR-32368 itself, which have suppressive effect on COL1A1 production, was up-regulated in SSc dermal fibroblasts. piR-32368 overexpression in SSc fibroblasts may therefore be negative feedback against collagen up-regulation, suggesting that piRNAs could be considered as a potential therapeutic target.

Serum piRNA has been reported to be a novel biomarker in human cancers. Serum exosomal piR-hsa-26925 and piR-hsa-5444 could, for example, serve as potential biomarkers for diagnosis of lung adenocarcinoma (*23*). However, amplification of the piRNA could not be detected in the human serum samples using real-time PCR in the current study. Further studies are needed to detect serum piR-32368 and to evaluate its usefulness in the diagnosis of SSc.

In conclusion, among piRNAs predicted to target SSc-related molecules, we first found significant piR-32368 up-regulation in SSc dermal fibroblasts, likely due to intrinsic TGF-β signaling. Forced piR-32368 overexpression in normal fibroblasts significantly reduced the expression of COL1A1 both at the mRNA levels and protein levels, but not COL1A2. piR-32368 overexpression in SSc fibroblasts may therefore be

negative feedback against collagen up-regulation, so piRNAs could be a potential therapeutic target.

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**Address correspondence to:*
Yutaka Inaba, Department of Dermatology, Graduate School of Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama, Wakayama 641-0012, Japan.
E-mail: ptfjk298@wakayama-med.ac.jp

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