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Liver Fibrosis by the Novel Gene Regulator
PI Polyamide Targeting
Transforming Growth Factor *β*1 Gene

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申請年 2016年

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Regular Article

Reduction of Dimethylnitrosamine-Induced Liver Fibrosis by the Novel Gene Regulator PI Polyamide Targeting Transforming Growth Factor β 1 Gene

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Received April 13, 2015; accepted September 7, 2015; advance publication released online September 16, 2015

Pyrrole–imidazole (PI) polyamide is a novel gene regulating agent that competitively inhibits transcription factor binding to the promoter of the specific target gene. Liver fibrosis is an integral stage in the development of chronic liver disease, and transforming growth factor β (TGF β) is known to play a central role in the progression of this entity. The aim of this study was to evaluate the effect of PI polyamide targeting TGF β 1 on rat liver fibrosis. PI polyamide was designed to inhibit activator protein 1 (AP-1) transcription factor binding to the TGF β 1 gene promoter. The effect of PI polyamide on hepatic stellate cells was evaluated by real time polymerase chain reaction (PCR) in RI-T cells. To determine the effect of PI polyamide *in vivo*, PI polyamide was intravenously administered at a dose of 3 mg/kg/week in dimethylnitrosamine (DMN)-induced rat model of liver fibrosis. Treatment of RI-T cells with 1.0 μ M PI polyamide targeting TGF β 1 significantly inhibited TGF β 1 mRNA expression. Azan staining showed that DMN treatment significantly increased areas of fibrous materials compared with controls. PI polyamide targeting TGF β 1 significantly decreased the fibrous area compared with DMN group. mRNA expression levels of α -smooth muscle actin and matrix metalloproteinase-2 were significantly increased in DMN-treated group compared with control. Treatment with TGF β 1 PI polyamide significantly decreased mRNA expression of these genes compared with DMN group. The novel gene regulator PI polyamide targeting TGF β 1 may be a feasible therapeutic agent for the treatment of chronic liver disease.

Key words pyrrole–imidazole (PI) polyamide; liver fibrosis; transforming growth factor (TGF) β 1

Liver cirrhosis is the end stage of all chronic liver diseases. Hepatic fibrosis is an integral phase in the development of chronic liver disease, and often precedes liver cirrhosis. Hepatic fibrosis is the common pathological basis of numerous chronic liver diseases including viral liver disease, alcoholic, non-alcoholic steatohepatitis, autoimmune hepatitis, primary biliary cirrhosis, and metabolic disease. Liver cirrhosis is characterized by the increase and excessive deposition of the liver extracellular matrix (ECM).^{1–3)}

Excessive activation of transforming growth factor β (TGF β) increases the synthesis and decreases the degradation of ECM proteins with a gradual destruction of organ tissue and structure.^{4–8)} The major source of ECM deposition in the liver is hepatic stellate cells (HSCs). After liver injury, TGF β promotes the activation and proliferation of hepatic HSCs.^{2,3,9–11)} High levels of TGF β 1 are often found in liver fibrosis and there may be a positive correlation between the elevation of TGF β 1 mRNA level and fibrogenic activity.^{12–14)} Thus, synthesis of ECM proteins increases in the liver due to excessive activation of the TGF β signal transduction pathway. Therefore, this pathway has become a potential target for the treatment of the liver fibrosis.

Pyrrole–imidazole polyamide (PI polyamide) is a novel gene regulator composed of *N*-methylpyrrole (Py) and *N*-methylimidazole (Im). PI polyamide can inhibit DNA–protein

interaction by binding to the minor groove of double-strand DNA with high affinity and sequence specificity. Antiparallel pairing of imidazole with pyrrole (Im/Py) recognizes G-C base pair, whereas a Py/Py pair recognizes either an A-T or T-A base pair. Interference of DNA–transcription factor protein interaction by PI polyamides inhibits the regulation of the gene transcription and modulates target protein expression.^{15–18)} Various types of sequence specific PI polyamides have been developed to control gene expression.^{19–25)} In a previous study, we designed PI polyamide targeting the consensus sequence of the activator protein 1 (AP-1) binding site on the TGF β 1 promoter and demonstrated that this had an anti-fibrogenic effect in the rat model of renal fibrosis.^{20,21)}

This study was undertaken to evaluate the effect of PI polyamide targeting TGF β 1 in inhibiting liver fibrosis in the rat model of chronic liver disease.

MATERIALS AND METHODS

Synthesis of PI Polyamides PI polyamide targeting TGF β 1 was designed to span the boundary of the AP1 binding site of the TGF β 1 promoter. PI Polyamides were synthesized according to previously described methods.^{26,27)} A mismatch polyamide that did not bind to the transcription binding sites of TGF β 1 promoter was also designed and synthesized.

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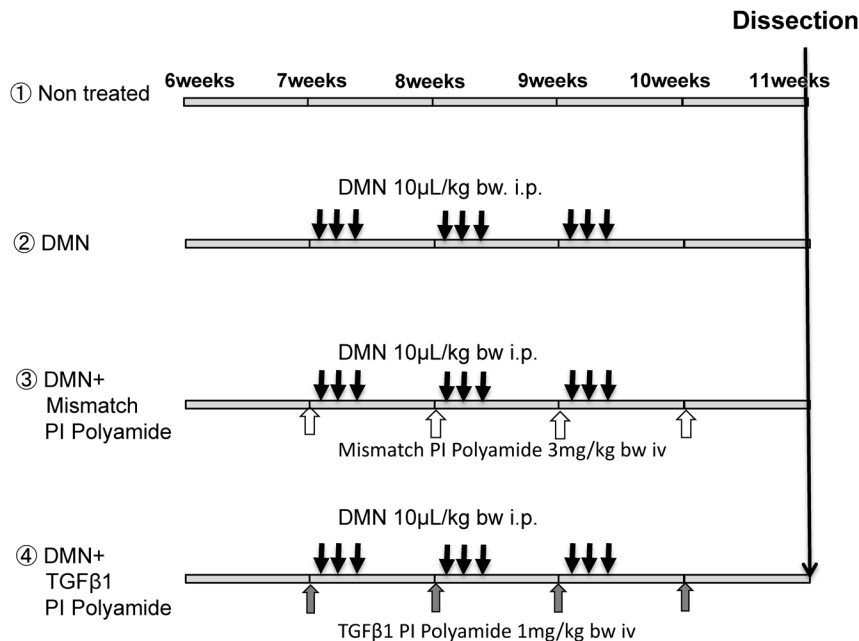


Fig. 1. Experimental Protocol for Administration of PI Polyamide That Targets TGF β 1 to Sprague-Dawley Rats

Determination of mRNA Expression For *in vitro* experiments, RI-T cells (JCRB1088) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). After a 24-h culture with DMEM containing 0.5% FCS, the cells were incubated with 1.0, 0.1 or 0.01 μ M PI polyamide targeting the TGF β 1 gene or 1.0 μ M mismatch PI polyamide in DMEM with 0.5% FCS for 8 h. Four hours after the initiation of PI polyamide treatment, 0.01 μ M phorbol-12-myristate-13 acetate (PMA) was added into medium to stimulate TGF β expression. Six hours after PMA stimulation, total RNA was isolated and reverse-transcribed to cDNA as described previously.²⁸⁾ Real-time quantitative polymerase chain reaction (RTq-PCR) was performed with the cDNA (diluted 4 times) using TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) and an ABI 7500 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. Assay-on-Demand primers and probes were purchased from Applied Biosystems (TGF β 1, Rn01475963). 18S ribosomal RNA (rRNA) expression (Applied Biosystems 4319413E) was quantified for normalization of mRNA expression level among samples. RT-PCR data were analyzed using a standard curve. In all cases, the correlation coefficients for the standard curves were >0.90 .

For the *in vivo* experiments, total RNA was isolated from 20 mg of liver tissue of a Sprague-Dawley rat and reverse-transcribed. RTq-PCR was performed with the cDNA. Assay-on-Demand primers and probes were purchased from Applied Biosystems (α -smooth muscle actin: Rn01759928_g1, collagen type IV: Rn01482927, and TGF β 1: Rn01475963).

In Vivo Experiments This study conformed to the guidelines published in the *Guide for the Care and Use of Laboratory Animals* of the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by Nihon University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (7-weeks old; CLEA Japan, Tokyo, Japan) were used in all of the experiments. Rats were fed normal chow diet (Oriental Yeast, Tokyo, Japan) *ad libitum* for 2 weeks.

Biochemical Examinations Serum levels of total protein, albumin, total bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by the spectrophotometric method.

Definition of liver fibrosis was based on the relevant literature.²⁹⁾ Liver fibrosis was induced by intraperitoneal injections of dimethylnitrosamine (DMN; Sigma, MO, U.S.A.) on 3 consecutive days a week for 3 weeks. PI polyamide (1 mg/kg body weight) targeting TGF β 1 or mismatch polyamide was dissolved in 100 μ L of 0.1% acetic acid and injected *via* the tail vein weekly for 4 weeks (Fig. 1).

Twenty-four rats were randomly divided into 4 groups: (1) normal control group ($n=6$, without DMN), treated with 0.1% acetic acid, (2) DMN group ($n=6$, treated with 1 μ L of 0.5% DMN/kg, and 0.1% acetic acid), (3) mismatch group ($n=6$, treated with 1 μ L of 0.5% DMN/kg, and 1 mg/kg mismatch PI polyamide in 0.1% acetic acid), (4) PI Polyamide group ($n=6$, treated with 1 μ L of 0.5% DMN/kg, and 1 mg/kg PI polyamide targeting TGF β 1 in 0.1% acetic acid).

The rats were euthanized by a lethal injection of sodium pentobarbital (IP, 100 mg/kg body weight) 4 weeks after PI polyamide administration.

Histological Examination and Immunohistological Staining Liver tissue sections were stained with haematoxylin-eosin (HE) for histopathological examination and Azan staining was used for determination collagen area. The areas of collagen stained blue with Azan were quantitated using the image J software (NIH, Bethesda, MD, U.S.A.)³⁰⁾ by analyzing 15 random fields per slide and calculating the ratio of connective tissue to the whole area of the liver tissue.

Immunohistochemical staining was carried out to detect the expression of various marker proteins using mouse primary antibodies against α -smooth muscle actin (α -SMA) (1:100; M0851, DAKO, Glostrup, Denmark), collagen type IV (1:100; ab6586, Abcam, Cambridge, U.K.) and TGF β (1:100; sc-146, Santa Cruz Biotechnology, Carlsbad, CA, U.S.A.) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) was used as the secondary antibody in each case.

Statistical Analysis Results are given as the mean \pm standard error of mean (S.E.M.). The statistical significance of differences between mean values was evaluated by Student's *t*-test for unpaired data. A *p* value ≤ 0.05 was considered statistically significant.

RESULTS

Reduction of TGF β 1 mRNA Expression by PI Polyamide in RI-T Cells Treatment of RI-T cells with 0.1 μ M PMA increased the TGF β 1 mRNA expression level by about 2.6 fold. When 1.0 μ M PI polyamide was added, the TGF β 1 mRNA expression level was significantly decreased by about 0.6 fold compared with the control cells. Cells treated with 0.01 and 0.1 μ M PI polyamide or 1 μ M mismatch PI polyamide did not show a significant difference in the TGF β 1 mRNA expression level compared with the non treated cells (Fig. 2).

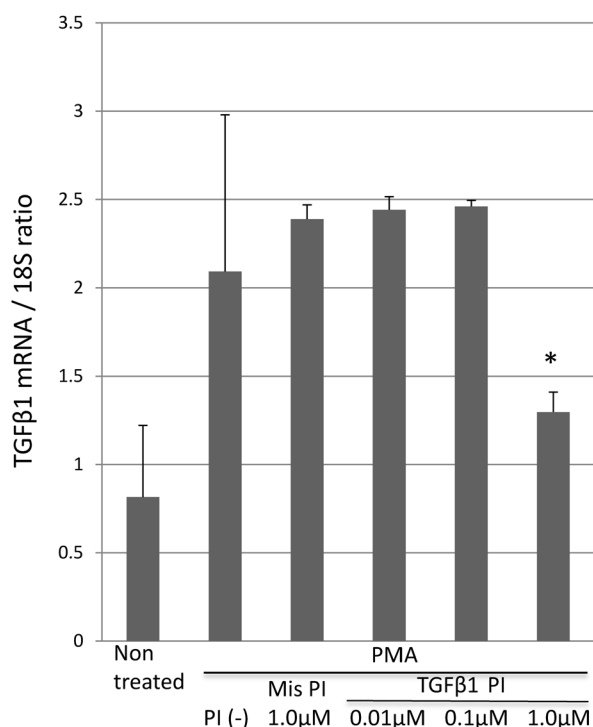


Fig. 2. Effect of PI Polyamide Targeting TGF β 1 on TGF β 1 mRNA Expression in RI-T Cells

**p* < 0.05 vs. PMA(+)/PI(-) group. PMA: phorbol-12-myristate-13 acetate, PI: PI polyamide, Mis: mismatch.

Anti Fibrotic Effect of PI Polyamide Targeting TGF β 1 on DMN-Induced Rat Hepatic Fibrosis Intravenous administration of PI polyamide or mismatch did not affect the body weight and organ weights of Sprague-Dawley rat. There were no significant changes between the serum values of total protein, albumin, total bilirubin ALT and AST of DMN treated rats and PI polyamide treated rats. Serum ALT levels in DMN treated group, mismatch PI polyamide group and TGF β 1 PI polyamide group were significantly higher compared with that in control group (Table 1).

In this rat model, hepatic fibrosis was induced by DMN treatment. HE and Azan staining were performed to determine whether the extent of the DMN-induced liver damage was reduced by PI polyamide targeting TGF β 1.

The livers of the control group rats showed normal lobular architecture and no fibrotic space. However, the DMN group showed dramatic changes consisting of the destruction of normal liver lobular architecture, bridging necrosis, inflammatory cell infiltration, fibrotic septae and pseudolobule formation. PI polyamide treatment suppressed these DMN-induced fibrosis including bridging necrosis, inflammatory cell infiltration, fibrotic septae and pseudolobule formation (Fig. 3). Figure 4 shows typical Azan staining results in which fibrous materials are stained blue. Little or no blue staining was observed in the controls. In contrast, the livers injured by DMN treatment displayed considerable accumulation of fibrous materials. DMN treatment for 3 weeks caused excessive deposition of collagen fibrils that was sufficient for the evaluation of the antifibrogenic effect of PI polyamide targeting TGF β 1. Based on the result of Azan staining, PI polyamide treatment clearly suppressed collagen accumulation. To quantitatively evaluate the effect of PI polyamide on fibrogenesis, we measured areas of fibrous materials stained blue using the Image-J software. DMN treatment significantly increased areas of fibrous materials compared with that in controls. PI polyamide targeting TGF β 1 significantly suppressed the fibrous area compared with that in DMN group (Fig. 5).

The immunostaining experiments using α -SMA and collagen type IV antibody was performed to evaluate the effect of PI polyamide targeting TGF β 1 on liver fibrosis. Immunohistochemical result showed that DMN-induced accumulation of α -SMA, which was stained brown, was decreased by intravenous administration of 3 mg/kg of PI polyamide targeting TGF β 1 (Fig. 6). DMN-induced accumulation of collagen type IV, which was stained red, was also decreased by the administration of PI polyamide targeting TGF β 1 (Fig. 7).

Table 1. Body Weight, Organweights and Plasma Measurements in DMN Treated Sprague-Dawley Rats

| | Body weight (g) | Liver weight (%) | Spleen weight (%) | Total protein (g/dL) | Albumin (g/dL) |
|--------------------|-------------------------|------------------|-------------------|----------------------|----------------|
| A. Control | 504.2 \pm 69.2 | 41.8 \pm 4.2 | 2.3 \pm 0.8 | 6.4 \pm 0.5 | 4.1 \pm 0.1 |
| B. DMN | 489.0 \pm 20.2 | 44.1 \pm 8.8 | 3.1 \pm 0.6 | 6.1 \pm 0.2 | 4.1 \pm 0.2 |
| C. DMN-mismatch PI | 406.0 \pm 17.7 | 40.3 \pm 5.1 | 3.3 \pm 0.6 | 6.0 \pm 0.5 | 3.9 \pm 0.2 |
| D. DMN-TGF PI | 442.3 \pm 24.7 | 40.9 \pm 0.7 | 3.4 \pm 0.7 | 5.6 \pm 0.3 | 3.7 \pm 0.3 |
| | Total bilirubin (mg/dL) | AST (IU/L) | ALT (IU/L) | | |
| A. Control | 0.05 \pm 0.01 | 123.0 \pm 19.7 | 41.4 \pm 8.1 | | |
| B. DMN | 0.06 \pm 0.01 | 165.3 \pm 79.3 | 53.8 \pm 2.9* | | |
| C. DMN-mismatch PI | 0.07 \pm 0.03 | 144.8 \pm 26.9 | 60.6 \pm 12.9* | | |
| D. DMN-TGF PI | 0.06 \pm 0.01 | 159.8 \pm 31.1 | 51.5 \pm 2.5* | | |

**p* < 0.05 vs. Control. Liver weight: Liver weight/body weight (%). Spleen weight: Spleen weight/body weight (%).

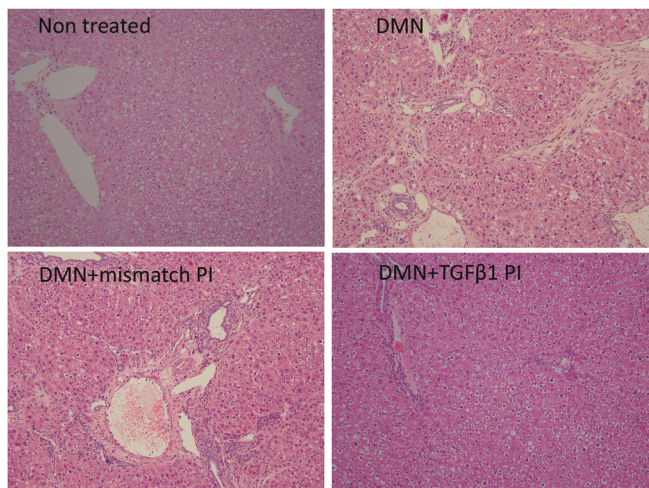


Fig. 3. Effect of PI Polyamide on DMN-Induced Liver Fibrosis (HE Stain)

DMN: dimethylnitrosamine, PI: PI polyamide.

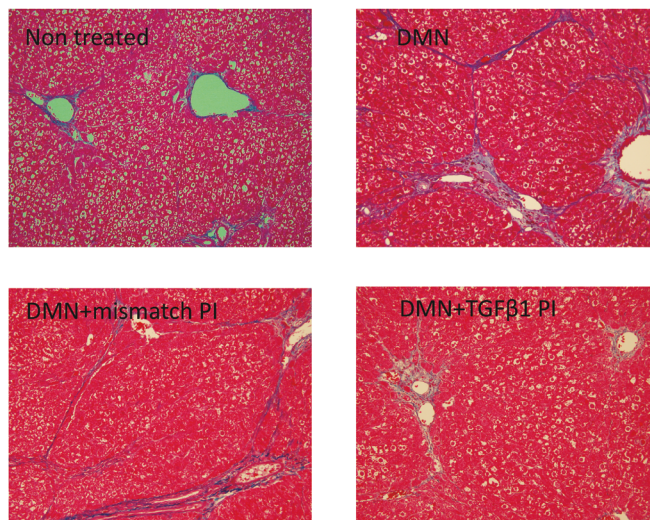


Fig. 4. Effect of PI Polyamide on DMN-Induced Liver Fibrosis (Azan Stain)

DMN: dimethylnitrosamine, PI: PI polyamide.

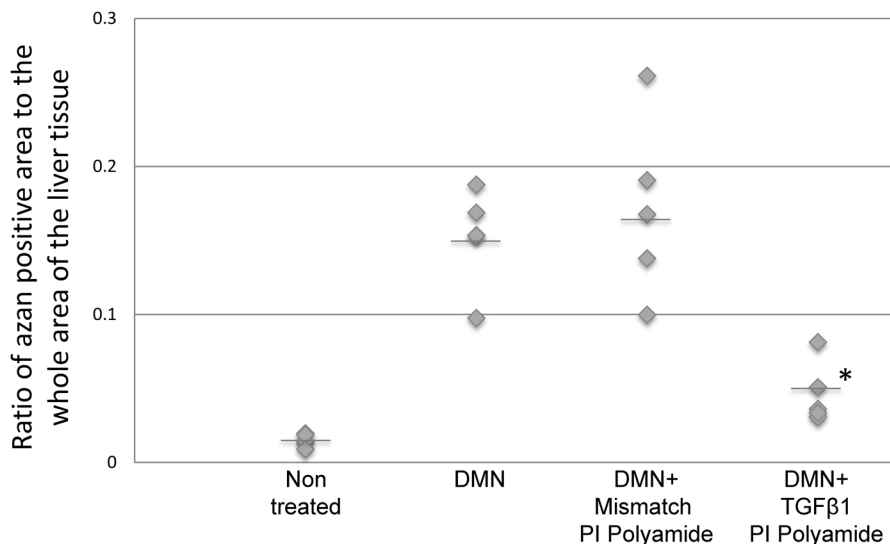


Fig. 5. Quantitative Evaluation of the Effect of PI Polyamide Targeting TGFβ1 on DMN-Induced Liver Fibrosis

* $p < 0.05$ vs. DMN group. DMN: dimethylnitrosamine, PI: PI polyamide.

Effect of TGFβ1 PI Polyamide on mRNA Expression in Rat Liver

The mRNA expression levels of α -SMA, collagen type I α 1, collagen type IV, matrix metalloproteinase-2 (MMP2), TGFβ1, and Tissue inhibitor of metalloproteinase-1 (TIMP1) were significantly increased in DMN treated group compared with that in control group. Treatment with TGFβ1 PI polyamide significantly decreased α SMA and MMP2 compared with that in DMN groups. Collagen type I α 1, collagen type IV, TGFβ1, and TIMP1 mRNA levels were lower in TGFβ1 PI polyamide group compared with that in DMN group, but they were not statistically significantly different from control (Fig. 8).

DISCUSSION

In the present study, the PI polyamide targeting the TGFβ1 gene promoter significantly inhibited the expression of TGFβ1 mRNA in cultured rat HSCs stimulated with PMA, suggest-

ing this polyamide has the potential to control TGFβ1 gene expression. The DMN-induced rat model of liver fibrosis was suitable to test this effect *in vivo*. Administration of PI polyamide targeting the TGFβ1 promoter reduced the DMN-induced liver fibrosis without any significant adverse effects. PI polyamide targeting the TGFβ1 promoter might be a novel promising therapeutic agent for treatment of progressive liver fibrosis in chronic liver diseases.

Hepatic fibrosis is characterized by scarring due to chronic inflammation from liver disease. Liver fibrosis is a complicated pathological process in which multiple components including HSCs, Kupffer cells, hepatocytes, various cytokines, and ECM proteins are involved. Among those, activated HSCs are the major producers of fibrotic matrix.^{31,32)} After liver injury, they become activated, developing a myofibroblast-like phenotype associated with increased proliferation and collagen synthesis. TGFβ1 is known to be the most potent fibrogenic cytokine that mediates HSC activation.³³⁾ Moreover, activated

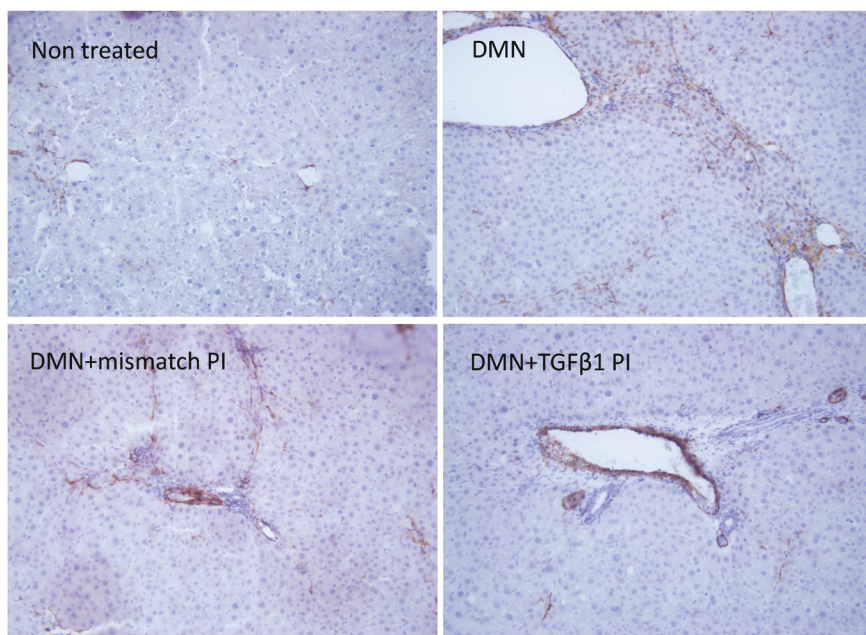


Fig. 6. Effect of PI Polyamide on α -Smooth Muscle Actin Expression in DMN-Induced Rat Liver Fibrosis (Immunohistochemical Staining)
DMN: dimethylnitrosamine, PI: PI polyamide.

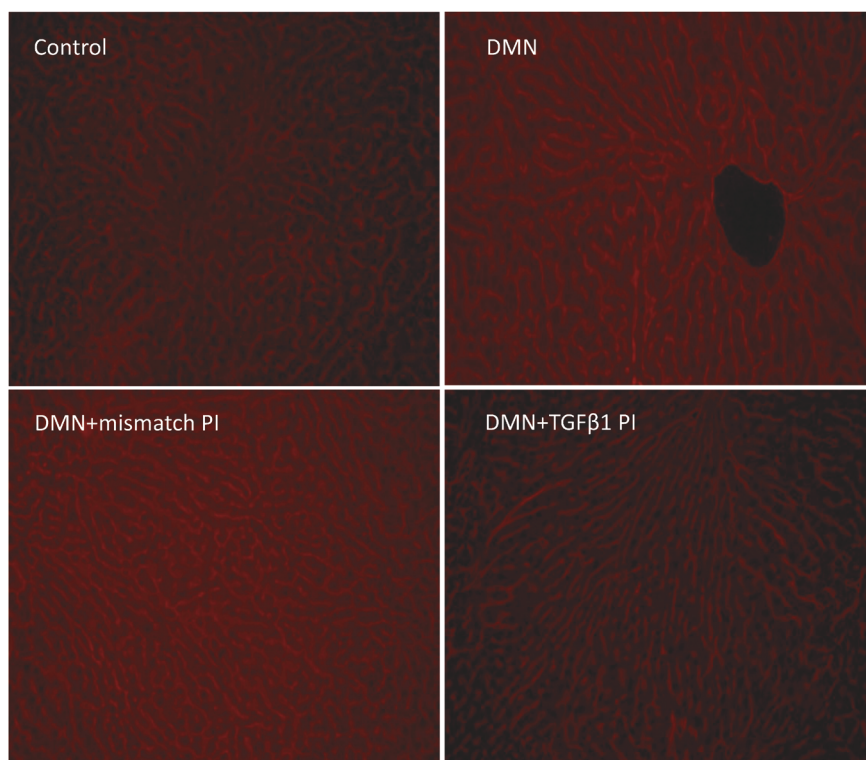


Fig. 7. Effect of PI Polyamide on Collagen Type IV Expression in DMN-Induced Rat Liver Fibrosis
DMN: dimethylnitrosamine, PI: PI polyamide (immunohistochemical staining).

HSCs secrete $TGF\beta$, and activate HSCs in turn by an auto-crine loop.³⁴⁾ Therefore, $TGF\beta 1$ is a promising target for the therapy of liver fibrotic disease.

In a previous study, we demonstrated that PI polyamide targeting $TGF\beta 1$ reduced urinary $TGF\beta$ excretion by 60%, resulting in marked inhibition of renal injury in an animal model of hypertension-induced renal injury.²¹⁾ PI polyamide targeting $TGF\beta 1$ is designed to inhibit the binding of AP-1 transcrip-

tion factor to the $TGF\beta 1$ promoter. Strong, fast and specific binding of this polyamide to the target sequence was shown in our previous study.¹⁹⁻²¹⁾ PI polyamide is a novel technology to control specific gene expression alike nucleic acid based medicines. PI polyamide binds to the minor groove of the double helical DNA with high sequence specificity in a hairpin shape forming an amide pairing. PI polyamide recognizes and binds to the target DNA sequence by the recognition rule. The par-

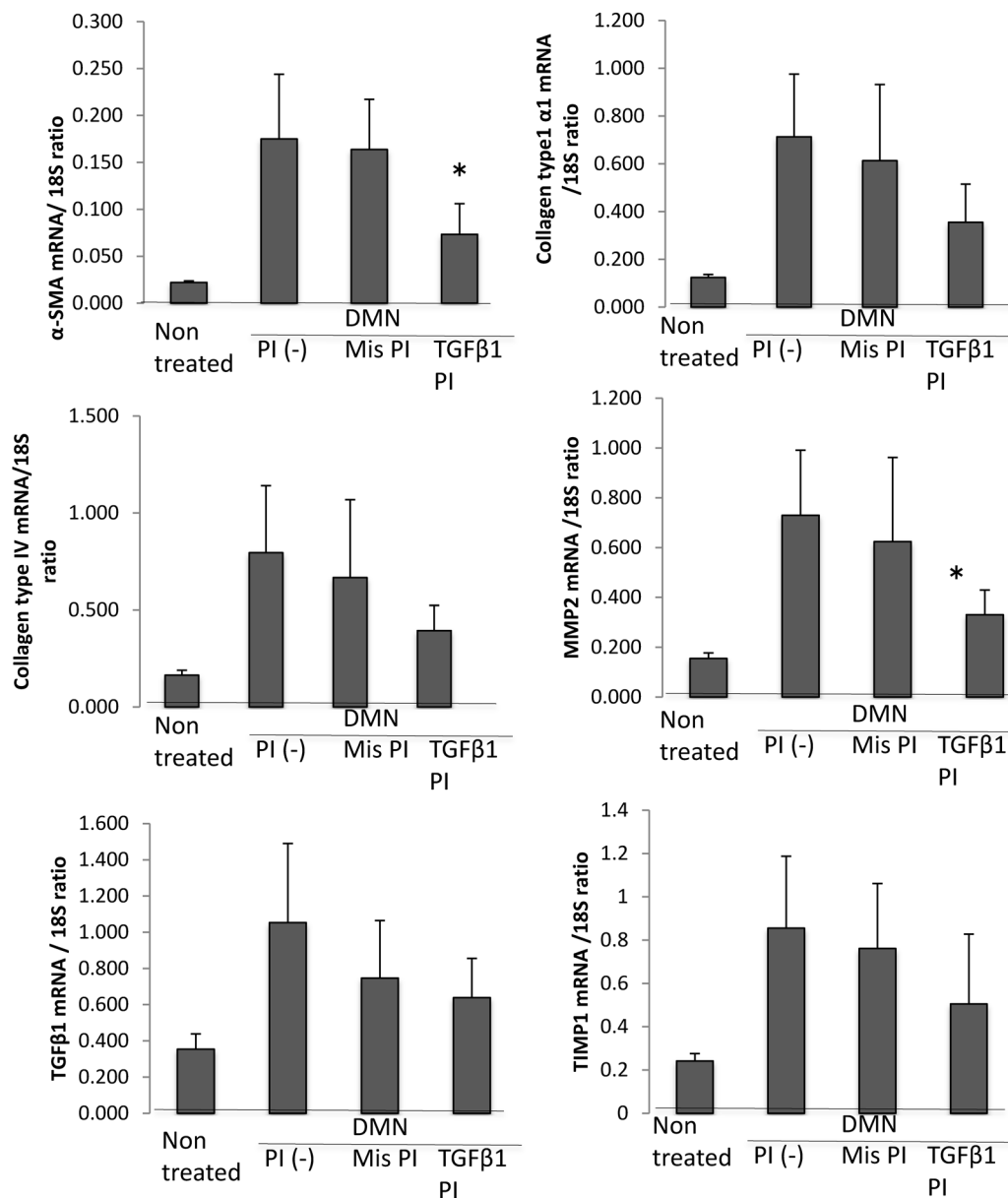


Fig. 8. Effect of PI Polyamide on α -Smooth Muscle Actin (α -SMA) Collagen Type IV, and TGF β mRNA Expression in DMN-Induced Rat Liver Fibrosis

* $p < 0.05$ vs. PI(-) group.

ing of an imidazole opposite a pyrrole targets G-C base pairs, and a pyrrole opposite an imidazole targets C-G base pairs. Pyrrole-pyrrole targets T-A and A-T base pairs.^{15,16} Following the recognition rules, we can design PI polyamides that bind to target genes playing key roles in diseases for which no effective treatment exists. We have previously reported PI polyamide targeting LOX1,³⁵ ABCA1,²³ FcR γ ³⁶ and CTGF.³⁷ Whereas nucleic acid medicines such as antisense DNA, ribozymes, and decoys are easily degraded by nucleases, PI polyamides are stable against these enzymes because of their non-nucleic acid based composition and structure. Nucleic acid based medicines require drug-delivery systems for their adequate distribution. By contrast, PI polyamides do not require any transfection reagents to incorporate into cells or distribute into target tissues.^{15,16} Because of these desirable properties of PI polyamide, this novel compound could be used as a gene regulating agent in the near future.

In this study, we did not observe any significant adverse effect due to PI polyamide targeting TGF β 1. However, pharmacokinetics of PI polyamides has not been well examined. Moreover, safety and toxicity of this novel compound has not been fully evaluated. Therefore, further examination of the chemical and biological features of PI polyamides are required in order to develop this compound as a therapeutic agent for human disease.

In conclusion, PI polyamide targeting TGF β 1 suppressed the DMN-induced liver fibrosis. This new compound may be a novel gene regulator for the prevention of liver fibrosis in chronic liver disease.

Acknowledgment This work was partially supported by a Grant from the "Strategic Research Base Development" Program for Private Universities subsidized by MEXT (2014).

Conflict of Interest The authors declare no conflict of interest.

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