Article

HtrA1 Is Specifically Up-Regulated in Active Keloid Lesions and Stimulates Keloids Development

Satoko Yamawaki [1], Motoko Naitoh [2,*], Hiroshi Kubota [3], Rino Aya [2], Yasuhiro Katayama [2], Toshihiro Ishiko [4], Taku Tamura [3], Katsuhiro Yoshikawa [5], Tatsuki Enoshiri [2], Mika Ikeda [6] and Shigehiko Suzuki [2]

[1] Department of Plastic and Reconstructive Surgery, Japanese Red Cross Fukui Hospital, 2-4-1 Tsukimi, Fukui-city, Fukui, 918-8501, Japan; satokoy@kuhp.kyoto-u.ac.jp
[2] Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Sakyo-ku, Kyoto city, 606-8507, Japan; Motoko Naitoh: mnaitoh@kuhp.kyoto-u.ac.jp; Rino Aya: rinok@kuhp.kyoto-u.ac.jp; Yasuhiro Katayama: hemim@kuhp.kyoto-u.ac.jp; Tatsuki Enoshiri: enotatsu@kuhp.kyoto-u.ac.jp; Shigehiko Suzuki: ssuzuk@kuhp.kyoto-u.ac.jp
[3] Department of Life Science, Faculty of Engineering Science, Akita University, 1-1 Tegata Gakuenmachi, Akita, 010-8502, Japan; Hiroshi Kubota: hkubota@gipc.akita-u.ac.jp ; Taku Tamura: taku@gipc.akita-u.ac.jp
[4] Department of Plastic and Reconstructive Surgery, Japanese Red Cross Otsu Hospital, 1-1-35, Nagara, Otsu city, Shiga, 520-8511, Japan; ishiko@otsu.jrc.or.jp
[5] Department of Plastic and Reconstructive Surgery, Shiga Medical Center for Adults, 5-4-30, Moriyama, Moriyama city, Shiga, 524-8524, Japan; khiro@kuhp.kyoto-u.ac.jp
[6] Department of Plastic and Reconstructive Surgery, Kobe City Medical Center General Hospital, 2-1-1, Minatojima minami-machi, Cyuou-ku, Kobe city, Hyogo, 650-0047, Japan; mikaring@crux.ocn.ne.jp
* Correspondence: mnaitoh@kuhp.kyoto-u.ac.jp; Tel.: +81-75-751-3613

Abstract: 1) Background: Keloids occur after the failure during the wound-healing process, persist the inflammation and are refractory to various treatments. The pathogenesis of keloids is still unclear. We previously analyzed the gene expression profiles in keloid tissue using microarray and Northern blot analysis and found that HtrA1 was markedly upregulated in the keloid lesions. HtrA1 is a member of the HtrA family of serine protease, has been suggested to play a role in the pathogenesis of various diseases including age-related macular degeneration and osteoarthritis by modulating proteins in extracellular matrix or cell surface. We focused on HtrA1, analyzed the localization and the role in keloid pathogenesis. 2) Methods: Twenty seven keloid patients and seven unrelated patients were enrolled in this study. We performed in situ hybridization analysis, immunohistochemical analysis, western blot analysis and cell proliferation assay. 3) Results: First, the fibroblast-like cells expressed HtrA1 higher in the active keloid lesions than in the surrounding lesions in situ hybridization. Second, the proportion of HtrA1-positive cells in keloid was higher than that of in normal skin significantly in immunohistochemical analysis. Third, HtrA1 protein was up-regulated, relative to normal skin tissue samples in western blot analysis. Finally, silencing of HtrA1 gene expression suppressed the cell proliferation significantly. 4) Conclusion: HtrA1 was highly expressed in keloid tissues and the suppression of HtrA1 gene inhibited the proliferation of keloid-derived fibroblasts. HtrA1 may promote keloid development through accelerating cell proliferation and remodeling keloid-specific extracellular matrix or cell surface molecules. HtrA1 is suggested to have an important role in keloid pathogenesis.

Keywords: keloids; fibroproliferative disorder; HtrA1; inflammation

1. Introduction

Keloids are a dermal fibrotic disease characterized by abnormal accumulation of extracellular matrix and fibroproliferation in the dermis[1,2]. They appear as raised, red, and inflexible scar tissue developing during the wound-healing process, even from tiny wound including vaccination and insect bites. Keloid lesions expand over the boundaries of the initial injury site, and the lesions

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continue to develop and become larger than original size during the lifetime[3,4]. There are many treatments for keloids; steroid injections, steroid tape, surgery with postoperative irradiation and so on. Even if we carry out the operation and postoperative radiation to the keloid patients, the cure rate is 28~89%[3,5-8], it depends on the individuals. To clarify the keloid pathogenesis will improve the treatments results.

In our previous report, to study molecular mechanism of keloid pathogenesis, cDNA microarray and Northern blot analysis comparing gene expression patterns in keloid lesions and normal skin were performed[9]. We found that HtrA1, a member of the HtrA family of serine protease and a mammalian homolog of *E. coli* HtrA (DegP), was markedly upregulated in the keloid lesions. As human HtrA1 has multiple domains including protease domain, IGFBP domain and PDZ domain, HtrA1 has been expected to be a multifunctional protein. Several cellular and molecular studies suggest that HtrA1 plays a key role in regulating various cellular processes via the cleavage and /or binding of pivotal factors that participate in cell proliferation, migration, and cell fate[10-12]. HtrA1 has been suggested to closely associate with the pathology of various diseases including osteoarthritis, age-related macular degeneration (AMD), familial cerebral small vessel disease (CARASIL), and malignant tumors. HtrA1 was suggested to stimulate progression of arthritis through degrading cartilage matrix in osteoarthritis[13]. Recently, increased expression of human HtrA1 in the mouse retinal pigment epithelium (RPE) was shown to induce vasculogenesis and degeneration of the elastic lamina and tunica media of the vessels, similar to that observed in AMD patients [14,15]. These observations implicate that HtrA1 play a role in the pathogenesis of various diseases by modulating proteins in extracellular matrix (ECM) or cell surface.

In the present study, we examined expression and localization of HtrA1 in keloid tissues, using *in situ* hybridization and immunohistochemical studies, and found that HtrA1 is strongly up-regulated at both the mRNA and protein levels in the hypercellular and active keloid lesions. Furthermore, we demonstrate that silencing *HtrA1* gene expression in keloid fibroblasts significantly inhibits cell proliferation. Taken together, we propose that HtrA1 may be a pivotal molecule in the keloid pathogenesis, and our discussion centers on the possible roles of HtrA1 in the molecular mechanism of keloid development.

2. Results

2.1. In situ hybridization of HtrA1 mRNA in keloid lesions and normal skins

To confirm the up-regulation of mRNA level for HtrA1 in our previous microarray and Northern blot analyses, and to analyze localization of *HtrA1* mRNA in keloid lesions, in situ hybridization was performed against five keloid and five unaffected skin samples. One specimen (No.27 in Table 1) was carried out in situ hybridization on the several parts of lesions which differed in the activity of keloid. Expression of the *HtrA1* gene was clearly detected in the fibroblastic cells in the hypercellular and actively growing area of keloid lesions (Figure 1A, Supplementary figure A1-A, C and E), but not in those of unaffected skin (Figure 1B). In the sections hybridized with sense probe, no signal was observed (Supplementary figure A1-B, D and F), demonstrating specific staining by the antisense probe. All keloid sections were hard and elevated portion in keloid lesions, and in this part, antisense probe provided strong signals (Figure 1A, Supplementary figure A1-A, C and E). We show the clinical findings and the results of in situ hybridization of the sample 27 (Figure 2). The activity of keoid falls in order of A, B, C. the higher in the activity of the part of the lesion is, the more the cells proliferated and the more HtrA1 was up-regulated (Figure 2). Therefore, *HtrA1* mRNA is strongly up-regulated in keloid lesions. Moreover, as the part of the higher activity of keloids revealed the higher expression of HtrA1.

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[1] **Table1.** List of all samples used in this study. Assays to which the samples were subjected. A, immunohistochemical staining; B, in situ hybridization; C, cell proliferation assay with silencing HtrA1 gene expression; D, western blotting.

	No.	age	sex	region	HtrA1-possitve cells (%)	assays
Keloid	1	75	M	shoulder	18.83	A
	2	51	F	chest	12.40	A
	3	49	M	neck	38.74	A, B
	4	67	F	abdomen	43.52	A
	5	32	M	chest	24.77	A
	6	34	M	abdomen	40.64	A
	7	67	M	abdomen	32.80	A
	8	16	F	chest	41.90	A, C
	9	64	M	shoulder	25.82	A
	10	28	M	chest	24.85	A
	11	24	F	chest	28.36	A, C
	12	20	F	shoulder	35.43	A
	13	62	F	chest	12.68	A
	14	30	M	shoulder	35.34	A
	15	20	F	chest	48.37	A
	16	65	M	back	35.55	A
	17	38	F	chest	39.68	A
	18	39	F	abdomen		B
	19	75	M	chest		B
	20	21	M	back		B
	21	20	M	back		B
	22	31	M	shoulder		D
	23	20	F	shoulder		D
	24	20	F	chest		C, D
	25	58	F	shoulder		D
	26	24	M	chest		C
	27	41	F	abdomen		B
Normal skin	1	51	F	back	3.76	A, D
	2	45	F	abdomen	2.83	A
	3	47	F	shoulder	2.60	A
	4	51	F	thigh	2.13	A
	5	88	M	back		D
	6	49	F	abdomen		D
	7	51	F	abdomen		D

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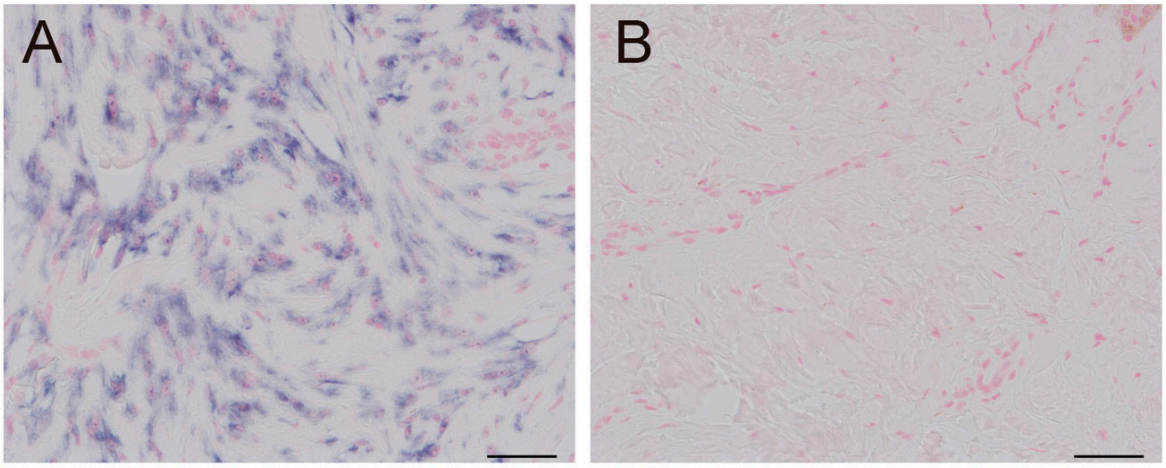

Figure 1. In situ hybridization for *HtrA1* mRNA in keloid and normal skin. Sections from active keloid lesions (A) or unaffected region (B) (patient no. 18 in Table 1) were hybridized with a probe specific to *HtrA1* mRNA. Positive signals are visualized in blue. Bar = 50 μm.

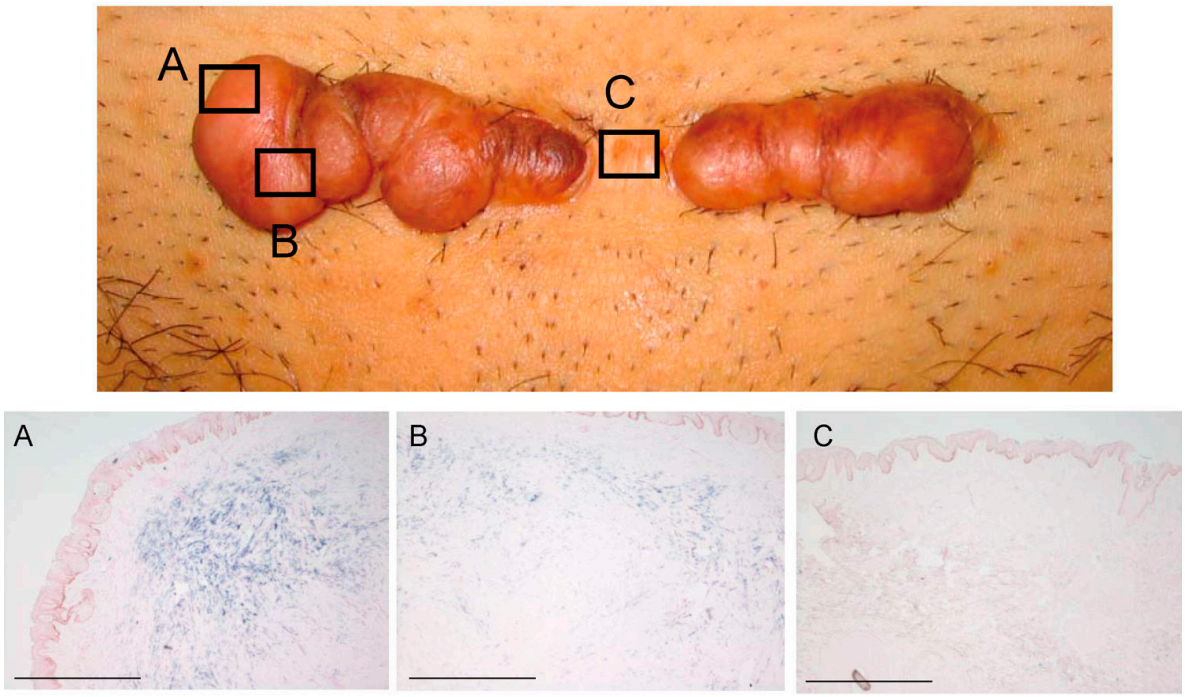

Figure 2. The abdominal keloid after laparoscopic surgery. The activity of keloid falls in order of A, B, C. The higher in the activity the part of the lesion is, the more the cell proliferated and the more HtrA1 was up-regulated. (A>B>C)

2.2. Immunohistochemical staining and Western blot analysis of HtrA1

To examine whether the up-regulation of HtrA1 at mRNA level leads to increase in protein level, we performed immunohistochemical analysis to detect HtrA1 (Figure 3A, B, Supplementary figure A2-A-F). HtrA1 was clearly detected by immunostaining in keloids (Figure 3A, Supplementary figure A2-A, C and E), while no signals were observed in normal skins (Figure 3B). These data are consistent with the results of *in situ* hybridization. No positive signal was found in

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controls untreated with the primary antibody (Supplementary figure A2-B, D, and F). Therefore, HtrA1 is strongly up-regulated at protein level in active area in keloid lesions. To confirm up-regulation of HtrA1 protein, Western blot analysis was performed. In all keloid tissue samples from four patients, HtrA1 protein was up-regulated, relative to four normal skin samples (Figure 4). Counting HtrA1-positive cells after immunohistochemical staining indicated that the proportion of cells expressing detectable levels of HtrA1 in keloid tissue was ranging from 12.4% to 48.4%, with an average of 31.9±10.5% (Figure 5). In contrast, the proportion of HtrA1-positive cells in normal skin was only ranging from 2.1 to 3.8%, with an average of 2.8±0.6%. The proportion of HtrA1-positive cells is significantly higher in keloids than in normal skin ($p<0.001$). The total number of fibroblasts was much less in normal skin relative to keloid tissue (Figure 3), as previously reported. These results indicate that keloid tissue exhibits an increase in the number of fibroblasts producing HtrA1, as well as an increase in the total number of fibroblasts.

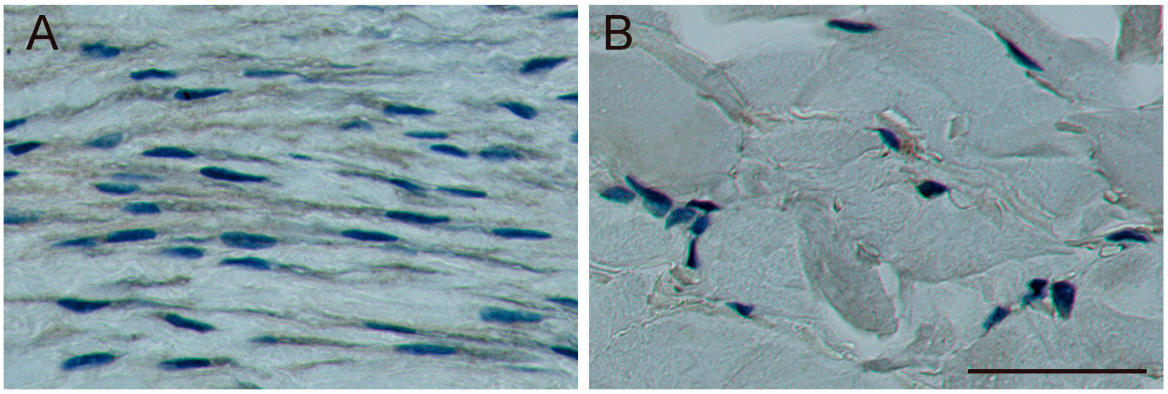

Figure 3. Immunohistochemical staining of *HtrA1* protein in keloid (A) and normal skin tissue (B). Sections from active keloid lesions (A) or normal skin (B). A; Patient no. keloid-3 in Table 1, B; Patient no. normal skin-1 in Table 1. Positive signals are visualized in brown. Bar = 50 μm.

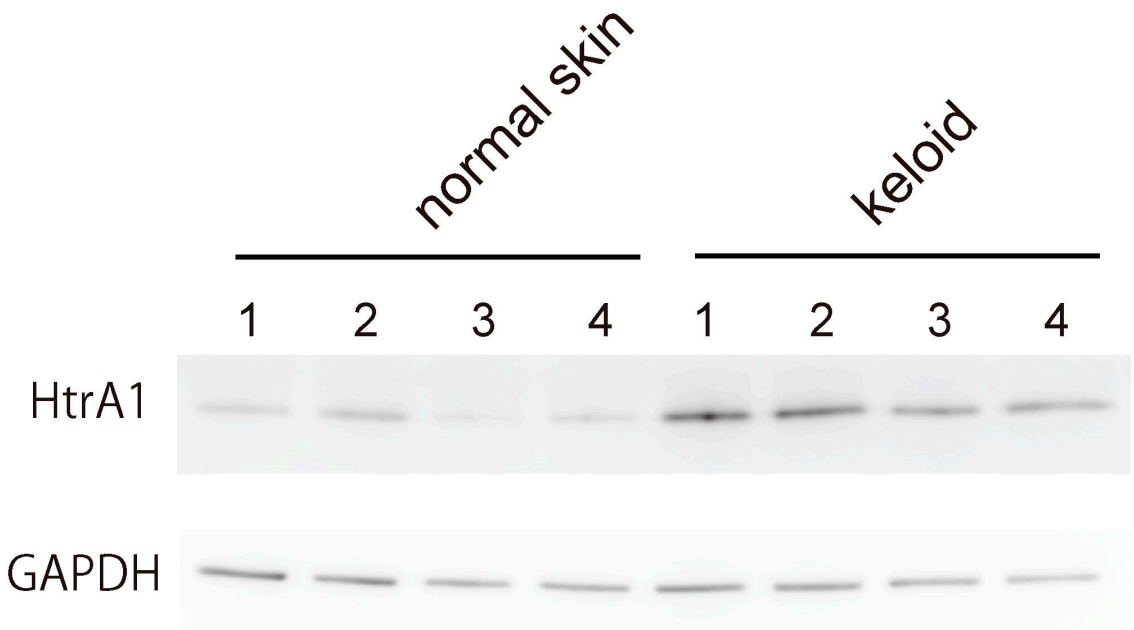

Figure 4. Western blot analysis of HtrA1 in keloid lesions and normal skin tissues. Soluble protein extract (8 μg/lane) was analyzed using specific antibodies against HtrA1 or GAPDH. Keloid and normal skin samples from four different patients were analyzed.

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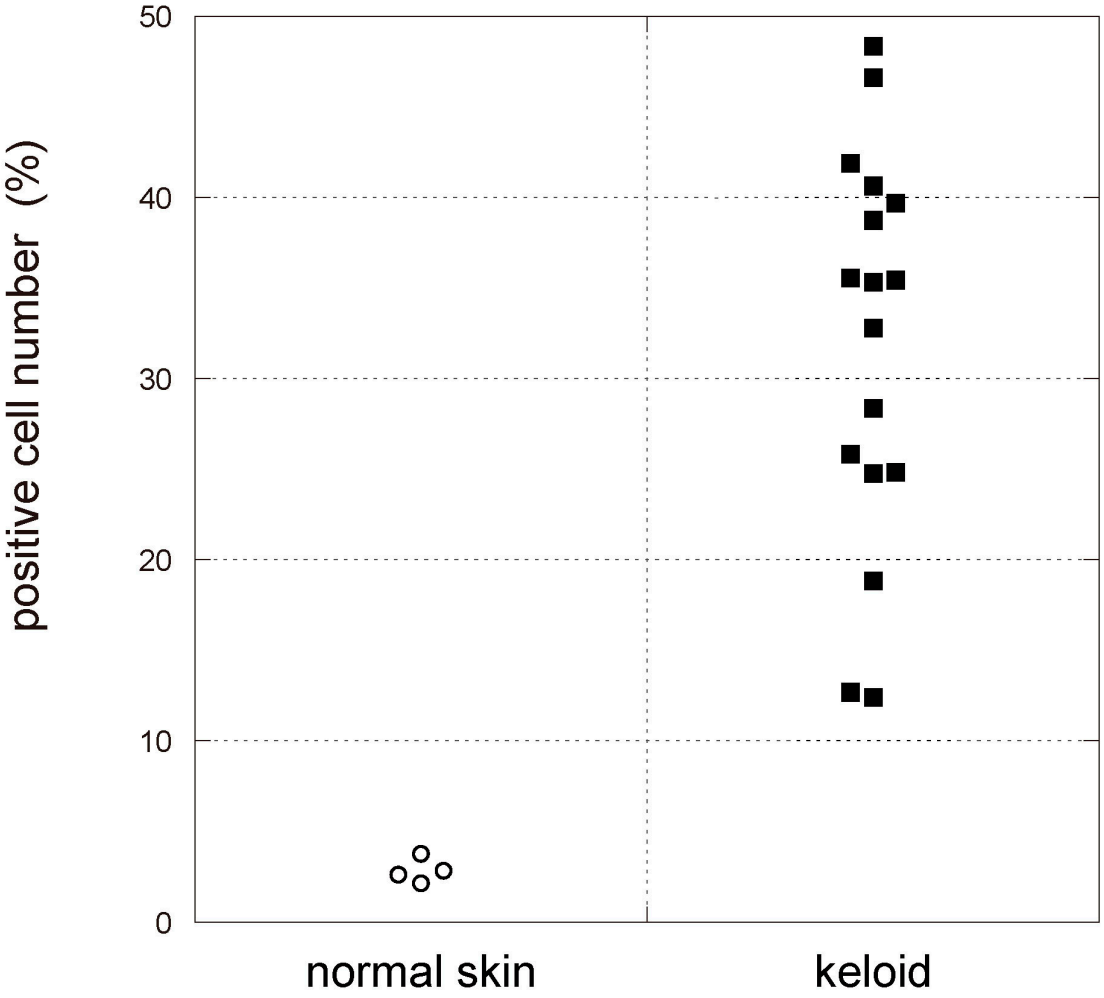

Figure 5. Proportion of fibroblasts expressing HtrA1 protein in keloid lesions and normal skin. The number of fibroblasts with positive signals was counted after immunohistochemical staining of HtrA1 using samples from 17 keloid and 4 unrelated patients. Ten high-power (×400) fields were selected at random from a section and numbers of total and stained fibroblasts were counted. Patients' information is described with proportion of HtrA1-positive cells in Table 1.

2.3. HtrA1 knockdown inhibits keloid cell proliferation

To investigate role of HtrA1 in keloid pathogenesis, we examined whether HtrA1 affect cell proliferation by silencing *HtrA1* gene expression using specific siRNA. Keloid fibroblasts treated with HtrA1 siRNA exhibited significantly slow proliferation rate relative to those treated with control siRNA (Figure 6). These results suggest that HtrA1 play an important role in keloid-cell proliferation.

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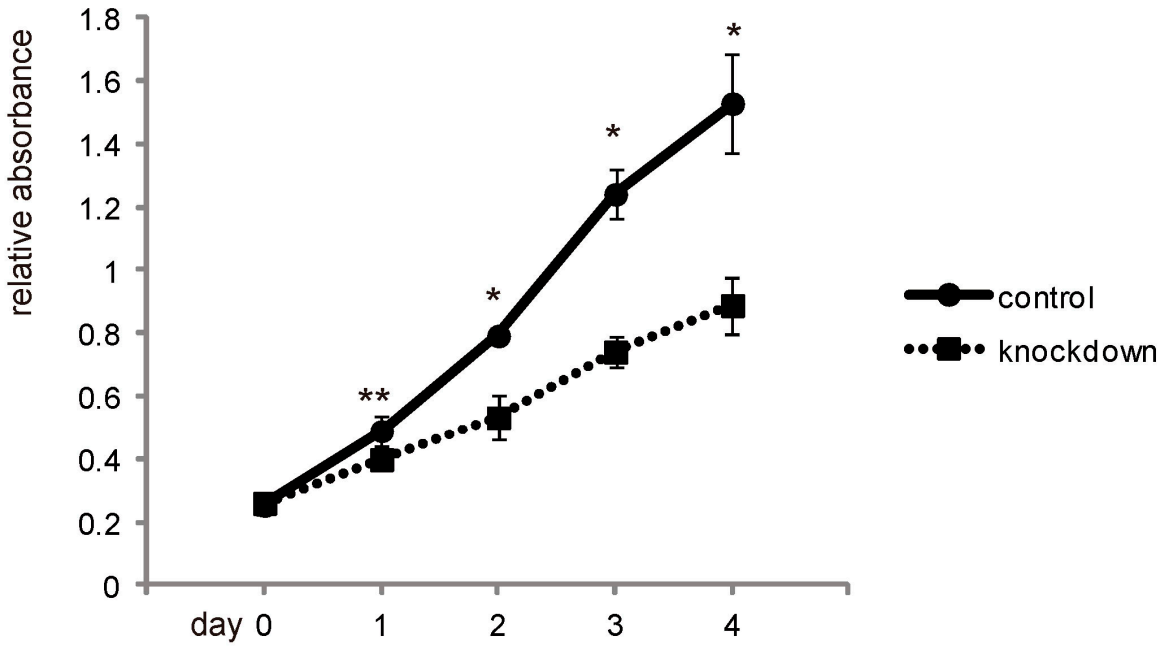

Figure 6. Proliferation rate of keloid fibroblasts transfected with HtrA1 siRNA or control siRNA. Proliferation curve of keloid fibroblasts obtained from No.26 keloid sample in Table 1 transfected with HtrA1 siRNA (HtrA1 KD) or control siRNA (control). Cell proliferation was analyzed by a colorimetric assay using water soluble tetrazolium salt as a substrate. Error bars represent standard deviations (n = 3). ＊, P< 0.01, ＊＊, P< 0.001.

3. Discussion

In the present study, we demonstrated that the expression of HtrA1 was strongly upregulated in active keloid legions as analyzed by *in situ* hybridization and immunohistochemical staining. Previous studies suggested in arthritis that HtrA1 stimulates the disease through digesting extracellular matrix[13]. In arthritis, synovial fibroblasts produce abundant HtrA1, and HtrA1 digests cartilage extracellular matrix, including fibronectin, collagens, and proteoglycans. ECM fragments produced by HtrA1 digestion was reported to activate synovial fibroblasts and to induce the remodeling of cartilage extracellular matrix. We propose that HtrA1 functions as a matrix protease stimulating keloid development because keloid matrix consists mainly of collagens, fibronectin and proteoglycans which are the substrate for HtrA1. HtrA1 may degrade keloid matrix and accelerates remodeling the ECM in keloid lesions. Matrix protein fragments produced by HtrA1 may activate keloid cells, leading to further progress of disease. Consistent with the notion, we found in the present study that HtrA1-knockdown inhibits proliferation of keloid fibroblasts.

Recently, Beaufort et al reported that HtrA1 facilitates TGF-ß signaling through processing latent TGF-ß binding protein (LTBP) [16]. In embryonic fibroblasts from HtrA1 knockout mice and skin fibroblasts from CARASIL patients caused by *HtrA1* mutations, reduction of TGF-ß activity was observed. These observations suggest a role of HtrA1 in facilitating TGF-ß signaling. LTBP functions as a part of the large latency complex (LLC) that anchors TGF-ß to the ECM[16-20]. Proteolysis of LTBP-1 results in its detachment from ECM, leading to TGF-ß release and activation [18,21-23]. HtrA1 cleaves LTBP-1 in the fibronectin binding domain, and this processing occurs in a site-specific manner, distinct from other proteases previously reported [18,21-23]. TGF-ß1 is overexpressed and activated in keloid lesions and plays a key role in keloid pathogenesis [24,25]. TGF-ß1 stimulates production of abundant ECM including fibronectin and collagens. HtrA1 may facilitate keloid pathogenesis through the activation of TGF-ß1 mediated by LTBP-1 cleavage.

HtrA1 has been reported to be a crucial molecule in AMD, a leading cause of irreversible blindness in the elderly[14,15]. AMD is accompanied with choroidal neovascularization and polypoidal choroidal vasculopathy. Analysis of HtrA1 transgenic mice indicated that increased

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HtrA1 is sufficient to cause hyper-vascularization and degeneration of elastic laminae in choroidal vessels[14]. Zhang et al demonstrated that HtrA1 promotes angiogenesis by regulating GDF6, a TGF-ß family-protein, using HtrA1 knock-out mice[10]. As in AMD, abundant microvessels are observed in keloid lesions [9]. Thus, HtrA1 may play a role in keloid hypervascularity by modulating TGF-ß family signaling.

Taken together, these observations suggest that HtrA1 contribute to the development of keloid lesions as matrix protease by remodeling keloid-specific extracellular matrix or cell surface molecules. HtrA1 may be useful as a target of keloid treatment, although further study is required.

4. Materials and Methods

Tissue specimens

Between September 2007 and September 2013, twenty seven keloid patients (aged 16-75) and seven unrelated patients (aged 45-88) undergoing surgical treatments were enrolled in this study. With approval from the Institutional Reviewing Board in Kyoto University Faculty of Medicine, which adheres to the ethical standards as formulated in the Helsinki Declaration, a written informed consent was obtained from all of the patients. All patients were diagnosed as keloid based on the clinical findings and definitive diagnosis that was obtained histopathologically by the operative specimens[3,4]. The skin tissue samples were obtained as the surplus skin at the plastic surgery. Samples' information is shown in Table 1.

Antibodies

Monoclonal anti-human HTRA1 antibody (MAB2916, R&D systems, Minneapolis, MN) was used for Western blotting. The antibody used in immunohistochemical staining, was developed in rabbit using a synthetic peptide corresponding to the C-terminal region of human HtrA1 as immunogen.

2.1. In situ hybridization

For *in situ* hybridization, keloid and surrounding unaffected skin tissue specimens were obtained from the keloid patients at the time of surgical treatment and fixed in 4% paraformaldehyde at 4°C, and paraffin sections (6 μm) were prepared. Deparffinized sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes and washed with PBS. Sections were treated with 3 μg/ml proteinase K in PBS for 30 minutes at 37°C, washed with PBS, refixed with 4% paraformaldehyde in PBS, washed again with PBS, and placed in 0.2 N HCl for 10 minutes. After washing with PBS, sections were acetylated by incubation in 0.1 M tri-ethanolamine-HCl (pH 8.0)/ 0.25% acetic anhydride, for 10 minutes. After washing with PBS, sections were dehydrated through a series of ethanol solutions. Hybridization was performed with 1558-2066 of human *HtrA1* gene (Accession # NM_002775) at concentrations of 300 ng/ml in Probe Diluent-1 (Genostaff, Tokyo, Japan) at 60°C for 16 hours. After hybridization, sections were washed in 5× HybriWash (Genostaff) at 60°C for 20 minutes, and in 50% formamide with 2× HybriWash at 60°C for 20 minutes, followed by RNase treatment with 50 μg/ml RNase A in 10 mM Tris-HCl (pH 8.0)/1 M NaCl/1 mM EDTA for 30 minutes at 37°C. Sections were then washed twice with 2× HybriWash at 60°C for 20 minutes, twice with 0.2× HybriWash at 60°C for 20 minutes. After treatment with 0.5% blocking reagent (Roche Diagnostics, Tokyo, Japan) in TBST (0.05M Tris-HCl / 0.15M NaCl / 0.05% Tween 20 for 30 minutes, sections were incubated for 2 hours at room temperature with anti-DIG AP conjugate (Roche) diluted 1:1,000 with TBST. Sections were washed twice with TBST and then incubated in 100 mM NaCl/50 mM $MgCl_2$/0.1% Tween20/100 mM Tris-HCl (pH 9.5). Coloring reactions were performed with NBT/BCIP solution (Sigma-Aldrich, Saint Louis, MO) overnight, followed by washing with PBS. Sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan), dehydrated, and mounted with Malinol (Muto Pure Chemicals).

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224 *2.2.1. Immunohistochemical analysis*

225 All keloid and normal skin tissue specimens were obtained from the surgical treatment and
226 fixed in 4% paraformaldehyde at 4°C, and paraffin sections (3 μm) were prepared. Deparffinized
227 sections were incubated at 90°C for 10 minutes in target retrieval solution (pH9, 1:10, DAKO,
228 Glostrup, Denmark). After blocking endogenous peroxidase and non-specific protein binding
229 activities, the sections were incubated with antibodies against human HtrA1 (1:400) using
230 LSAB™2kit/HRP (Dako). After incubation with a peroxidase-conjugated anti-rabbit IgG antibody,
231 sections were stained using a LSAB/HRP kit (Dako) and counterstained with hematoxylin.
232 Microscopic images of sections were obtained by a Biorevo BZ-9000 microscope (Keyence, Osaka,
233 Japan) and counting of total and stained fibroblasts was performed using ten microscopic fields at
234 high-power (×400). The number of the each cell of ten fields was added up and stained
235 fibroblasts/total fibroblasts were assumed as the proportion of HtrA1-positive cells per each
236 specimen.

237 *Statistical analysis*

238 Significance of difference was analyzed by the Student's *t*-test. A *p* value of less than 0.05 was
239 taken as an indication of statistical significance.

240 *2.2.2. Western blot analysis*

241 Tissue samples were homogenized in RIPA buffer (Takara Bio, Otsu, Japan) containing protease
242 inhibitors at 4°C, using a Polytron homogenizer (Kinematica, Luzern, Switzerland). Following
243 centrifugation (12,000 prm, 4°C, 20 min), soluble proteins in the supernatant were separated by
244 SDS-PAGE (gradient gels) and then blotted onto PVDF filters. Filters were then blocked with 5%
245 Block Ace (DS Pharma Biomedical, Osaka, Japan) in PBS containing 0.05% Tween 20, prior to
246 incubation with anti HtrA1 antibody (1:500, R&D systems). Specific antibody binding was detected
247 by LAS-3000 (Fuji Photo Film, Tokyo, Japan).

248 *2.3. Knockdown of HtrA1 gene expression and cell proliferation assay*

249 Keloid fibroblasts were extracted by explants method from surgical specimens. These were
250 cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10%
251 fetal bovine serum, 10,000 U/ml penicillin G, and 10 mg/ml streptomycin sulfate. One day before
252 transfection, keloid fibroblasts were plated at 40% confluence at 3rd passage in DMEM without
253 antibiotics on 10 cm dishes, followed by transfection with HtrA1 siRNA using Lipofectamine
254 RNAiMAX Reagent, (Life technologies, Carlsbad, CA). After 48 hours, cell proliferation assay was
255 performed using WST assay reagent (Nacalai Tesque, Kyoto, Japan). Expression level of target gene
256 was analyzed by real time polymerase chain reaction.

257 *Real-Time PCR Analysis*

258 Total RNA was extracted from cells after the transfection using RNeasy Mini Kit (Qiagen,
259 Venlo, Netherlands). First-strand cDNA was synthesized using Prime Script RTreagent Kit with
260 gDNA Eraser (Takara Bio, Otsu, Japan). RT-PCR was performed with cDNA using TaqMan Probe
261 Assay (Applied Biosystems, Foster City, CA). Glyceraldehydes-3-phosphate dehydrogenase was
262 used as a housekeeping control gene. Relative expression was calculated by calibration curve
263 method.
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5. Conclusions

In Summary, the expression of HtrA1 was revealed especially in keloid active lesions and the silencing of HtrA1 suppressed the proliferation of keloid fibroblasts. HtrA1 may have a key role in keloid development.

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Author Contributions: Satoko Yamawaki, Motoko Naitoh, and Toshihiro Ishiko performed histopathological experiments; Satoko Yamawaki, Motoko Naitoh, Yasuhiro Katayama, and Taku Tamura performed molecular and cellular biological experiments; Satoko Yamawaki and Motoko Naitoh analyzed the data and performed statistical analysis; Motoko Naitoh and Hiroshi Kubota conceived and designed the experiments; Satoko Yamawaki, Motoko Naitoh, Rino Aya, Yasuhiro Katayama, Toshihiro Ishiko, Katsuhiro Yoshikawa, Tatsuki Enoshiri, Mika Ikeda, and Shigehiko Suzuki recruited the patients; Satoko Yamawaki wrote the paper. Motoko Naitoh conducted the experimental design and writing.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

HtrA1	High Temperature Requirement Factor A1

Appendix B

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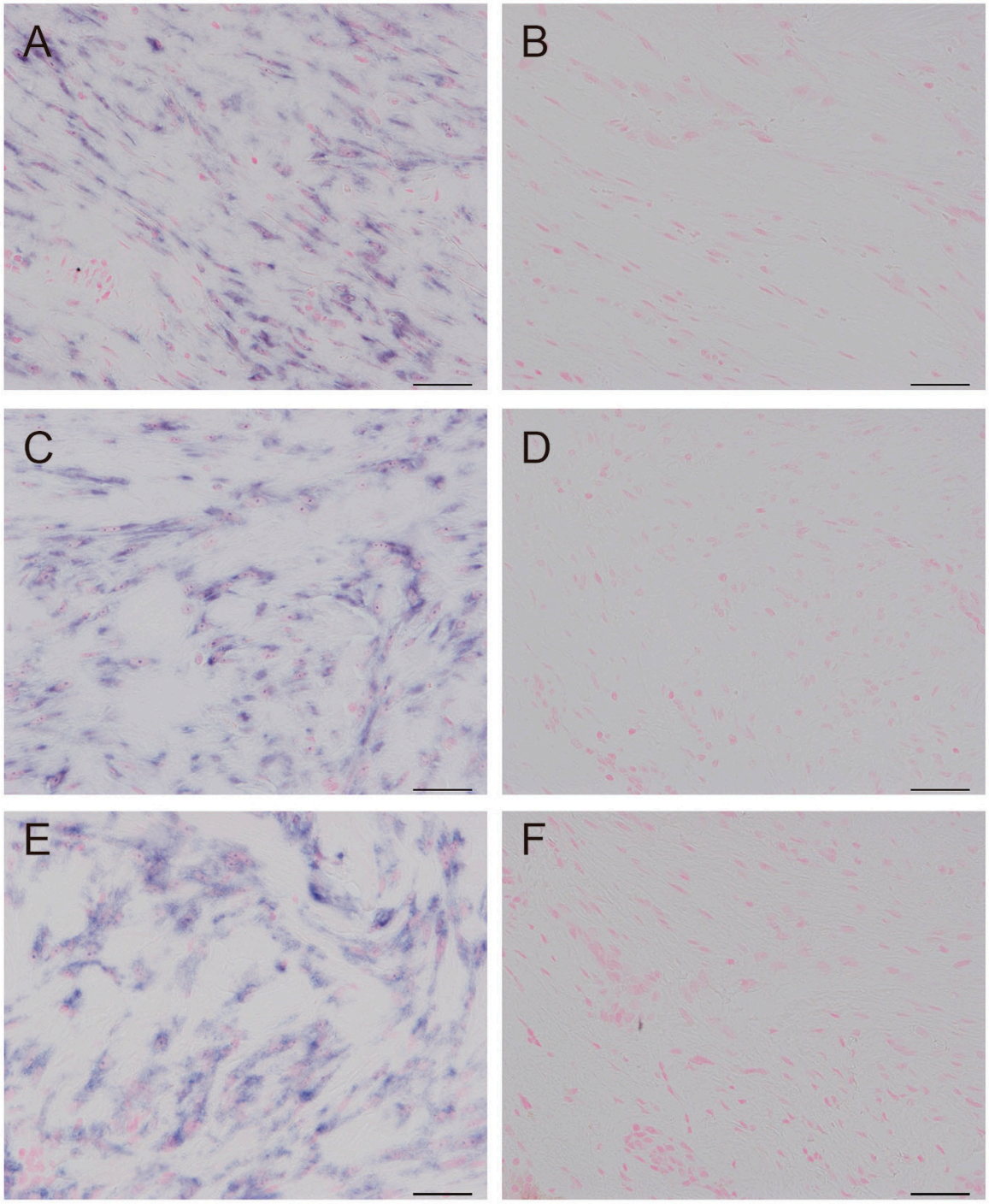

Suppl. Figure A1. In situ hybridization of *HtrA1* mRNA in keloid lesions. Sections were hybridized with HtrA1 antisense probe (A, C, and E) or sense probe (B, D, and F). Sections of keloid lesions from 3 different patients were analyzed, and sections shown in A and B were obtained from the same patient (patient no. keloid-19 in Table 1). Sections in C and D (patient no. keloid-3 in Table 1), or E and F (patient no. keloid-20 in Table 1) were obtained from two other patients. Positive signals are visualized in blue. Bar = 50 μm.

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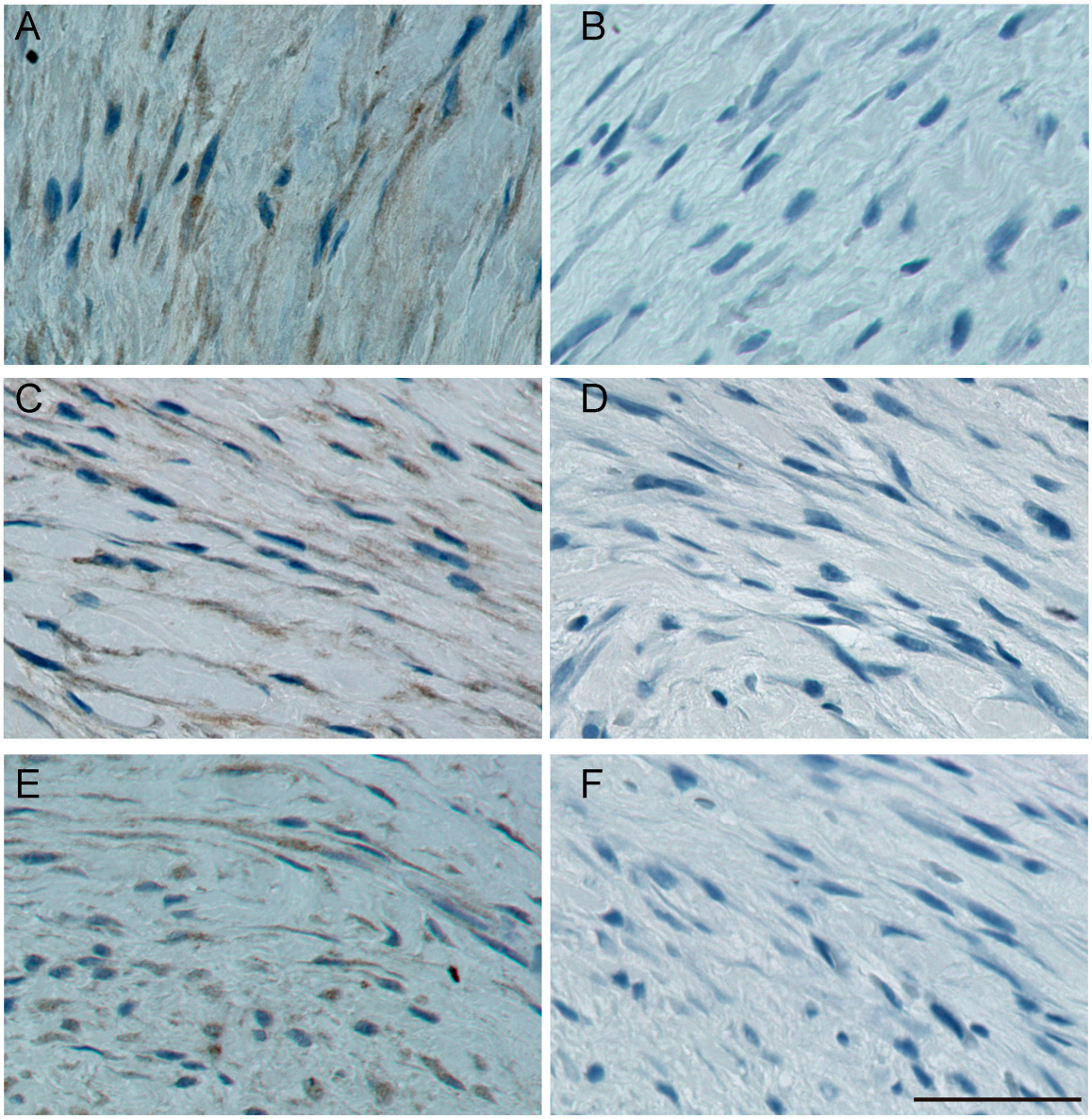

Suppli. Figure A2. Immunohistochemical staining of HtrA1 protein in keloid lesions. Sections were stained with anti HtrA1 antibody (A, C, and E) or without the primary antibody (B, D, and F). Sections of keloid lesions from 3 different patients were analyzed, and sections shown in A and B were obtained from the same patient (patient no. keloid-3 in Table 1). Sections in C and D (patient no. keloid-4 in Table 1), or E and F (patient no. keloid-5 in Table 1) were obtained from two other patients. Positive signals are visualized in brown. Bar = 50 μm.

References

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