Effect of *Artemisia capillaris* flower extract on the microRNA-regulated HYBID expression in human dermal fibroblasts

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Abstract

HYBID is known to play a great role in the degradation of hyaluronic acid (HA) into smaller fragments the functions, of which are unknown. Although the expression of HYBID has been reported in extrinsically aged skin, there is no available data in the case of intrinsic skin aging. In this study, we have investigated the regulation of HYBID expression modulated by intrinsic factors and searched for an effective material which can inhibit its expression in both intrinsic and extrinsic aging of skin. Histamine, a well-known factor released from mast cells upon UV irradiation, has been used as an extrinsic aging factor to induce HYBID expression in normal human dermal fibroblasts (NHDF) from newborn (NB). In search for intrinsic aging factor, miRNA which directly targets and inhibits HYBID expression has been investigated in NHDF from adult (AD). Screening of plant extracts has been carried out in NHDF-NB based on the inhibition of histamine-induced HYBID expression. In the presence of a potent proinflammatory factor (TNF-α), intermediate-size fragments of HA accelerate the expression of IL-1β, IL-6, and MMP-1 in newborn fibroblasts. Histamine dose-dependently increases the mRNA expression of HYBID in NHDF-NB. Also, the expression level of a selected miRNA, miR-600, decreases in NHDF-AD compared to NHDF-NB. *Artemisia capillaris* flower extract not only inhibits the histamine-induced mRNA expression of HYBID in NHDF-NB concentration-dependently, but also increases the expression of miR-600 in NHDF-

AD. Finally, the plant extract improves the condition of sagging and wrinkle in human clinical study. This plant extract could be useful for taking care of both extrinsic and intrinsic aging of skin.

Keywords: Hyaluronic acid; HYBID; Sagging; Wrinkle; miRNA-600; *Artemisia capillaris* flower extract

Introduction

Hyaluronic acid (HA), a naturally occurring non-sulfated glycosaminoglycan, is generally involved in many cellular and physiological processes (e.g., proliferation, differentiation, development, migration, lubrication, hydration balance, space filling, and matrix structure) [1, 2]. It is mainly synthesized by fibroblasts, synoviocytes and chondrocytes [3], and is distributed ubiquitously in the extracellular matrix of various connective tissues such as skin, cartilage, and others [2, 4]. However, approximately half of the total HA in the body is present in the skin [5], more particularly in the dermis, where it is synthesized by dermal fibroblasts [6].

The balance between synthesis and degradation of HA plays an important role in total skin homeostasis. Among the hyaluronidase enzymes (HYAL), HYAL1 and HYAL2 are usually known to be involved in the degradation of HA. But with the advancement of science, the concept of HA metabolism by HYAL has become somewhat elusive, and during the last decade, a new mechanism of HA degradation system has been proposed, which is independent of HYAL system. This novel system involves a protein called HYBID (HYaluronan-Binding protein Involved in hyaluronan Depolymerization) alias KIAA1199 which binds and degrades high molecular size (>1000 kDa) HA into intermediate size fragments with molecular weights ranging from 10 kDa to 100 kDa [7]. These authors have also demonstrated that, not HYAL enzymes, but HYBID is responsible for the degradation of HA in human skin fibroblasts.

Although high molecular size HA has been reported to promote anti-inflammatory responses, medium-to-low molecular size HA produces inflammatory

reactions in patients with osteoarthritis [8]. Cowman *et al*. demonstrate that degradation of HA in articular joint synovial fluid can reduce the viscosity and elasticity of the synovial fluid, and has also been shown to reduce its lubricating ability [9]. Moreover, the smaller size HA is commonly available in cancer cells, and induces inflammatory responses in mouse ovarian stromal cells [10], murine macrophages [11], and human dendritic cells [12]. However, the effect of this medium-to-small size HA has not been investigated so far in human skin fibroblasts.

HYBID is generally expressed in many cell types such as astrocytes [13], fibroblasts [7], osteoblasts [14], and various cancer cells [15, 16]. The regulation of HYBID expression in skin fibroblasts has been elucidated using many factors including cytokines and chemokines [7]. It has been demonstrated that its expression is significantly up-regulated by treatment with histamine, a well-known factor usually released from mast cells upon UV irradiation. Recently, it has been reported that the expression of HYBID is increased in photoaged skin, and this increase is probably due to the increased release of histamine from mast cells [17]. It has also been revealed that HYBID expression is negatively correlated with the levels of HA, and positively correlated with sagging and wrinkle in photoaged skin, suggesting that HYBID-mediated HA reduction in the dermis contributes to the development of wrinkle and sagging in this type of skin [17]. However, although the regulation of HYBID expression is well-documented in extrinsic skin aging, there is still no report on its expression in natural or intrinsic aging of skin.

MicroRNA (miRNA) is a small endogenous noncoding RNA (~22 nucleotides) which post-transcriptionally control the synthesis of many proteins by binding to partially complementary sequences of its target messenger RNA (mRNA). Bioinformatics data suggest that miRNAs may regulate more than 30-60% of the human genome and, thus be involved in regulating many cellular processes including cell proliferation, differentiation, metabolism, senescence, and apoptosis etc. [18]. Interestingly, the TargetScan software predicts miR-600 as a direct target of HYBID (https://www.targetscan.org/vert_80/). However, the expression and involvement of this miRNA has not been investigated in skin cells particularly in the case of natural aging.

We have characterized the functions of medium-to-small size fragments of HA in dermal fibroblasts for the first time. We also suggest that the reduced expression of miR-600, which directly targets HYBID, might be involved in the cause of natural aging. *Artemisia capillaris* (*A. capillaris*) flower extract could be a promising agent which could reduce HYBID-mediated sagging and wrinkle of skin caused by extrinsic and intrinsic factors.

Materials and Methods

Materials. Normal Human Dermal Fibroblasts (NHDF) from newborn (NB) and adult (AD) were purchased from Kurabo Industries Ltd., Osaka, Japan; D-MEM from FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan; Fetal Bovine Serum (FBS) and histamine from Sigma-Aldrich Corp., St. Louis, MO, USA; Hyaluronic Acid (HA) in the form of sodium hyaluronate (NAHA) was purchased from Iwai Chemicals Company, Tokyo, Japan; and TNF-α was purchased from HumanZyme Inc., Chicago, IL, USA.

Plant information. *A. capillaris* Thunb. (Compositae) is a medicinal plant, and is distributed worldwide. We purchased the flower part of the plant from Nagano, Japan, and was extracted in 30% butylene glycol followed by purification.

Effect of different sizes of HA on the expression of pro-inflammatory cytokines. NHDF-NB were cultured in 6-well plates (6x104 cells/well) using a defined medium (D-MEM) containing 10% FBS, and maintained at 370C under a humidified atmosphere of 95% air and 5% CO2. After the cells reached to about 75% confluent, the medium in each well was exchanged with a fresh medium containing 0.25% FBS and incubated for 24 hrs. After the incubation, cells were exposed with 10 μg/ml of different sizes of HA, namely, NAHA-H2 (MW: 1,200,000-1,600,000), NAHA-M2 (MW: 600,000-1,120,000), NAHA-S2 (MW: 40,000-80,000), and NAHA-U2 (MW: 5,000-10,000) for 1-2 hrs followed by direct addition of TNF-α (1 ng/ml). After 6 hrs incubation, total RNA was isolated using RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions for the measurement of IL-1β, IL-6, and MMP-1 expression.

Effect of histamine on the mRNA expression of HYBID. NHDF-NB were seeded

in 6-well plates (6x104 cells/well) using a defined medium (D-MEM) containing 10% FBS, and cultured until the cells reached to about 75% confluent. Then, the medium in each well was exchanged with a fresh medium containing 0.25% FBS and incubated for 24 hrs. After the incubation, cells were exposed with different concentrations (0.1, 1.0, and 10 μM) of histamine for 24 hrs. Then, total RNA was isolated using RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions for the measurement of HYBID mRNA expression.

Effect of A. capillaries flower extract on histamine-induced HYBID expression. NHDF-NB were cultured in 6-well plates (6x104 cells/well) using a defined medium (D-MEM) containing 10% FBS, and maintained at 370C under a humidified atmosphere of 95% air and 5% CO2. After the cells reached to about 75% confluent, the medium in each well was exchanged with a fresh medium containing 0.25% FBS and incubated for 24 hrs. After the incubation, cells were exposed with different concentrations (0.125, 0.25, 0.5, and 1.0%) of *A. capillaries* flower extract for around 3 hrs followed by substitution of the medium with fresh one including 10 μM histamine. After 24 hrs of histamine challenge, total RNA was isolated using RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions for the measurement of HYBID mRNA expression.

Expression of miRNA in NHDF-NB and NHDF-AD, and effect of A. capillaries flower extract. NHDF-NB and NHDF-AD were cultured in 6-well plates (6x104 cells/well) using a defined medium (D-MEM) containing 10% FBS, and maintained at 370C under a humidified atmosphere of 95% air and 5% CO2. After the cells reached to about 75% confluent, the medium in each well was exchanged with a fresh medium containing 0.25% FBS and incubated for 24 hrs. Then, the cells were further incubated for 6 hrs in the absence or presence of *A. capillaries* flower extract (0.5%). After the incubation, total RNA was isolated using miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions for the measurement of miR-600 expression.

Real-time polymerase chain reaction (PCR). For the measurement of mRNA, total RNA was reverse transcribed to complementary DNA (cDNA) using a PrimeScript

RT reagent kit (Takara Bio, Otsu, Japan). Relative semi-quantitative real-time PCR was carried out in a Thermal Cycler Dice Real Time System TP800 (GE Healthcare, Buckinghamshire, UK) using the SYBR Premix Ex Taq II system (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. The thermal cycling conditions were as follows: 30s at 95^0C, followed by 40 cycles of two-step PCR at 95^0C for 5s and 60^0C for 30s, followed by a single cycle of dissociation steps performed at 95^0C for 15s, 60^0C for 30s, and 95^0C for 15s. Ribosomal Protein S18 (RPS18) mRNA was used as control. The primer sequences were as follows: for IL-6, 5′-GCCTTCGGTCCAGTTGCCTT -3′ (forward) and 5′-AGTGCCTCTTTGCTGCTTTCAC-3′ (reverse); and, for MMP-1, 5′-AAGGCCAGTATGCACAGCTT-3′ (forward) and 5′-TGCTTGACCCTCAGAGACCT-3′ (reverse). The primer set ID for HYBID is HA243626 (Takara Bio, Otsu, Japan) and that for IL-1β is HA106116 (Takara Bio, Otsu, Japan). The delta-delta-CT method was used to compare the differences of mRNA expressions among different experimental groups.

For the measurement of miRNA, total RNA was reverse transcribed to complementary DNA (cDNA) using a Mir-X miRNA First-Strand Synthesis Kit (Takara Bio, Otsu, Japan). Relative semi-quantitative real-time PCR was carried out in a Thermal Cycler Dice Real Time System TP800 (GE Healthcare, Buckinghamshire, UK) using the Mir-X miRNA qRT-PCR SYBR Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. The thermal cycling conditions were as follows: 30s at 95^0C, followed by 40 cycles of two-step PCR at 95^0C for 5s and 60^0C for 30s, followed by a single cycle of dissociation steps performed at 95^0C for 15s, 60^0C for 30s, and 95^0C for 15s. U6 snRNA was used as control. The sequence for miR-600 specific primer was 5′-ACTTACAGACAAGAGCCTTGCTC-3′, and was used as a forward primer. A universal primer, mRQ 3′Primer supplied with the PCR Kit, served as a reverse primer. The delta-delta-CT method was used to compare the differences of miRNA expressions among different experimental groups.

Human clinical study. This study, consisting of a total of 13 male and female participants, was conducted for a short period of 4 weeks under the guideline of Helsinki

Declaration, and was approved by the Ethical Review Committee. The one half of the face of each participant served as control (placebo) and the other half of the face served as test (lotion containing 1% *A.* capillaries flower extract). Antera 3D® (Miravex) was used for wrinkle measurement and wrinkle imaging. For measurement of sagging, an adhesive tape with a circular hole was fixed on the cheek and then 5-20 g of weight was set with a string with one end holding the weight and other end holding the circular hole. The weight was determined in advance in such a way that the cheeks would move downwards by 1-2 mm due to gravity. The actual distance travelled by the cheeks due to gravity was measured by VISIA® Evolution (Canfield Scientific) before and after the weight load in the absence or presence of *A.* capillaries flower extract at 0- and 4-week.

Statistical analysis. Data were expressed as mean ± standard deviation (M±SD) as well as mean ± standard error (M±SE), where applicable, and analyzed by using a statistical software JMP8 (SAS Institute, North Carolina, USA). Statistical analyses were performed by Dunnett's test, Student's t-test, and Wilcoxon Rank-Sum Test, where applicable. A value of $p<0.05$ was considered statistically significant.

Results

Effect of different sizes of HA on the expression of pro-inflammatory cytokines. We have investigated whether any size of HA (total 4 different sizes of HA) has any sort of effect on the expression of pro-inflammatory cytokines in NHDF-NB. It has been found that no HA of any size can induce the expression of inflammatory markers (data not shown). But, in the presence of different sizes of HA at a concentration of 10 μg/ml, if the cells are induced with just 1 ng/ml of TNF-α, a potent inflammatory agent, the expressions of IL-1β and IL-6 are accelerated in a HA-size-dependent manner. Whereas the largest size of HA (NAHA-H2) shows no effect on the acceleration of IL-1β and IL-6 expression, the medium size (NAHA-M2) shows an increasing tendency, and the small (NAHA-S2) and ultra-small (NAHA-U2) sizes show significant accelerating effects (Fig. 1a, b). Not only that, MMP-1 expression also follows the same pattern as cytokines have shown under the same experimental condition (Fig. 1c).

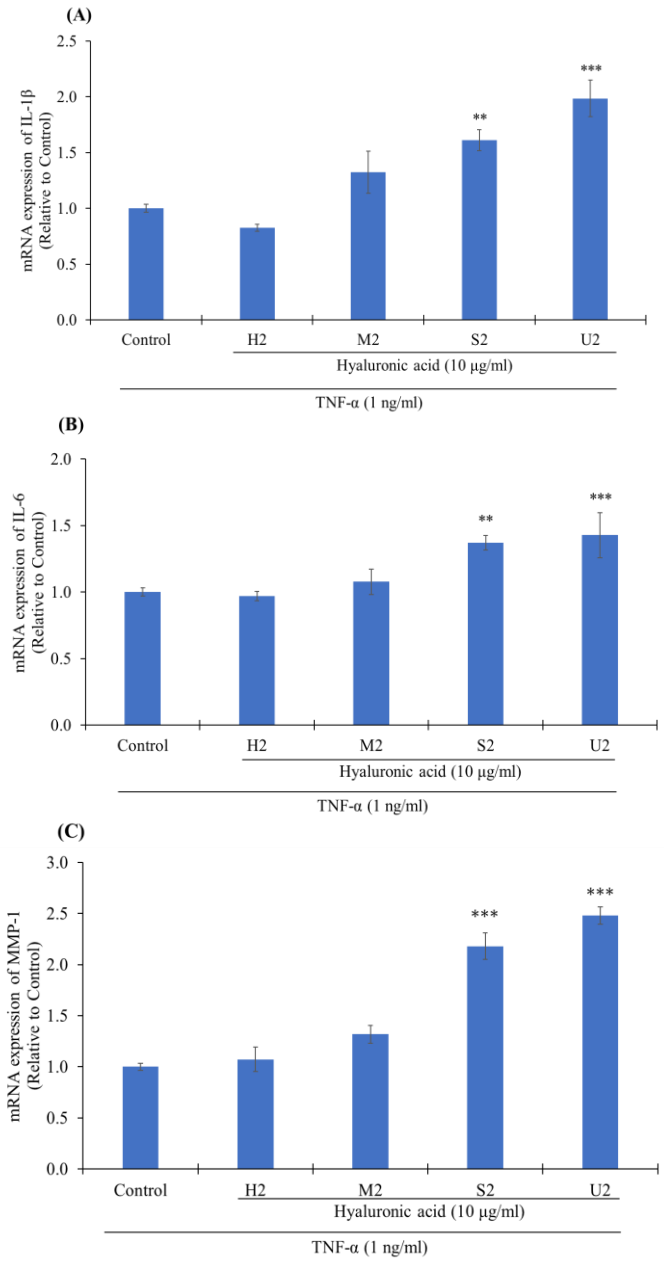

Figure 1. Effects of different sizes of HA on the acceleration inflammation in the presence of TNF-α. NHDF-NB were exposed with HA (10 μg/ml) for 1-2 hrs followed by challenge with TNF-α (1 ng/ml) for 6 hrs. Then, PCR was carried out for the measurement of mRNA expression of (A) IL-1β, (B) IL-6, and (C) MMP-1. H2: 1,200,000-1,600,000; M2: 600,000-1,120,000; S2: 40,000-80,000; U2: 5,000-10,000. Data were expressed as mean±SD (n=3), and analyzed by Dunnett's test (**$p<0.01$, and ***$p<0.001$ vs control).

Effect of histamine on HYBID expression in NHDF. As HYBID is a key molecule involved in the degradation of HA into intermediate size fragments, the regulation of HYBID expression has been tested in the presence of different concentrations of histamine, and considered as an extrinsic aging model. Although histamine has been reported to be a potent inducer of HYBID [7], we have established and reconfirmed the same protocol in our own laboratory condition. It has been found that histamine dose-dependently increase the mRNA expression of HYBID in NHDF-NB (Fig. 2).

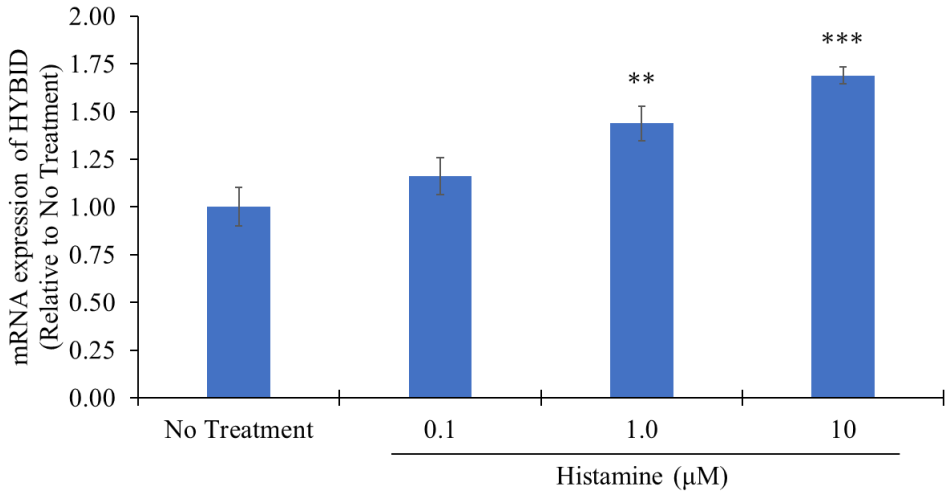

Figure 2. Effect of histamine on the upregulation of HYBID expression. Different concentrations (0.1, 1.0, and 10 μM) of histamine were treated with NHDF-NB for 24 hrs followed by PCR for the measurement of HYBID mRNA expression. Data were expressed as mean±SD (n=3), and analyzed by Dunnett's test (**p<0.01, and ***p<0.001 vs No Treatment).

Effect of A. capillaries flower extract on the histamine-induced expression of HYBID. To search for a potent inhibitor of HYBID, we have screened as many as 400 plant extracts, and finally selected only one, *A. capillaries* flower extract, based on its excellent efficacy. It has been found that the plant extract inhibits the mRNA expression of HYBID induced by histamine in NHDF-NB in a concentration-dependent manner (Fig. 3).

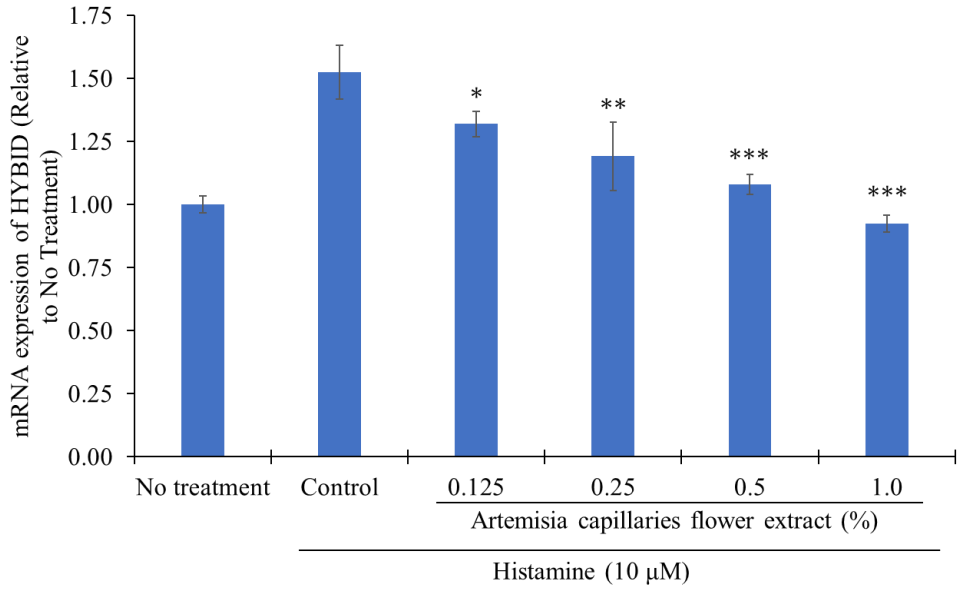

Figure 3. Effect of *Artemisia capillaris* flower extract on the inhibition of histamine-induced upregulation of HYBID. Different concentrations (0.125, 0.25, 0.5, and 1.0 %) of plant extract were treated with NHDF-NB for 3 hrs followed by challenge with histamine (10 μM) for 24 hrs. Then, PCR was performed for the measurement of HYBID mRNA expression. Data were expressed as mean±SD (n=3), and analyzed by Dunnett's test (*$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs Control).

Expression of miR-600 in NHDF. To investigate the regulation of HYBID in natural or intrinsic aging, we have identified the expression of a specific miRNA, miR-600, in both NHDF-NB and NHDF-AD. It has been found that the expression of miR-600 has been reduced significantly ($p<0.05$) in AD compared to NB (Fig. 4).

Effect of A. capillaries flower extract on the expression of miR-600 in NHDF. As the expression of miR-600 has been found to be reduced in NHDF-AD, we have investigated whether *A. capillaries* flower extract has any effect on the expression of this miRNA in AD cells. It has been found that the extract significantly ($p<0.05$) increases the expression of miR-600 in NHDF-AD when compared with the untreated cells (Fig. 4). Moreover, the extract has been shown to increase the same miRNA significantly ($p<0.05$) in NHDF-NB as well (Fig. 4).

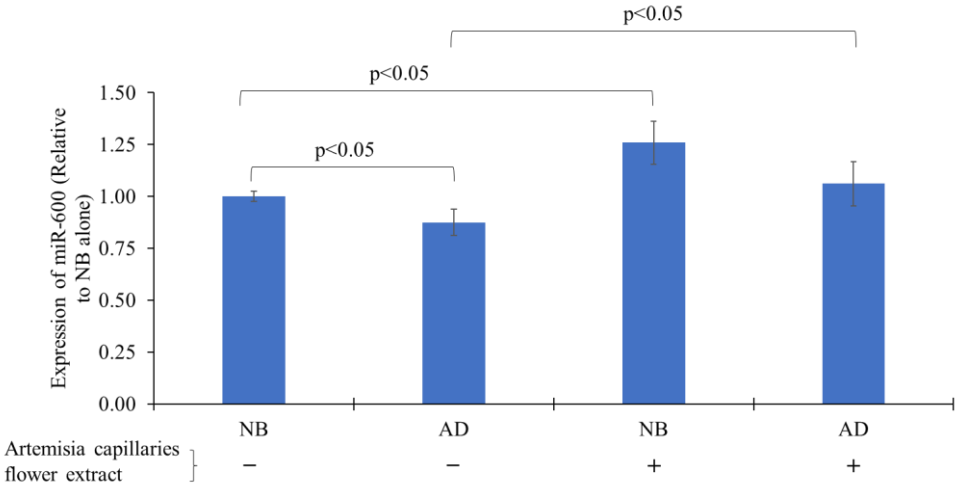

Figure 4. Expression of miR-600 in NHDF-NB and NHDF-AD, and the effect of *Artemisia capillaris* flower extract on its expression. NHDF-NB and NHDF-AD were cultured in the absence or presence of Artemisia capillaris flower extract (0.5%) for 6 hrs followed by PCR for the measurement of miR-600 expression. Data were expressed as mean±SD (n=3), and analyzed by Student's t-test.

Effect of A. capillaries flower extract on skin wrinkle and sagging. As HYBID has been found responsible for the degradation of HA which is one of the key components of dermal matrix, the efficacy of *A. capillaries* flower extract has been tested against wrinkle and sagging. The investigation has been carried out on 13 male and female subjects who topically applied the test formulation containing 1% *A. capillaries* flower extract on one side of the face and the placebo formulation (control) on the other side of the face twice daily for 4 weeks. The extract formulation has been shown to decrease sagging significantly ($p<0.05$) when compared between week-0 (baseline) and week-4 (Fig. 5A). Moreover, *A. capillaries* flower extract-formulated lotion decreases significantly (<0.05) the extent of sagging after 4 weeks (Fig. 5B), when compared with control. In case of wrinkle, the extract formulation, compared to placebo formulation, has been shown to reduce significantly ($p<0.05$) the maximum depth of eye lid wrinkle after 4 weeks (Fig. 6).

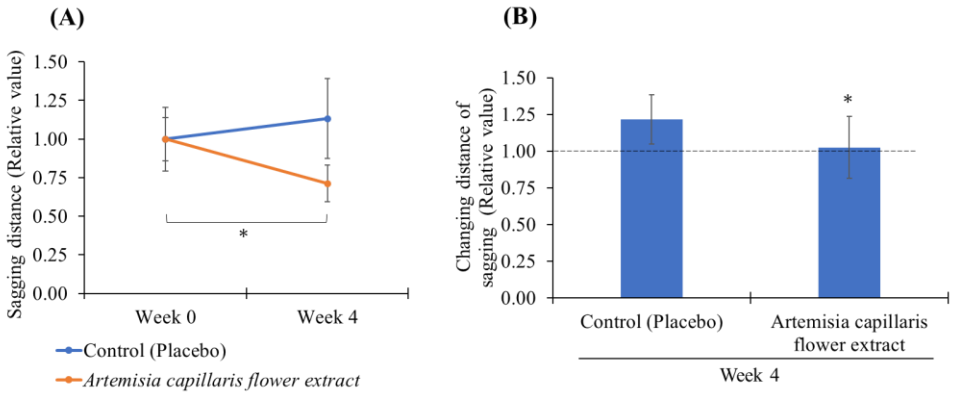

Figure 5. Effect of *Artemisia capillaris* flower extract on the inhibition of sagging. (A) Decrease of sagging after 4 weeks by the extract compared to Week 0. Data were expressed as mean±SE (n=13), and analyzed by Wilcoxon Rank-Sum Test (*p<0.05). (B) The data of the change of sagging distance represents the ratio between Week 4 to respective Week 0. Data were expressed as mean±SE (n=13), and analyzed by Wilcoxon Rank-Sum Test (*p<0.05 vs Control).

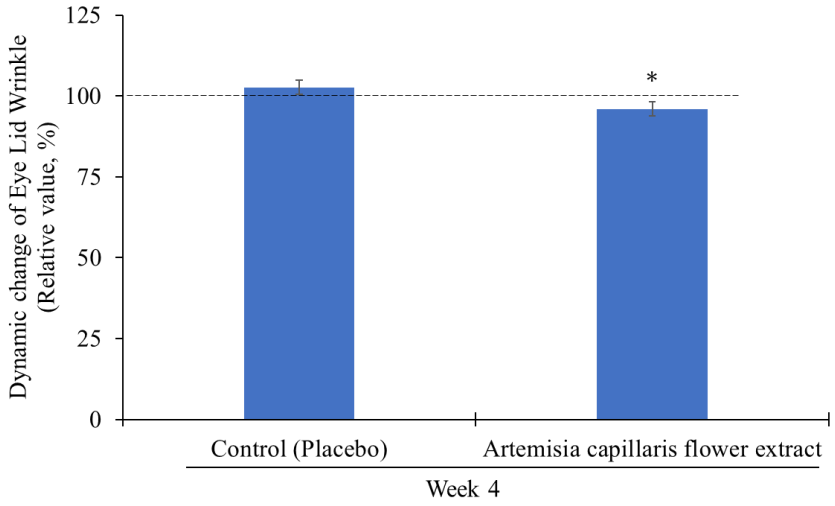

Figure 6. Effect of *Artemisia capillaris* flower extract on the inhibition of wrinkle. The data of the change of eye lid wrinkle represents the ratio between Week 4 to respective Week 0. Data were expressed as mean±SE (n=13), and analyzed by Wilcoxon Rank-Sum Test (*p<0.05 vs Control).

Discussion

To the best of our knowledge, this study is the first to demonstrate the molecular size-dependent effect of HA on accelerating the immune response in human skin fibroblasts under pathophysiological condition caused by the presence of a strong inflammatory agent, TNF-α. Although high-molecular-size HA has been recognized and approved by the FDA for the treatment of various inflammatory conditions including joint [3], medium-to-low molecular size HA has been reported to cause inflammatory reactions in patients with osteoarthritis [8], and reduce the viscosity, elasticity, and lubricating ability of the synovial fluid [9]. We have showed that this medium-to-small size HA does not induce inflammatory response in fibroblasts under normal physiological condition, but can accelerate the expression of certain pro-inflammatory cytokines, IL-1β and IL-6 (Fig. 1A, 1B), under pathological condition.

This study also suggest that HA may somehow get involved in inducing wrinkle once it is degraded into different intermediate size fragments which lead to accelerate MMP-1 expression (Fig. 1C) under the same pathophysiological condition. HA has long been used for its beneficial effect in joint disease, such as osteoarthritis, and it has been suggested that this beneficial effect may be due to the pharmacological effects rather than to physico-chemical properties [1]. Recently, HA is being used as a dermal filler to improve skin condition including wrinkle, but the exact mechanism is not clear. However, it is important to think about the cautions during its use so that the injection of small-size HA to dermis may be restricted in the case of pathological or already inflamed skin.

We have, for the first time, identified the expression of miR-600 in human skin fibroblasts, and suggested its involvement in the regulation of HYBID expression, at least, in this cell type (Fig. 4). The lower expression of this miRNA in NHDF-AD compared to NHDF-NB implies that HYBID expression level could be increased in dermis due to chronological aging. However, there is a limitation of this study that the comparison of miR-600 expression data between NB and AD fibroblasts has been carried out using cells from single donor. Further experiments are necessary to be carried out in cells from at least 3 donors to come to a concrete decision.

Furthermore, we report that *A. capillaries* flower extract has the potential to inhibit histamine-mediated HYBID expression in human skin fibroblasts in culture (Fig. 3), and this inhibition may lead to decreased degradation of HA. Histamine is a released factor from mast cells under UV irradiation, and HYBID expression has been reported to be increased in photo-aged skin [17]. So, there is a possibility that histamine is released due to extrinsic aging of skin and causes the degradation of HA by inducing the expression of HYBID. In this aspect, our extract may be said to have potential to inhibit extrinsic aging. We also report that *A. capillaries* flower extract increases the expression of miR-600, which directly targets HYBID and inhibits its expression, in NHDF-AD (Fig. 4) meaning that our extract may also be involved in inhibiting intrinsic aging of skin. However, the decreased expression of miR-600 in NHDF-NB as well by the extract implies that the decrease of this miRNA alone is not associated with the HYBID-mediated intrinsic aging of skin, probably any additional mechanism exits there. We also report that this plant extract decreases the extent of wrinkle (Fig. 6) and sagging in clinical study (Fig. 5). Yoshida et al have demonstrated that there are strong correlations between HYBID-mediated HA degradation and the indices of wrinkle and sagging [17]. Our report implies that the effect of *A. capillaries* flower extract on the decrease of wrinkle and sagging in a human clinical study might be provided through the inhibition of HYBID-mediated HA degradation.

In conclusion, it is suggested that although HA with its intact size provides strong structural and functional support against aging, it may somehow get involved in inducing wrinkle when degraded into different intermediate size fragments. We have revealed this characterization of intermediate size fragments in dermal fibroblasts for the first time. We have also confirmed the miRNA-regulated expression of HYBID in skin fibroblast. The decreased level of miRNA in NHDF-AD compared to NHDF-NB suggest that it may be associated, at least partially, in the cause of intrinsic aging of skin. We have proposed a specific plant ingredient, *A. capillaris* flower extract, with interesting properties for skin aging care. This plant extract may be able to take care of both extrinsic and intrinsic skin aging by downregulating the stress-induced expression of HYBID and by upregulating

the chronologically decreased expression of miRNA, respectively.

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Conflict of interests

NONE.

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