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

Kazal Boron Biswas[1,2], Teruaki Masutani[1], Shintaro Inoue[2], Arunasiri Iddamalgoda[1,2]
[1]Department of Research and Development, Ichimaru Pharcos Co. Ltd., Motosu, Gifu, Japan;
[2]Department of Cosmetic Health Science, Gifu Pharmaceutical University, Gifu, Japan

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Introduction

Hyaluronic acid (HA) is one of the most important components of the extracellular matrix of dermis. It is generally accepted that the proper balance between the synthesis and degradation of HA plays a great role in maintaining total skin homeostasis. The conventional mechanisms of HA degradation involve the hyaluronidase (HYAL) enzymes, mainly HYAL1 and HYAL2, which depolymerize the large HA into individual sugar units following lysosomal action. These free sugar units then extrude into the cytoplasm and are used up by the cells for different biological reactions [1]. On the other hand, a new mechanism of HA degradation system has recently 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 [2]. These intermediate size fragments are known to cause harmful effects including inflammation and pain in various tissues, but their effects in skin fibroblasts have not yet been determined.

In the case of extrinsic skin aging, the increase of HYBID level has already been reported [3]. We have suggested the microRNA (miR-600)-based regulation of HYBID expression in intrinsic aging, probably for the first time. Also, we have attempted to identify a promising material which may inhibit the expression of HYBID in human dermal fibroblasts, and thereby, suppress skin wrinkling and sagging.

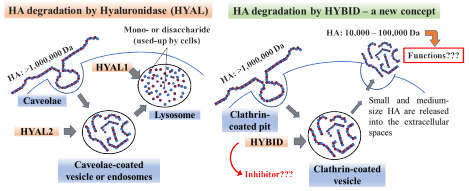

Figure 1: Major pathways of HA degradation in dermis. The new concept was proposed by Yoshida *et. al.* [2].

Materials & 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; TNF-α from HumanZyme Inc., Chicago, IL, USA, and Hyaluronic Acid (HA) in the form of sodium Hyaluronate (NAHA) was purchased from Iwai Chemicals Company, Tokyo, Japan. There were 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).

Plant information: *Artemisia 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.

Cell culture: NHDF-NB and-AD were cultured in 6-well plates ($6x10^4$ cells/well) and maintained in D-MEM (10%) at 37^0C under a humidified atmosphere of 95% air and 5% CO2. Before exposure and during incubation of cells with any chemical or plant material, the cells were maintained under starved condition with medium containing 0.25% FBS.

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 using a SYBR Premix Ex Taq II system (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. 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 using the Mir-X miRNA qRT-PCR SYBR Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. The delta-delta-CT method was used to compare the differences of mRNA and miRNA expressions among different experimental groups.

Human clinical study: This study consisted of a total of 13 male and female participants, was conducted for a period of 4 weeks. 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 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.

Results & Discussion

First, we have investigated whether different sizes of HA has any 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, if the cells are induced with TNF-α, the expressions of IL-1β and IL-6 as well as MMP-1 are accelerated depending on the molecular sizes (Fig. 2a, b, c).

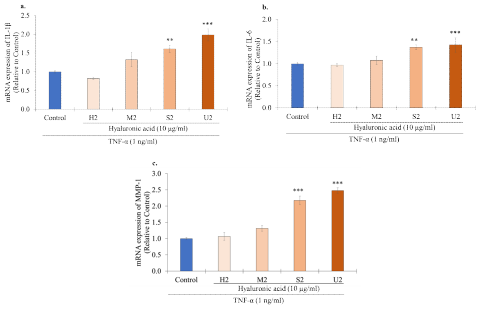

Figure 2: Effect of different sizes of HA (large, medium, small, and ultra-small) on the expression of pro-inflammatory cytokines and MMP-1. Data were expressed as mean±SD (n=3), and analyzed by Dunnett's test (**p<0.01, and ***p<0.001 vs control).

Then, we have checked the regulation of HYBID expression in the presence of histamine, a mast cell-derived factor which is involved in skin aging, and found that the mRNA expression of HYBID is dose-dependently increased in NHDF-NB (Fig. 3a). We have also found that *Artemisia capillaris* flower extract down-regulates the histamine-induced HYBID expression in a concentration-dependent manner (Fig. 3b).

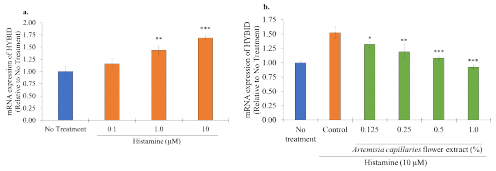

Figure 3: Effect of histamine on the expression of HYBID (a) and role of *Artemisia capillaris* flower extract on the inhibition of histamine-induced expression of HYBID (b). Data were expressed as mean±SD (n=3), and analyzed by Dunnett's test. **p<0.01, ***p<0.001 vs No Treatment (a); and *p<0.05, **p<0.01, ***p<0.001 vs Control (b).

Next, we have investigated the expresssion of miR-600, which directly targets HYBID to inhibit its expression. It is found that the expression of miR-600 is signicantly reduced in NHDF-AD, but the use of *Artemisia capillaris* flower extract increases that level (Fig. 4).

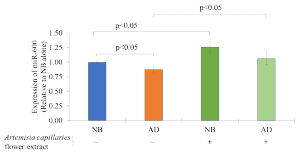

Figure 4: Expression of miR-600 in NHDF-NB and NHDF-AD, and the effect of *Artemisia capillaris* flower extract (0.5%) on its expression. Data were expressed as mean ± SD (n=3), and analyzed by Student's t-test. NB: Newborn, AD: Adult.

Finally, we have evaluated the function of *Artemisia capillaris* flower extract in inhibiting wrinkling and sagging in a human clinical study. It has been found that sagging is significantly decreased when compared between week-0 (baseline) and week-4 (Fig. 5a, d). Moreover, the extract decreases the extent of sagging after 4 weeks (Fig. 5b) when compared with placebo. 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. 5c).

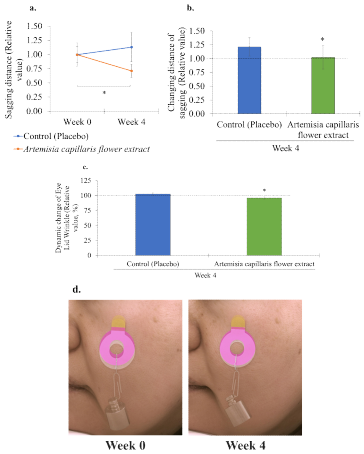

Figure 5: Effect of *Artemisia capillaris* flower extract on the inhibition of sagging (5a, b) and wrinkle (c). Data were expressed as mean ± SE (n=13), and analyzed by Wilcoxon Rank-Sum Test. *p<0.05 vs Week 0 (5a), and *p<0.05 vs Placebo (5b, c). A representative picture showing the efficacy of the extract is also shown (Fig. 5d).

Conclusions

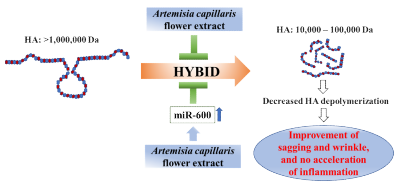

Figure 6: *Artemisia capillaris* flower extract inhibits HYBID expression directly as well as indirectly by increasing miR-600, and thereby, improves the sagging and wrinkle by inhibiting HA degradation.

References

1. *Joy RA et. al. Drug Metabol Pers Ther 2018; 33: 15-32.*
2. *Yoshida H et. al. Proc Natl Acad Sci USA 2013; 110: 5612-5617.*
3. *Yoshida H et. al. Br J Dermatol 2018; 179: 136-144.*