Expression of cyclophilin A and CD147 during skin aging

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Abstract: Objective To investigate the role of cyclophilin A (CypA) and CD147 in the process of skin aging. **Methods** Twenty cases of tissue samples from junior group(<15 years old), middle age group(30—40 years old)or old age group (>65 years old) were collected from photophobic and exposal parts of skin, respectively. Immunohistochemistry (IHC) and in situ hybridization (ISH) were carried out to semi-quantitatively detect the expression level of CyPA and CD147. **Results** IHC demonstrated that both CyPA and CD147 were expressed in both photophobic and exposal parts of normal human skin in all 3 groups. The expression levels of both CyPA and CD147 were increased with increase in age. There were significant differences in both CyPA and CD147 expression among 3 groups ($P<0.05$). CyPA and CD147 were also positively correlated in all 3 groups. Similar results were achieved by ISH. **Conclusion** The interaction between CD147 and CyPA might play an important role in the process of skin aging.

Key words: CyPA; CD147; skin aging

亲环素 A 和 CD147 在皮肤衰老过程中表达的研究

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[摘要] **目的:**检测不同年龄组正常人避光部位及曝光部位表皮组织中亲环素 A(CyPA)和 CD147 的表达,初步探讨 CyPA 和 CD147 在皮肤衰老过程中的意义。**方法:**收集少年组(<15 岁)、中年组(30—40 岁)和老年组(>65 岁)避光部位和曝光部位正常皮肤各 20 例,运用免疫组织化学及原位杂交 2 种方法检测表皮中 CyPA 和 CD147 蛋白和 mRNA 的表达水平,并分析各年龄组 CyPA 和 CD147 的表达水平的差异及相关性。**结果:**免疫组织化学结果显示 CyPA 和 CD147 2 种蛋白在曝光和避光部位都为老年组最高,中年组次之,少年组最低,各组间差异具有统计学意义(均 $P<0.05$),且各组中 CyPA 和 CD147 表达呈正相关(避光部位 $r=0.899$,曝光部位 $r=0.945$)。组织原位杂交结果显示 *CyPA* 和 *CD147* mRNA 在曝光和避光部位都为老年组最高,中年组次之,少年组最低,各组间差异具有统计学意义(均 $P<0.05$),且各组中 CyPA 和 CD147 表达呈正相关(避光部位 $r=0.792$,曝光部位 $r=0.782$)。**结论:**CyPA 和 CD147 相互作用可能在皮肤衰老过程中起重要作用。

[关键词] 亲环素 A; CD147; 皮肤衰老

DOI:10.3969/j.issn.1672-7347.2011.03.003

Date of reception 2010—10—25

Biography LI Ji,M.D.,attending physician,mainly engaged in the study of skin aging and epidermal stem cells.

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Foundation items This work was supported by the National Natural Science Foundation of China for Youth(30800992) and Scientific Research Fund from the Department of Science and Technology of Hunan Province,P.R.China (2010FJ3155).

Skin aging is classified into endogenous and exogenous aging[1]. It is shown in the existing research[2] that the oxidative stress caused by the reactive oxygen species (ROS) generated in the process of cell metabolism is one of the most important causes of skin intrinsic aging. As the skin is the outermost layer of the body, it is directly affected by environmental factors such as oxygen, ultraviolet (UV) and so on. The skin cells can be oxidized directly, or absorb UV and convert the luminous energy into chemical energy, producing large amount of ROS and causing oxidative stress[3]. The excessive ROS produced by the endogenous and exogenous factors will cause direct damages to various biomacromole cules such as protein, lipid, and nucleic acid[4], and finally lead to dysfunction and aging of cells[5-7]. It can be concluded from the above facts that skin intrinsic aging has the same molecular mechanism as photoaging[3, 8-9]. Both of them are affected by oxidative stress caused by ROS, which brings a series of subsequent effects[10], and influences the skin aging.

Cyclophilin A (CyPA), a member of the immunophilin family, is the main receptor of the immune inhibitor cyclosporine A (CsA). It has the activity of peptidyl prolyl cis-trans isomerase, and can catalyze protein folding and transference in cells. In recent years, it has been found that the increase of CyPA in cancer cells can reduce the cell apoptosis induced by oxidative stress[11]. CyPA can protect cells from oxidative stress in various ways[12-14]. In the state of oxidative stress, the expression of CyPA increases so as to protect the body from oxidative damages and prolong cell life[15]. So CyPA is a potent molecule against oxidative stress[16]. It has been found that CyPA may serve as a ROS scavenger. It can increase the activity of antioxidant enzymes, alleviate oxidative stress, and protect cells[17]. Then, whether CyPA slows down skin aging by inhibiting oxidative stress?

CD147 is a kind of transmembrane glycoprotein Ⅰ, belonging to the immunoglobulin superfamily (IGSF), which has a molecular weight of about 58 kD[18]. CD147 distributes widely in body, and is involved in various physiological and pathological processes[19-21]. CD147 is the cell surface receptor of CyPA, and plays an important role in CyPA-mediated signal transmission and chemotactic activity[22]. Recent study[23] proved that CyPA/CD147 can protect neurons from oxidative damages by way of extracellular regulated protein kinases (ERK) 1/2 signaling pathway. It was found in our early study[24] that CD147 can protect the malignant melanoma cell line A375 from oxidative stress caused by H_2O_2. Therefore, we speculated that the combined action of CyPA and CD147 plays an important part in the process of slowing down skin aging by inhibiting oxidative stress. However, no relevant reports have been published yet.

The aging of epidermis is thought to be one of the most important reasons for skin aging because it plays an important role in skin moisturizing and skin barrier. In our early study[25], change of expression level of many molecules was found in aged skin. In the present research, in order to study the possible effect of CyPA and CD147 in the process of skin aging, we detected their protein and mRNA expression levels in normal skin keratinocyte (KC) from shaded and exposed body parts of young, middle-aged, and old people, using immunohistochemical method and in-situ hybridization.

1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Subjects

We collected the normal skin from the skin-grafting or skin flap grafting surgeries of 120 patients who had been diagnosed of no chronic systemic or local skin diseases. All the patients were from Department of Plastic Surgery and Department of Skin Surgery, Xiangya Hospital, Central South University (Changsha, China). The 120 patients were divided into the junior group (<15 years old), the middle age group (between 30 and 40 years old), and the old age group (>65 years old), respectively. The skin was taken from the shaded parts of 20 patients in each group, and exposed parts of the other 20 patients. Here, the shaded parts referred to abdomen and the inner side of the thigh, and the exposed parts referred to face and neck.

In the junior group, providers of skin from shaded parts included 10 males and 10 females,

with age of (9.2±3.4) years old, and providers of skin from exposed parts included 11 males and 9 females, with age of (9.8±4.5) years old; in the middle age group, providers of skin from shaded parts included 11 males and 9 females, with age of (34.8±3.1) years old, and providers of skin from exposed parts included 11 males and 9 females, with age of (34.5±3.0) years old; in the old age group, providers of skin from shaded parts included 10 males and 10 females, with age of (71.0±3.2) years old, and providers of skin from exposed part included 10 males and 10 females, with age of (69.8±3.6) years old. In each group, the age of providers of skin from shaded parts was not significantly different from those from exposed parts ($P>0.05$), and the ratio of male to female in each group was not significantly different ($P>0.05$).

1.1.2 Main equipments and reagents

Immunohistochemical primary antibody rabbit anti-human CD147 polyclonal antibody and SP immunohistochemical staining kit were purchased from Beijing Zhongshan Goldenbrige Biotechnique Co. Ltd. (China); rabbit anti-human CyPA polyclonal antibody was from Abcam Corporation in the USA;CD147 in-situ hybridization kit was bought from Wuhan Boster Bio-engineering Co. Ltd. (China); CyPA kit (multiphase oligonucleotides probe and hypersensitive labeling technique was used to synthesize CyPA oligonucleotides probe labeled by digoxin at 5′) was bought from Wuhan Boster Bioengineering Co. Ltd. (China); and BX61 Upright optical microscope was purchased from OLYMPUS company in Japan.

1.1.3 Processing of specimens

The specimens in each group were divided into 4 parts, 2 of which were fixed in 4% formalin, and the other 2 were put into tubes and preserved in liquid nitrogen.

1.2 Methods

1.2.1 SP immunohistochemical staining

The staining was conducted strictly following the instruction of the SP immunohistochemical staining kit. The specimens were embedded in paraffin and made into 4 μm-thick slices. The slices were underwent routine deparaffinating, hydration, and PBS rinsing. Then, they were put into citrate buffer solution for antigen retrieval, and washed with PBS for 3 times, 3 min each time. Then normal goat serum was dripped into the tissue slides to block for 20 min at room temperature. After the blocking, rabbit anti-human CD147 or CyPA antibody of working concentration were dripped into the slice, and then the tissue slices were incubated at 4 ℃ overnight. The slices were washed with PBS for 3 times, 5 min each time and then added with enzyme-labeled anti-rabbit polymer and incubated at 37 ℃ for 20 min. The slices were washed with PBS for 3 times, 5 min each time again before DAB color development was performed. After color was developed, the tissue slices were washed with running water and counterstained with hematoxylin (HE) for 3 min. Then hydrochloric acid/alcohol differentiation was done followed by routine washing, dehydration, vitrification, and slide covering. The result was observed with optical microscope, the positive cells were counted, and photographs were taken. PBS buffer in replacement of the primary antibody serves as the negative control.

1.2.2 In-situ hybridization

The hybridization was performed strictly following the instructions of the kit. The skin tissues preserved in liquid nitrogen was embedded with OCT embedding medium, series-sectioned at −20 ℃ into a thickness of 8 μm, and set on the dedicated slides. The tissue slices were fixed with 4% paraformaldehyde at room temperature for 20−30 min, carefully washed with distilled water, and then treated with the mixture of 30% H_2O_2 and methanol (1∶50) for 30 min. They were washed with distilled water 3 times before digested with 3% pepsase at 37 ℃ for 5−120 s, and then washed with PBS for 5 min 3 times and distilled water once. Then they were fixed with paraformaldehyde at room temperature for 10 min, and washed with distilled water 3 times. After that, 20 μL of prehybridization solution was added, and the tissues were incubated at 37 ℃ for 4 h. Excess liquid was wiped away, and the slides were kept unwashed. Hybridization solution (20 μL) was added into each slice, which were covered with dedicated cover slips and hybridized at 37 ℃ overnight. Uncovered the slices and the slices were washed with 2×SSC solution (37 ℃) for 5 min twice,

and then with 0.2×SSC solution (37 ℃) for 15 min once. Blocking reagent was added and the tissue slices were incubated at 37 ℃ for 30 min. Excess liquid was discarded, and the slides were kept unwashed. The tissue slices were incubated at 37 ℃ for 60 min after biotinylated rat anti-digoxin was added, and washed with PBS for 4 times, 5 min each. They were added with SABC, incubated at 37 ℃ for 20 min, and washed with PBS for 3 times, 5 min each. Then they were added with biotinylated peroxidase, incubated at 37 ℃ for 20 min, and washed with PBS for 4 times, 5 min each. Following that, DAB color development was performed and the slices were carefully washed with water. Then hematoxylin counterstain, hydrochloric acid/alcohol differentiation were done followed by routine washing, dehydration, vitrification, and slide covering. The result was observed with optical microscope, and the positive cells were counted. The negative control is probe-free hybridization solution.

1.3 Criteria of judge

1.3.1 Immunohistochemical staining

Double-blind method that 2 people assess the staining results separately was applied in this study. Staining of CD147 was determined as positive when yellow brown or brown particles presented in cell membrane, and the staining of CyPA was determined as positive when yellow brown or brown particles presented in the cytoplasm. The staining results were observed in non-overlapping field of views of optical microscope (10×40 magnification). Five fields were picked out randomly in each slice, 200 cells were counted in each field, and the immunoreactivity intensity distribution index (IRIDI) values of the cells were calculated. According to the assessment of Bayramgurler, et al.[26], the staining degrees were classified into none, weak, medium, and strong, which were marked with 0−3 points respectively; the ratio of positive cells were classified into 0, 1%−25%, 26%−50%, and >50%, which were marked with 0−3 points respectively. The IRIDI values were obtained by multiplying the 2.

1.3.2 In-situ hybridization

Double-blind method that 2 people assess the stain result separately was applied in this study. The staining of CD147 and CyPA was determined as positive when yellow brown or brown particles presented in the cytoplasm. The staining results were observed in non-overlapping field of views of optical microscope (10×40 magnification). Five fields were picked out randomly in each slide, 200 cells were counted in each field and the values of expression level were calculated. The staining degrees were classified into none, weak, medium and strong, which were marked with 0−3 points respectively; the ratios of positive cells were classified into 0, 1%−25%, 26%−50%, and >50%, which were marked with 0−3 points respectively. Then the values of expression level were obtained by multiplying the 2.

1.4 Statistical analysis

Data are presented as mean ± standard deviation($\bar{x}\pm s$). The statistical analyses were processed by use of the statistical software SPSS15.0. The comparison of CyPA and CD147 expression level in different groups was tested with Kruskal Wallist test, and the correlation analysis of CyPA and CD147 in different body parts was tested with Spearman's rho method. $P<0.05$ was considered as having statistical significance.

2 RESULTS

2.1 Results of the SP immunohistochemical staining

It was showed by SP immunohistochemical staining that, the expression of CyPA and CD147 existed in cells of the normal skin from all the patients in the 3 groups. CyPA was mainly distributed in the cytoplasm, and CD147 existed mainly in the cell membrane (Fig. 1, 2). The IRIDI values of CyPA expression in the skin from the shaded parts were 1.35±0.49, 2.15±0.67, and 3.55±0.76 in the junior, middle age, and old age group, respectively and the difference between the 3 groups had statistical significance ($P<0.05$, Fig. 3); the IRIDI values of CD147 expression in the skin from the shaded parts were 1.20±0.41, 2.35±0.67, and 3.80±1.06 in the junior, middle age, and old age group, respectively and the difference between the 3 groups had statistical significance ($P<0.05$, Fig. 3). The IRIDI values of CyPA expres-

sion in the skin from the exposed parts were respectively 1.40±0.50, 2.60±0.75, and 3.80±0.89 in the junior, middle age, and old age group, and the difference between the 3 groups had statistical significance ($P<0.05$, Fig. 4); the IRIDI values of CD147 expression in the skin from the exposed parts were 1.30±0.47, 2.75±0.79 and 4.10±1.07 in the junior, middle age and old age group, respectively and the difference between the 3 groups had statistical significance ($P<0.05$, Fig. 4).

The expression of CyPA and CD147 in shaded parts was positively correlated, and the correlation coefficient was 0.899; the expression of CyPA and CD147 in exposed parts was positively correlated, and the correlation coefficient was 0.945.

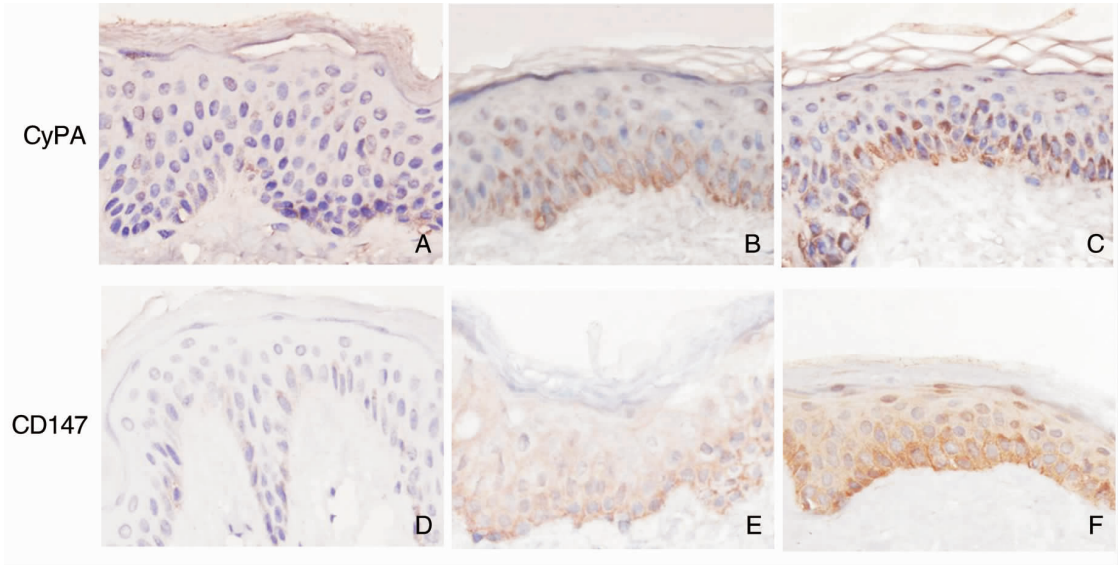

Fig. 1 SP immunohistochemical staining showing the expression of CyPA and CD147 in the shaded normal skin (×400). A, B, and C represented the expression of CyPA in the junior, middle age, and old age group, respectively; D, E, and F represented the expression of CD147 in the junior, middle age, and old age group, respectively.

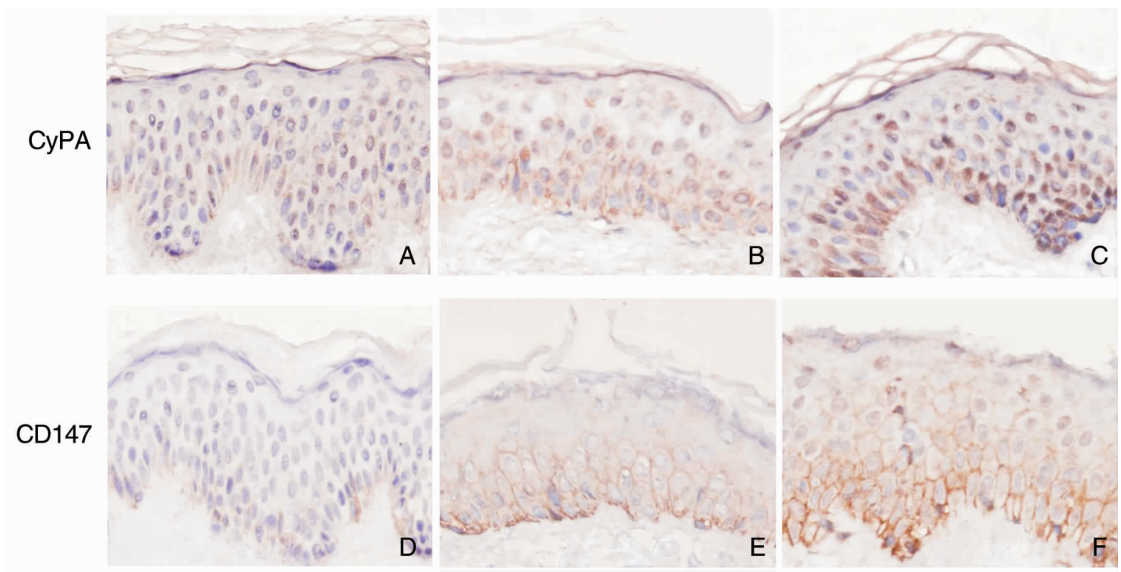

Fig. 2 SP immunohistochemical staining showing the expression of CyPA and CD147 in the exposed normal skin (×400). A, B, and C represented the expression of CyPA in the junior, middle age, and old age group, respectively; D, E, and F represented the expression of CD147 in the junior, middle age, and old age group, respectively.

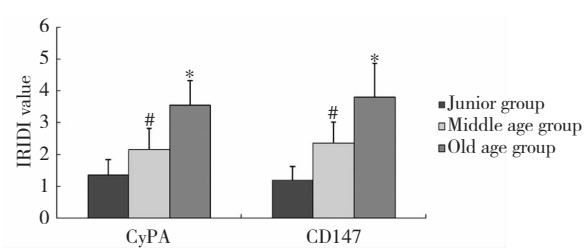

Fig. 3 IRIDI values of CyPA and CD147 in the shaded parts of each group. Compared with the junior group and middle age group, * $P<0.05$; compared with the junior group and old age group, # $P<0.05$.

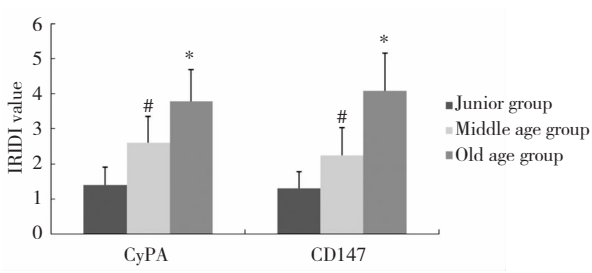

Fig. 4 IRIDI values of CyPA and CD147 in the exposed parts of each group. Compared with the junior group and middle age group, * $P<0.05$; compared with the junior group and old age group, # $P<0.05$.

2.2 Results of in-situ hybridization

It was showed by in-situ hybridization that the expression of CyPA and CD147 existed in the KC of the normal skin from all the patients in the 3 groups. CyPA was mainly distributed in the cytoplasm, and CD147 existed mainly in the nucleus and cytoplasm (Fig. 5,6). The expression levels of CyPA expression in the skin from shaded parts were respectively 1.67±0.75,2.83±1.07, and 3.83±1.67 in the junior, middle age, and old age group, and the difference between the 3 groups had statistical signi-ficance ($P<0.05$, Fig. 7); the expression level of CD147 expression in the skin from shaded parts were respectively 1.83±1.07, 2.67±0.94, and 3.5±1.38 in the junior, middle age, and old age group, and the difference between the 3 groups had statistical significance ($P<0.05$, Fig. 7). The average expression level values of CyPA expression in the skin from exposed parts were respectively 1.33±0.71, 2.67±1.11, and 3.67±1.25 in the junior, middle age and old age group, and the difference between the 3 groups had statistical significance ($P<0.05$, Fig. 8); the expression level values of CD147 expression in the skin from exposed parts were respectively 1.5±0.86, 2.69±1.73, and 3.63±1.46 in the junior, middle age, and old age group, and the difference between the 3 groups had statistical significance ($P<0.05$, Fig. 8).

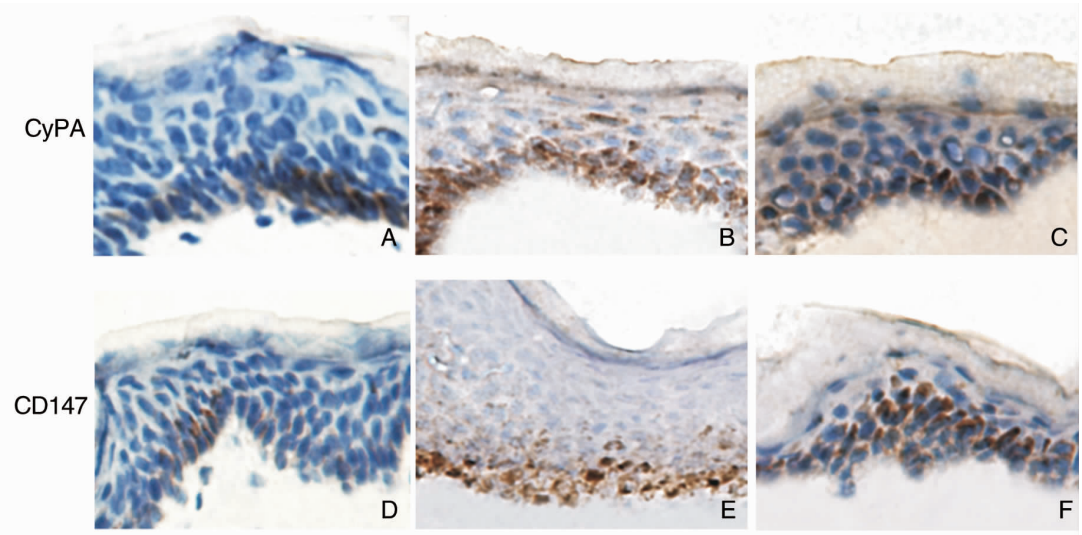

Fig. 5 In-situ hybridization showing the expression of *CyPA* and *CD147* mRNA in the shaded normal skin (×400). A, B, and C represented the expression of CyPA in the junior, middle age, and old age group, respectively; D, E, and F represented the expression of CD147 in the junior, middle age, and old age group, respectively.

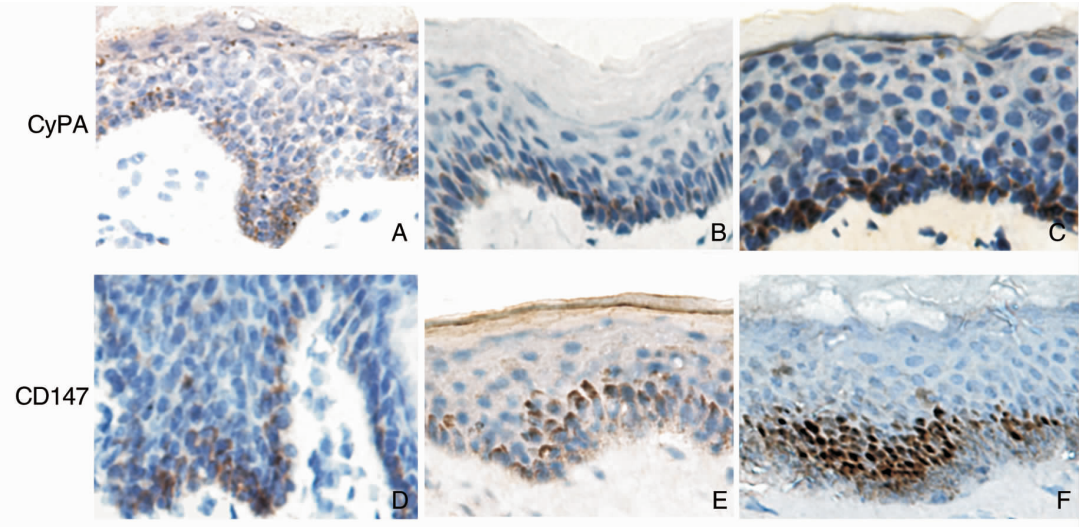

Fig. 6 In-situ hybridization showing the expression of *CyPA* and *CD147* mRNA in the exposed normal skin (×400). A, B,

and C represented the expression of CyPA in the junior, middle age, and old age group, respectively; D, E, and F represented the expression of CD147 in the junior, middle age, and old age group, respectively.

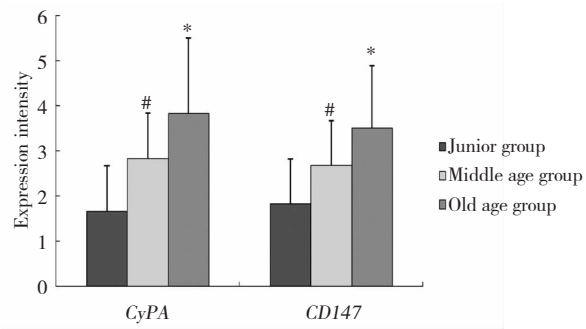

Fig. 7 mRNA expression levels of *CyPA* and *CD147* in the shaded parts of each group. Compared with the junior group and middle age group, $*P<0.05$; compared with the junior group and old age group, $\#P<0.05$.

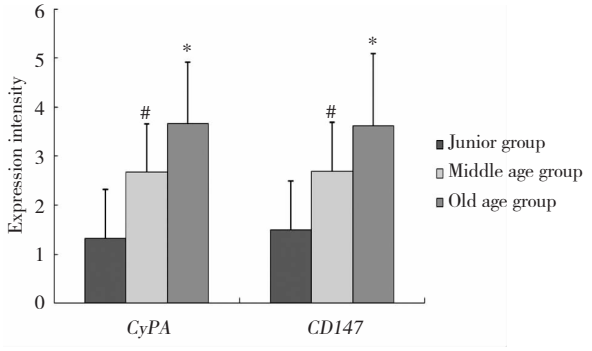

Fig. 8 mRNA expression levels of *CyPA* and *CD147* in the exposed parts of each group. Compared with the junior group and middle age group, $*P<0.05$; compared with the junior group and old age group, $\#P<0.05$.

The expression of CyPA and CD147 in shaded parts was positively correlated, and the correlation coefficient was 0.792; the expression of CyPA and CD147 in exposed parts was positively correlated, and the correlation coefficient was 0.782.

3 DISCUSSION

The mechanism of skin aging is very complica-ted. So far, theories on skin aging include free radical theory, theory of heredity, telomere theory and so on. It is held by the free radical theory that, in the process of aging, ROS increases and the activity of antioxidase decreases, and that the accumulated of damages caused by excessive ROS is the molecular basis of aging[27]. The imbalance between the generation and elimination of ROS will leads to oxidative stress[28], and the irreversible accumulation of damages caused by ROS is the key to aging[29].

CyPA is a highly conserved protein of 18 kD, which mainly exists in the cytoplasm and is expressed in a wide range of tissues. It has the activity of peptidyl prolyl cis-trans isomerase, which enables it to promote protein folding and transference, activate signaling pathway, and regulate the activity of receptors. It has been found in the previous research[30] that the expression level of CyPA is higher in the skin of aged people than in young people, and significantly higher in old rats than in young ones[31]. The result of our research showed that the expression of CyPA increased with the increasing of age in normal skin no matter it was exposed or shaded, which indicated that CyPA acted in the whole process of aging, and was closely related to aging. Then, dose the increase of CyPA expression in the process of aging slow down or promote the aging progress?

In some scholars' opinion, CyPA is a kind of antioxidant which plays an important role in the oxidation-reduction regulation of human body[17] because CyPA can combine with various peroxidase to increase their activity, increase the viability of Cu/Zn superoxide dismutase (SOD)-1 mutated cells[12], improve the activity of SOD and GSH-Px, and serves as the ROS scavenger to relieve oxidative stress[16]. CyPA can activate the ERK1/2 signaling pathway, increase the viability of neurons in the situation of oxidative stress and blood/oxygen-deprivation, and protect cells from oxidative damages[32-34]. The over-expression of CyPA in cancer cells can protect cells from apoptosis induced by oxidatiue stress through inhibiting ROS genenation and relieving the depolarization of mitochondrial membrane potential. So, we deduces that the oxidative stress accumulated in the process of skin intrinsic aging and photoaging may promote the generation of CyPA, which starts the protective function of the body and slows down the aging by inhibit the oxidative stress[35]. In addition, CyPA can enhance the proliferation of embryo cerebral cells and various cancer cells, and inhibit apoptosis[36-38]. So we deduced that CyPA may also take part in anti-aging activity by enhancing cell proliferation and inhibiting apopto-

sis.

CD147 is a trans-membrane glycoprotein belonging to the IGSF, which exists in various tissues and organs. CD147 is the cell surface receptor of CyPA, and plays an important role in CyPA-mediated signal transmission and chemotactic activity[22]. The combined action of CD147 and CyPA activates the ERK1/2 signaling pathway, protects the neurons from oxidative damages[32-34], promote cell proliferation and inhibit apoptosis[36-38]. Therefore, CD147 and CyPA are closely related, and the combined action of them can protect cells from oxidative stress, promote cell proliferation and inhibit apoptosis. The result of our research showed that the protein and mRNA of CD147 were expressed in the normal skin of people of different ages. The expression level increased with the increase of age, and is positively correlated to the expression of CyPA. Because that the expression of CD147 increased in aged skin cells, and that CyPA has an obvious effect on the inhibition oxidative stress, we deduced that CD147 may inhibit oxidative stress, slow down skin aging, promote cell proliferation, and inhibit apoptosis by interacting with CyPA.

In conclusion, we found that the expression of CyPA and CD147 was closely related to skin aging, and deduced that the effect of CyPA and CD147 in slowing down skin aging may be realized by inhibiting oxidative stress. However, the mechanism of the anti-aging function of CyPA and CD147 is not clear yet, so we will continue the study in order to provide ideal target molecules for research and treatment of skin aging.

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(Edited by GUO Zheng)