Report

Study of telomerase reverse transcriptase (hTERT) expression in normal, aged, and photo-aged skin

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Abstract

Background Telomerase is a ribonucleoprotein enzyme capable of extending chromosome ends with telomeric DNA sequences. It protects the germline and stem cells from senescence by preventing telomere attrition. Cutaneous aging includes intrinsic aging, and photo-aging. Telomere-associated cellular senescence contributes to certain age-related cutaneous disorders, including increased cancer incidence. Premature skin aging in xeroderma pigmentosa (XP) is expected to show increased telomere attrition. We aimed to study human telomerase reverse transcriptase (hTERT) expression in normal, aged and photo-aged skin and to investigate its possible role in the pathogenesis of aging and photo-aging.

Methods hTERT expression using immunohistochemistry was studied in 75 subjects comprising four groups: group I, 10 subjects with aged skin; group II, 20 subjects with photo-aging; group III, Five patients with XP; and group IV, 40 subjects comprising the control groups.

Results We found positive hTERT in normal skin and in the basal and sometimes in supra-basal layers. We reported positive hTERT expression in dermal fibroblasts, histiocytes, and skin appendages (other than hair follicles) in some cases from all the studied groups. Photo-aged and prematurely photo-aged skin showed greater hTERT expression than young and aged skin.

Conclusion Telomeres rather than telomerase are involved in cellular senescence. Yet, telomerase is intimately related to photo-aging in which lifetime cumulative sun exposure is an important factor. However, genetic damage in XP is the decisive factor and not merely ultraviolet exposure.

Introduction

Telomeres are protective caps at the ends of chromosomes, which, together with an associated set of proteins, form a DNA–protein complex that is thought to maintain the integrity of chromosome end.[1] The shortening of telomere ends, with progressive rounds of cell division, has been proposed to serve as a mitotic clock by which cell divisions are counted, eventuating in a state of replicative arrest known as cellular senescence.[2]

To protect the germ line and the subpopulation of stem cells from senescence, mechanisms have evolved to prevent telomere attrition in these cellular compartments. The most common and best-studied mechanism involves the activation of telomerase. Telomerase is a ribonucleoprotein enzyme with reverse transcriptase activity that is capable of extending chromosome ends with specific telomeric DNA sequences by using a portion of its RNA component as a template.[3,4]

The highly regenerative tissues, such as the skin, likely express telomerase to slow the rate of shortening of telomere length in their cells.[5] However, the infiltration of lymphocytes into the skin may cause transient telomerase activity in skin biopsy samples.[6] This problem had only been offset by the introduction of morphology-preserving techniques, so that the histological distribution of telomerase can be precisely assessed.[7] Immunohistochemistry (IHC) using anti-human telomerase reverse transcriptase (anti-hTERT) monoclonal antibodies had been applied, which made it possible to get results with high specificity.[8–10]

Cutaneous aging includes two distinct phenomena: intrinsic aging, a universal, presumably inevitable change attributable to the passage of time alone; and photo-aging,

the superposition on intrinsic aging of changes attributable to chronic sun exposure, which are neither universal nor inevitable.[11] One might anticipate that this high turnover organ, the skin, would be more adversely affected because of an accelerated loss of telomere repeats.[12] Thus, it had been suggested that telomere-associated cellular senescence might contribute to certain age-related cutaneous disorders, including an increase in cancer incidence, wrinkling, and diminished skin elasticity.[13]

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by congenital photosensitivity with premature photo-aging. These changes occur in sun-exposed areas, usually during childhood.[14] Premature photo-aging in patients with XP would be expected to show increased telomere attrition.[15]

The aim of this work was to study hTERT expression in normal, aged, and photo-aged skin, to investigate the possible role of telomerase in the pathogenesis of aging and photo-aging, thus raising the possibility of the use of telomerase targeting in their treatment. The study was conducted with the specific use of IHC, by which we can exactly determine the cells of considerable telomerase activity.

Subjects and Methods

Subjects

The study was conducted on 75 subjects selected from the Dermatology and the Geriatrics Outpatient's clinics of Ain Shams University Hospitals. The study comprised four groups:

- Group I: 10 subjects from whom sun-protected aged skin biopsies were taken (age group ranging from 65 to 76 years) (United Nations tabulations provided figures for both 60 and 65 years of age and older.[16]
- Group II: 20 subjects from whom photo-aged skin biopsies were taken (age group ranging from 65 to 80 years), 10 of them with actinic keratosis (Ak), from whom lesional skin biopsies were taken.
- Group III: Five patients with XP representing premature photo-aging (age group ranging from 3 to 16 years), from whom both sun-exposed and sun-protected skin biopsies were taken.
- Group IV: Control group: including:
  - Group IVa: 10 normal adults from whom sun-exposed skin biopsies were taken (age group ranging from 21 to 31 years).
  - Group IVb: 10 normal adults from whom sun-protected skin biopsies were taken (age group ranging from 23 to 32 years).
  - Group IVc: 10 normal children from whom sun-exposed skin biopsies were taken (age group ranging from 3 to 14 years).
  - Group IVd: 10 normal children from whom sun-protected skin biopsies were taken (age group ranging from 2 to 13 years).

For all subjects, detailed history was taken with special attention to smoking and to the presence of special habits, occupations and/or hobbies requiring prolonged sun exposure. The administration of drugs, whether prescribed or self-administrated, and the use of sunscreens and cosmetics were considered. Systemic review of the subjects to exclude any internal systemic disease or chronic debilitating condition was asked about. Family history of similar condition and family pedigree for patients with XP were performed.

General examination was carried out to exclude any systemic disease or chronic debilitating condition. Thorough dermatological examination was performed including skin photo-typing determination according to cutaneous sun-burning and tanning tendency following UV exposure.[17]

Methods

Skin biopsies were fixed in 10% buffered formalin, embedded in paraffin, and sectioned in 5-micron thick sections to be prepared for: routine hematoxylin and eosin (H&E) stain for conventional histopathology, and IHC using the Strept-avidin-biotin method for studying the expression of hTERT.

For IHC, antigen retrieval was carried out using a high temperature unmasking technique for paraffin sections.[18] Incubation of the sections in pre-diluted blocking serum for 10 min was performed. Excess serum was blotted from the sections to be prepared for addition of primary antibody. For each section, 100 microns of the Telomerase catalytic unit mouse monoclonal antibody (NCL-hTERT clone 44F12 – Novocastra, UK, pre-diluted 1 : 20 in buffer containing 1.5% blocking serum) were applied and the sections were incubated overnight in a humid chamber. The secondary antibody was then applied for 20 min (biotinylated goat antibody reacting with mouse and rabbit immunoglobulins (from Novocastra: Strept-avidin-biotin complex/Quick Kit), pre-diluted at 1 : 50 in TRIS buffer saline). Sections were then incubated in a Strept-avidin-biotin complex pre-diluted at 1 : 50 for more than 20 min. Color development was obtained using Diamino-benzidine (DAB), and the slides were counter-stained with Mayer's hematoxylin.

For IHC scoring for hTERT, the expression of the antigen was studied in the epidermis and dermis. Only nuclear or nucleolar staining for telomerase was considered positive.

- In the epidermis, the IHC score was as follows (percentage of cells with positive staining): 0 = no staining (negative), +1 = 1–10% of cells are positive, +2 = 11–30% of cells are positive, and +3 = >30% of cells are positive.
- In the dermis, the expression of hTERT in different dermal structures was localized as present (positive) or absent (negative).

Statistical analysis

SPSS version 12 was used for analysis of data. Mann–Whitney and Fisher (SPSS Inc., Chicago, IL, USA) exact tests were

used for comparison between two variables. Kruskal-Wallis test was used for comparison between more than two groups. Kruskal test was used to relate variables to each other. Significance level (*P*-value) was considered insignificant if >0.05, significant if <0.05, and highly significant if <0.001.

Results

Immunohistochemistry data of different study groups are summarized in Table 1.

In group I, the epidermis of aged subjects showed positive hTERT in 5/10 aged subjects (50%) (+1), and in the dermis, 3/10 (30%) of aged subjects showed positive hTERT in dermal fibroblasts ± lymphocytes, histiocytes, and sweat gland ducts (Fig. 1).

In the epidermis of photo-aged subjects (group II), 16/20 (80%) of cases showed positive hTERT expression [in 2 (10%) there was +3 expression (Fig. 2), in 6/20 (30%) expression was +2, and in 8/20 (40%) expression was +1 (Fig. 3)]. In the dermis, hTERT was positive in 10/20 (50%) of photo-aged subjects in dermal fibroblasts ± lymphocytes, and histiocytes, as well as in sebaceous glands (Fig. 4) and the lining of the cysts of Favre Racouchot. In patients with AK, the epidermis of lesional skin showed positive hTERT in 7/10 patients (70%) [4/10 (40%) showed +1 expression, and 3/10 (30%) showed +2 expression (Fig. 5)]. In regard to other subjects with photo-aging, 8/10 (80%) showed positive expression [4/10 (40%) showed +1 expression, 3/10 (30%) showed +2 expression, and 1/10 (10%) showed +3 expression].

In group III (XP), 4/5 (80%) of exposed skin biopsies showed positive hTERT in the epidermis [3/5(60%) showed +1 expression and 1/5 (20%) showed +2 expression], and 3/5(60%) showed positive hTERT in dermal fibroblasts ± lymphocytes and histiocytes. On the other hand, 3/5 (60%) of sun-protected skin biopsies showed +1 epidermal hTERT, while it was not expressed in the dermis.

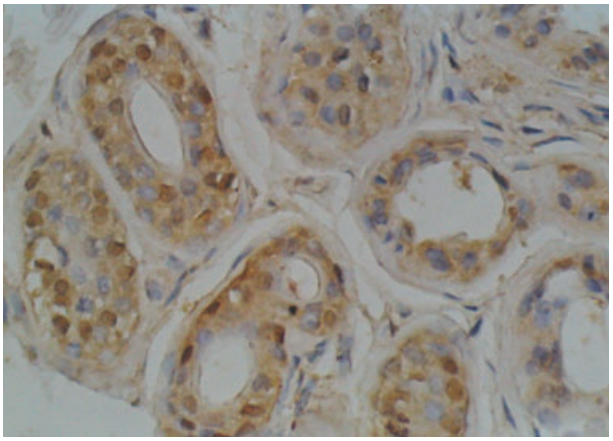

Figure 1 Positive hTERT expression in sweat gland ducts, in aged skin (group I) (Streptavidin biotin, Diamino-benzidine (DAB) chromogen ×400)

In the epidermis of group IVa (adult exposed skin), 4/10 (40%) of skin biopsies, was positive for hTERT (+1 in 2 and +3 in two biopsies), and in the dermis, 2/10 (20%) of skin biopsies showed positive hTERT in dermal fibroblasts, lymphocytes, histiocytes, and skin appendages.

In the epidermis of 4/10 (40%) of skin biopsies of adult protected skin (group IVb), there was positive hTERT (+1), while in 6/10 (60%) it was negative (Fig. 6). On the other hand, in the dermis, all skin biopsies showed no expression.

In the epidermis of 2/10 (20%) of skin biopsies of children exposed skin (group IVc), hTERT was positive

Table 1 Immunohistochemistry (IHC) results of the study groups

	hTERT expression											
	Epidermal								Dermal			
	0		+1		+2		+3		Positive		Negative	
Group	No	%	No	%	No	%	No	%	No	%	No	%
Group I: Aged (Total 10)	5	50	5	50	0	0	0	0	3	30	7	70
Group II: Photo-aged (Total 20)	4	20	8	40	6	30	2	10	10	50	10	50
Group III: XP exposed (Total 5)	1	20	3	60	1	20	0	0	3	60	2	40
Group III: XP protected (Total 5)	2	40	2	40	1	20	0	0	0	0	5	100
Group IVa: Adult exposed (Total 10)	6	60	2	20	0	0	2	20	2	20	8	80
Group IVb: Adult protected (Total 10)	6	60	4	40	0	0	0	0	0	0	10	100
Group IVc: Children exposed (Total 10)	8	80	0	0	2	20	0	0	0	0	10	100
Group IVd: Children protected (Total 10)	6	60	2	20	2	20	0	0	2	20	8	80

XP, xeroderma pigmentosa.

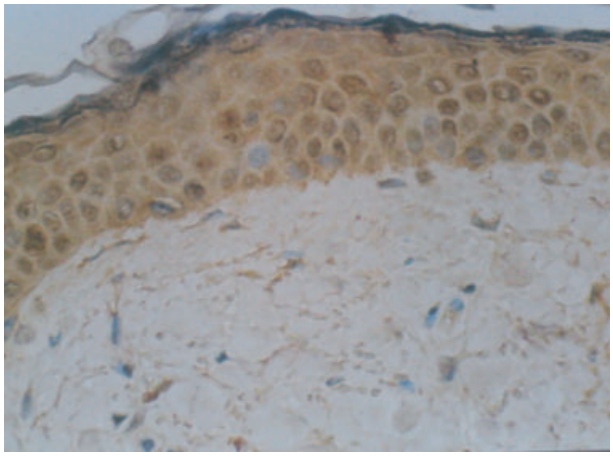

Figure 2 Positive telomerase expression (+3) in the epidermis, and negative dermal expression in photo-aged skin (Streptavidin biotin, Diamino-benzidine (DAB) chromogen ×400)

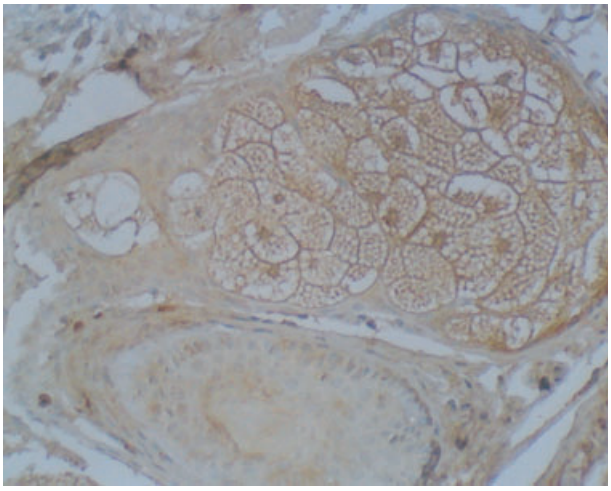

Figure 4 Positive hTERT expression in pilosebaceous follicles in photo-aged skin (Favre Racouchot) (Streptavidin biotin, Diamino-benzidine (DAB) chromogen ×400)

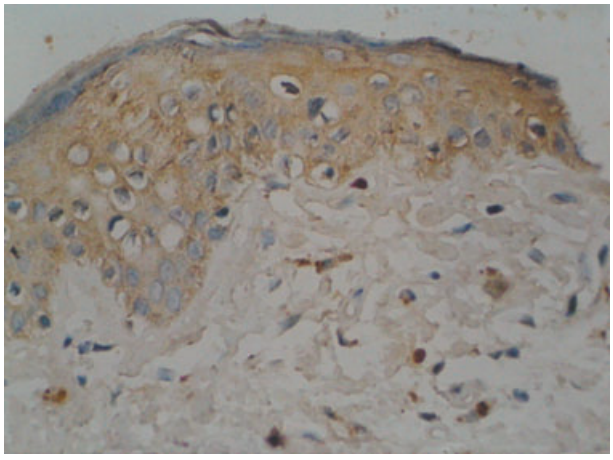

Figure 3 Positive telomerase expression in the epidermis of photo-aged skin (+1) as well as in dermal fibroblasts, lymphocytes and histiocytes (Streptavidin biotin, Diamino-benzidine (DAB) chromogen ×400)

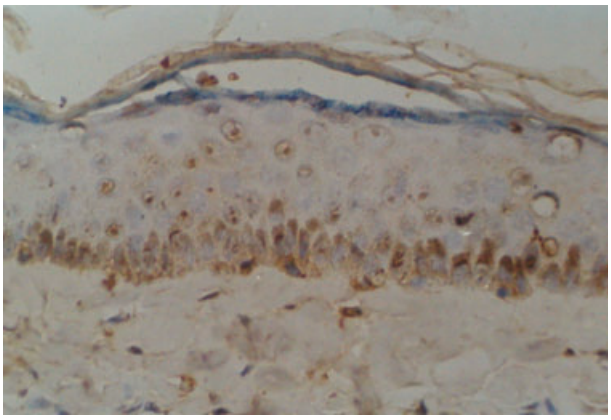

Figure 5 Positive hTERT expression (+2) in the epidermis of photo-aged skin with AK (Streptavidin biotin, Diamino-benzidine (DAB) chromogen ×400)

(nucleolar) (+2) (Fig. 7). On the other hand, in the dermis, all skin biopsies showed no expression.

In the epidermis of 4/10 (40%) of skin biopsies of children protected skin (group IVd), hTERT was positive (2/10 (20%) showed +1 expression and 2/10 (20%) showed +2 expression). On the other hand, in the dermis, 2/10 (20%) showed positive expression.

Comparing hTERT expression in aged skin, adult protected skin and children protected skin revealed that there was no statistically significant difference (Table 2). In addition, comparing hTERT expression in photo-aged and prematurely photo-aged skin (XP), they showed similar expression. On the other hand, comparing hTERT expression among photo-aged skin, adult exposed skin, and children exposed epidermis revealed that photo-aged skin showed statistically significant higher expression than both adult exposed and children exposed skin (Table 3). Comparison between hTERT expression in XP skin and children exposed skin also revealed that XP epidermis showed statistically significant greater expression than their age-matched group (Table 4).

Using Krouskal test, dermal positivity to hTERT was found to be highly related to epidermal expression ($P < 0.001$).

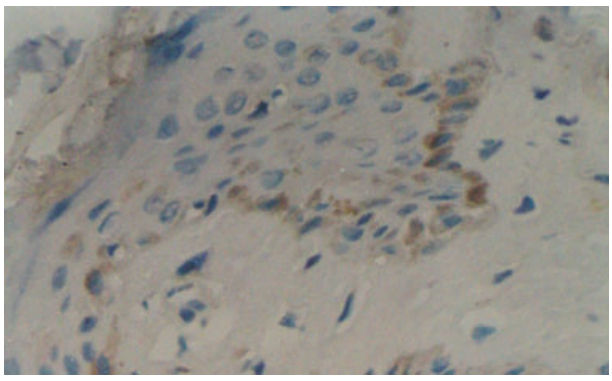

Figure 6 Negative telomerase expression in normal adult protected skin (Streptavidin biotin, Diamino-benzidine (DAB) chromogen ×400)

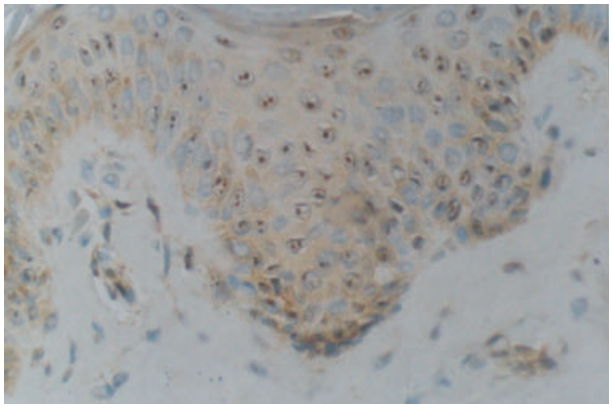

Figure 7 Positive nucleolar telomerase expression in the epidermis of normal children's sun-exposed skin (+2) (Streptavidin biotin, Diamino-benzidine (DAB) chromogen ×400)

Discussion

Stem cells are responsible for the maintenance of the proliferative capacity of the tissues. Although telomerase is repressed in most tissues during development, it is abundantly clear that many stem or stem-like cells in adult humans are able to exhibit some telomerase activity when stimulated to divide. This much lower level of activity is apparently sufficient to slow, but not prevent, telomere shortening as a function of aging.[3]

In this work, a study of hTERT expression in normal, aged, and photo-aged skin was performed, with the specific use of IHC. IHC is characterized by morphology preservation, exact determination of cells with considerable activity, and the ability to relatively quantitatively assess this activity. In group I, 5/10 (50%) of aged subjects showed positive hTERT expression, closely resembling the findings of Harle-Bachor and Boukamp (1996). Using TRAP assay, they found that total human skin was telomerase deficient in 55% of the samples.[19]

We found that the positive epidermal portion for hTERT, in aged skin, was the basal and sometimes the supra-basal layers. This finding is consistent with what was reported by Ogoshi *et al.* (1997)[20], who found that adult epidermis from sun-protected areas showed weak hTR (human Telomerase associated RNA) expression, with no difference in basal cells compared with supra-basal cells.

On the contrary, Harle-Bachor and Boukamp (1996) found that telomerase activity was restricted to the basal cell fractions, with good activity seen only in the populations consisting predominantly of proliferative basal cells (using TRAP assay). When differentiated supra-basal cells were present, the signal was diluted below the level of detection.[19] Similarly, Bickenbach *et al.* (1998)[21] found expression of hTR by ISH in the basal layer of the epidermis of normal human skin, although the level was generally low.

The presence of hTERT expression in basal and supra-basal layers could be attributed to the fact that primitive stem cells lack or have low levels of telomerase activity, because they are quiescent for much of their life span. But these stem cells, identified by the longest telomeres rather than telomerase activity,[22] give rise to specialized transient amplifying cells that are highly proliferative.[21] Thus, the source of most of the detected telomerase activity in the basal or supra-basal regions of the epidermis is likely to involve subsequent proliferating descendents of stem cells.

In group II, 15/20 (75%) of photo-aged subjects showed positive hTERT expression. Ueda *et al.* (1997)[23] demonstrated telomerase activity by TRAP assay in 21 of 39 (54%) normal skin samples from sun-exposed sites. Similarly, Parris *et al.* (1997) found one of 2 (50%) telomerase positive normal skin samples to be from chronically sun-exposed skin.[24] Later on, hTERT expression was detected in three of seven samples (42.9%) of photo-aged skin.[25]

One of the major differences between intrinsic aging and photo-aging is the up-regulation of telomerase, which is an early feature of skin carcinogenesis.[23] The activation of telomerase had been implicated as the major mechanism for attainment of cellular immortalization in the molecular pathogenesis of most malignant tumors.[26]

In 7/10 (70%) of AK lesions, the epidermis showed positive hTERT expression. Nevertheless, Ueda *et al.* (1997)[23] found that 100% (9 of 9) AK was telomerase positive, using TRAP assay. However, Parris *et al.* (1999)[24] found 10 out of 22 samples (45%) of AK to be telomerase positive. Similarly, Wu *et al.* (1999)[25] detected

Table 2 Comparison between aged skin, adult protected skin and children protected skin as regards to hTERT expression using Kruskal-Wallis test

hTERT Expression	Aged (Group I) number = 10 (%)	Adult protected skin (Group IVb) number = 10 (%)	Children protected skin (Group IVd) number = 10 (%)	χ^2	*P*-value	
Epidermal						
0	5 (50)	6 (60)	6 (60)	0.250	0.903	nonsignificant
+1	5 (50)	4 (40)	2 (20)			
+2	0 (0)	0 (0)	2 (20)			
+3	0 (0)	0 (0)	0 (0)			
Dermal				–	–	
Negative	7 (70)	10 (100)	2 (20)			
Positive	3 (30)	0 (0)	8 (80)			

Table 3 Comparison between photo-aged skin, adult exposed skin and children exposed skin as regards to hTERT expression using Kruskal-Wallis test

hTERT expression	Photo-aged (group II) number = 20 (%)	Adult exposed skin (group IVa) number = 10 (%)	Children exposed skin (group IVc) number = 10 (%)	χ^2	*P*-value
Epidermal					
0	4 (20)	6 (60)	6 (60)	7.035	0.030*
+1	8 (40)	2 (20)	4 (40)		
+2	6 (30)	0 (0)	0 (0)		
+3	2 (10)	2 (20)	0 (0)		
Dermal				12.024	0.013*
Negative	10 (50)	8 (80)	10 (100)		
Positive	10 (50)	2 (20)	0 (0)		

*Significant.

Table 4 Comparison between exposed XP and children exposed skin as regards to hTERT expression using Mann–Whitney test and Fisher exact test

hTERT expression	Exposed XP number = 5 (%)	Children exposed skin (group IVc) number = 10 (%)	Mann–Whitney test	
			Z	*P*-value
Epidermal				
0	1 (20)	8 (80)	2.26	0.024*
+1	3 (60)	2 (20)		
+2	1 (20)	0 (0)		
+3	0 (0)	0 (0)		
Dermal			Fisher's exact test	
Negative	2 (40)	10 (100)	*P*-value = 0.02*	
Positive	3 (60)	0 (0)		

XP, xeroderma pigmentosa.
*Significant.

hTERT expression in 1 of 2 AK (50%), although hTR expression was detected in the two cases.

Harle-Bachor and Boukamp (1996)[19] suggested that full expression of telomerase activity correlated with a very early stage in immortalization process and that it may represent a useful marker for the switch to immortality. However, the finding of Wu *et al.* (1999)[25] of hTERT expression in one of two AK (50%), and in three of seven samples (42.9%) of photo-aged skin, showed that there was no significant difference between both samples. Besides, the nature of AK has been a matter of argument. Some consider it pre-cancerous as it is the initial lesion in a disease continuum that progresses to invasive squamous cell carcinoma (SCC).[27] However, it is not possible to tell which AK will progress to SCC.[28]

When we compared hTERT expression in aged and photo-aged skin, photo-aged skin showed greater expression (15/20 (75%) compared to 5/10 (50%)), yet the

difference was not statistically significant. On the contrary, Taylor *et al.* (1996)[29] reported increased levels of telomerase activity in sun-damaged skin, compared with sun-protected skin. Besides, Wu *et al.* (1999)[25] found positive hTERT expression in three of seven samples (42.9%) of photo-aged skin and in none (0 of 9) of aged samples. However, the same study showed similar hTR expression using RT-PCR in both sun-exposed and covered skin. Studying this matter on a larger scale of cases can clarify the difference.

Apparently normal skin samples from XP patients were found previously to have strong telomerase activity.[23] In our study, 4/5 (80%) of the epidermis of exposed skin biopsies from XP showed positive hTERT, closely similar to that obtained from photo-aged subjects (75%), but statistically significantly higher than age-matched group. Explanations for the previous finding could be: anatomically, the number of telomerase-positive cells may be higher in skin from sun-exposed sites. Besides, XP patients are extremely susceptible to UV carcinogenesis,[23] thus, microscopically undetected tumor cells might remain, especially in normal skin adjacent to skin tumors.

Interestingly, 3/5 (60%) of skin biopsies taken from XP sun-protected skin, showed positive hTERT expression. This could be because of inadequate repair of oxidative damage resulting from normal metabolism.[30] This defective DNA repair leads to degenerative changes and may be telomerase expression in cells not exposed to UV. However, the inadequate number of our XP group makes it difficult to establish such a conclusion, particularly with our finding that their control group expressed positivity in 4/10 (40%) of biopsies.

In our study, comparing hTERT expression in photo-aged skin, adult exposed, and children exposed skin revealed that photo-aged skin had greater expression, based on cumulative UV-activation of telomerase. Ueda *et al.* (1997)[23] suggested that either activation of telomerase or clonal expansion of telomerase-positive cells by chronic sun exposure to be an early feature of skin carcinogenesis.

Comparing hTERT expression in aged skin, adult protected skin, and children protected skin revealed that there was no statistically significant difference. Similarly, TRAP assay of telomerase in keratinocytes isolated from donors of different ages (ranging from 17 to 90 years) showed that the enzyme activity was not age-dependent.[19] Moreover, no significant differences in telomerase activity levels were observed in anagen hair follicles with increasing age or among the different types of male pattern baldness.[31] Thus, telomerase expression does not differ by age, but increases by cumulative sun exposure to be significantly increased in photo-aged skin.

Gilhar *et al.* (2004)[32] compared telomerase activity in specimens of normal skin obtained from sun-protected unexposed areas of the thighs of 14 older persons and 14 younger persons. They used telomerase PCR enzyme linked immunosorbent assay in their study, and they found that telomerase activity of the epidermis did not correlate with donor age, suggesting that in a self-renewing tissue with slowly cycling stem cells, telomerase was not a limiting factor for aging.

Forsyth *et al.* (2002)[3] illustrated regions of telomerase activity in the skin in the regenerative basal layer of the epidermis in human skin, and at limiting levels in differentiating transiently amplifying keratinocytes, but absent in the dermis or stratum corneum. Only the infiltration of lymphocytes into the skin was reported to cause transient telomerase activity in the dermis of skin biopsy samples.[6] However, hair follicles, a specialized skin appendage, displayed a specific pattern of telomerase, where telomerase activity (measured by TRAP assay) correlated with proliferative state.[23,31] Hoogduijn *et al.* (2006)[33] stated that following appropriate stimulation, telomerase activity was identified in follicle dermal stem cells capable of clonal expansion.

We found that the dermis of some of the skin biopsies taken from all the studied groups showed positive hTERT expression. It was positive in dermal fibroblasts ± lymphocytes, histiocytes, sweat gland ducts, hair follicles, sebaceous glands, and the lining of the cysts of Favre Racouchot. On the contrary, Olsen *et al.* (2007)[34] found that mature sebocytes were telomerase negative by IHC technique, and Harle-Bachor and Boukamp (1996)[19] found all extracts from fibroblasts to be negative and suggested the presence of telomerase inhibitors in the dermis. The molecular mechanisms of telomerase inhibition and activation as well as the consequences remain to be elucidated.

We found that dermal expression of hTERT is highly related to epidermal expression indicating crosstalks between the two skin layers.

Taken all together, hTERT activity seems to be adjusted irrespective of the individual's age. Moreover, hTERT expression is increased in sun-damaged skin (in both photo-aging and premature photo-aging), compared with sun-protected skin. However, this increase is insignificant until transformation occurs with the abnormal cells containing telomerase-dependent telomere maintenance mechanisms. Thus, lifetime cumulative sun exposure is an important factor. hTERT activity was also detected in skin from sun-exposed as well as sun-protected sites in XP patients, indicating that genetic abnormality is the decisive factor and not merely UV exposure.

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