Field Cancerisation Improvement with Topical Application of a Film-Forming Medical Device Containing Photolyase and UV Filters in Patients with Actinic Keratosis, a Pilot Study

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Abstract

Background: Actinic keratoses (AKs) are considered to be a ‘field of cancerization’ consisting of a histologically abnormal epithelium adjacent to tumour tissue. Treatment of the ‘field of cancerization’ is important for the prevention of neoplasm progression. UV radiation, especially UVB, produces genotoxic photoproducts such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPPs) in DNA, being major players in skin cancerization. The potential use of DNA photolyases in skin cancer prevention is increasingly being demonstrated. Topical application of a liposome formulation containing CPD photolyase onto human skin provides protection against UV-B-induced damages.

Objectives: To assess the effects of topical application of a medical device (Eryf-AK) containing a DNA-repair enzyme, photolyase, encapsulated in liposomes and UV filters, on cancerization field in actinic keratosis (AK).

Methods: 13 AK patients were included. Clinical, dermoscopic, and reflectance confocal microscopy (RCM) assessments, as well as skin biopsies, before and after a 4-week treatment were performed. Patients used Eryf-AK twice daily or only a sunscreen (3:1) with a similar sun protection factor (SPF) for one month.

Results: Erythema and scaling improved with Eryf-AK. RCM showed a reduction in scaling, detached corneocytes and polygonal nucleated cells in the stratum corneum (p=0.004, p=0.018, and p=0.021), an improvement of the atypical honeycomb pattern, and a decreased number of round nucleated cells at the spinous granular layer (p<0.0005 and p=0.019) with Eryf-AK while no improvement was noted with the sunscreen product. The mean RCM score for AK significantly improved from 0.76 to 0.27 (p=0.002) with Eryf-AK. Histological clearance of AK in 4 cases and an improvement with a focal AK associated with inflammation in 3 additional patients were also observed with Eryf-AK. A decrease in p21 expression (p=0.042) and a tendency to decrease PCNA expression was also observed with Eryf-AK (p=0.076).

Conclusion: Our results show a benefit from Eryf-AK in the treatment of AK cancerization field. The improvement was demonstrated clinically, by RCM, histologically and by immunohistochemistry. An improvement was also observed in the two patients with xeroderma pigmentosum, suggesting a benefit from this topical treatment in patients with this rare genetic disorder.

Keywords: Actinic keratosis; Field of cancerization; Photolyase; Reflectance Confocal microscopy; p21

Introduction

Actinic keratoses (AKs) are skin lesions showing a mild degree of keratinocytic atypia and are confined to the lower part of epidermis; they are considered either precancerous lesions or an incipient form of squamous cell carcinoma (SCC) [1-4]. A number of studies have shown that about 20% to 27% of cutaneous SCCs arise on AK lesions and approximately 8% of all AKs can progress to invasive SCCs [5]. AKs are also considered to be a ‘field of cancerisation’ consisting of an histologically abnormal tissue adjacent to tumour tissue. AKs are common in elderly and middle-aged fair skin people, appearing as rough, dry, scaly lesions that occur primarily on the sun-exposed skin areas [6,7]. Although the exact mechanism of AK pathogenesis is unknown, the UV-induced DNA damage is responsible for the initiation of the pre-cancerous process. UV induces the formation of major dimeric configurations based on covalent bonds between two adjacent pyrimidines that interfere with biological processes (e.g., transcription and replication) that are critical for cell viability. If left unrepaired, such lesions can induce mutations and skin cancer.

The carcinogenic effect of UV radiation, especially UVB but also in a lesser extent UVA, relies on the production of genotoxic photoproducts such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPPs), which subsequently interfere with critical biological processes that are needed for cell viability; being major players in skin cancerization [8,9]. Nucleotide excision repair (NER) is the molecular system employed by mammal cells to remove UV-induced DNA damage [10]. However, whereas the NER system recognises 6-4PPs and removes them efficiently, the recognition and removal of CPDs is poor.
evaluate the effect of Eryf-AK in the treatment of the precancerous field that appears promising as a diagnostic aid in many dermatologic evaluations. RCM is a non-invasive imaging technique for eliminating the dimers from DNA is nucleotide excision repair, a poorly effective process.

At a cellular level, the p53 gene has been found to be essential in the maintenance of genomic integrity through a blockage of DNA replication in response to DNA damage due to exposure to agents like UV light. Bcl-2 and p53 proteins are altered in keratinocytic hyperproliferative lesions, and showed a gradual up-regulation in the pre-tumorigenic (AK) and tumorigenic (SCC) lesions as compared to normal skin and non-tumorigenic lesions [15]. Besides, Einspahr et al. provided evidence suggesting a differential p53 expression in the progression of UV-induced skin carcinogenesis [16]. Later on, the authors suggested a potential use of biomarkers such as p53 expression as predictive markers for skin cancer risk [17]. More recently, Mateou et al. concluded that the biomarkers bcl-2 and p53 are useful tools to assess the severity of BCCs [18].

Recognition and treatment of AK is important for the prevention of neoplasm progression [19,20]. The potential use of DNA photolyases in skin cancer prevention is increasingly being recognised. Furthermore, it has been demonstrated that topical application of a liposome formulation containing CPD photolyases onto human skin provides protection against UV-B-induced damages [11,21].

The present study aimed to assess the effects of topical application of Eryfotona® AK-NMSC (Eryf-AK), a film-forming medical device containing DNA Repairsomes®, the DNA-repair enzyme photolyase encapsulated in liposomes and UV filters, on carcinization field in AK, by clinical, dermoscopic, histological and immunohistological evaluation. Besides, reflectance confocal microscopy (RCM) imaging analysis was also used. RCM is a non-invasive imaging technique that appears promising as a diagnostic aid in many dermatologic conditions [22-25]. It helps to bridge the gap between dermoscopy and histological analysis, allowing a horizontal evaluation of a lesion while producing in vivo images of the epidermis and superficial dermis at a resolution that approaches the one in histopathological specimens. In a study by Rishpon et al., mosaic RCM images demonstrated an increased frequency of abnormal RCM features across the spectrum of keratinocytic neoplasms [26]. In addition, the presence of an atypical honeycomb or disarranged pattern of the spinous-granular layer, round nucleated cells at the spinous-granular layer, and round blood vessels traversing through dermal papillae are the key RCM features in SCC.

Subjects and Methods

Design
A pilot, prospective, controlled, interventional clinical study to evaluate the effect of Eryf-AK in the treatment of the precancerous field in AK patients was carried out. Clinical evaluations, dermoscopy and reflectance confocal microscopy (RCM) evaluations were performed during the treatment; and histopathology and immunohistochemistry assessments were done at the beginning and at the end of the treatment period (4 weeks).

Subjects
Patients older than 50 years with AK with an area larger than 3.6x3.6 cm affected by multiple AK lesions in a sun-exposed skin area, were included. A written informed consent was obtained from all patients after having read and understood the information approved by the ethics committee. The study was approved by the institutional research board and was conducted according to the Declaration of Helsinki Principles.

Methods and statistical analyses

The evaluation area was identified and followed through the study by using a plastic wrap. At the screening visit, four areas were selected within this evaluation area and marked in a body chart. The whole area under assessment was documented with clinical pictures (Canon G11), 4 dermoscopy (DermLite photo, 3gen, Dana Farber) pictures and 4 RCM (Vivascope 1500, Lucid Corp) (0.8×0.8 cm) along the study. At baseline, two 3-mm punch biopsies were obtained from two representative areas (1 and 2) and were also documented with images. The test products, Eryf-AK or a sunscreen containing UV filters only (3;1); both with an identical sun protection factor (SPF) were applied in the treatment evaluation area for 4 weeks, in the morning and 4-6 hours later. After two weeks, a first assessment was performed with imaging techniques of the 4 areas. At the end of the treatment (week 4), a final evaluation was performed and 2 punch biopsies were obtained from the 2 areas (3 and 4) where no biopsies were performed at inclusion.

Clinical and dermoscopic evaluation
The clinical assessment was based upon the scoring of erythema, scaling, pigmentation, and follicular plugs within AK lesions. Clinical data, clinical images of the lesions and 4 dermoscopy images (area 1, 2, 3 and 4) were taken into account for the assessment. Semi-quantitative scoring scales were defined (Table 1a).

Confocal microscopy evaluation
The following previously described RCM criteria were assessed in the present study (Table 1b: 1) [27-31]. Three RCM features were evaluated at the stratum corneum level: presence of scaling, presence of detached corneocytes and presence of polygonal nucleated cells. 2) Three RCM features were evaluated at the spinous-granular layer: presence of an atypical honeycomb pattern, presence of a disarranged epidermal pattern and presence of round nucleated cells. 3) Two RCM criteria were considered at the dermis level: presence of round blood vessels traversing dermal papillae and presence of inflammatory cells. A Total RCM Score was calculated as the mean of all the scores. At least 3 mosaics from each area (1, 2, 3 and 4) were evaluated per patient at each visit (12 mosaics per visit and per patient).

Histopathology evaluation
The final diagnosis for each biopsy was reported as a dichotomous variable: presence or absence of actinic keratosis. The following histopathological characteristics were evaluated by an independent pathologist, blinded to sample source (either taken before treatment or after treatment): 1) Epidermis thickness: normal, atrophic, or hypertrophic; 2) Stratum corneum morphology: normal,
hyperkeratosis, or parakeratosis; 3) Presence of cellular atypia: focal or diffuse atypia (in AK) or absent (normal skin); 4) Extension of cellular atypia: only 1/3 of the epidermis thickness, or 2/3 of the epidermis thickness (no lesion showed atypia involving the whole thickness; such a case, would have been considered an in situ SCC); and 5) Inflammation: focal (patches in the dermis), diffuse (present in the whole superficial dermis), or absent. Inflammation was also categorised as lympho-plasmocytic or lympho-eosinophilic.

**Immunohistochemistry evaluation**

Immunohistochemical detection of p53 was performed on formalin-fixed paraffin-embedded punch biopsies with the automated immunohistochemical system TechMate 500® (Dako Co, Carpinteria, CA), using the EnVision system (Dako).

Immunohistochemical studies for p16, Bcl2, Ki67, p27, p21, and PCNA were performed on punch biopsies fixed with formalin and embedded in paraffin with the automated immunohistochemical system Bond Max (Menarini). The primary antibodies used in the study were p16 (CINtec Histology V-Kit), Bcl2 (Bond ready to use, optimally diluted antibody), Ki67 leica concentrated (1/100 dilution), p27 leica concentrated (1/40 dilution), p21 leica (dilution 1/40), and PCNA leica (dilution 1/50). The criteria used for the evaluation of p53, p16, Bcl2, Ki67, p27, p21, and PCNA are reported in Table 1c.

The statistical analysis for categorical variables was performed with a chi-squared test, using a Fisher’s test correction (for 2×2 tables) when required due to sample size. For continuous variables, mean score for each parameter before treatment was compared with the score at the end of the study. Student t test for paired samples was used for normally distributed variables, and an ANOVA was used for multiple comparisons.

**Results**

Thirteen patients with AK (12 males and 1 female; 2 had xeroderma pigmentosum; mean age 72 years) were included; one patient refused the treatment after the first evaluation and before the first biopsy (ery 009) and was withdrawn from the study. Two patients refused the second biopsy due to concomitant personal reasons (ery 006 and ery 009) and three patients received the sunscreen cream. The characteristics of the study population are summarised in Table 2.

Clinical assessment, dermoscopy, confocal microscopy and histopathology evaluation showed an improvement in AK lesions after Eryf-AK therapy. In contrast, no improvement was noted in the 3 patients that used the sunscreen.

An example of the clinical and dermoscopic evaluation is shown in Figure 1a. Erythema and scaling improved significantly (p=0.03
and p=0.028, respectively) in patients receiving Eryf-AK, and an improvement close to significance (p=0.06) was observed in follicular plugs, with no changes in pigmentation after the 4-week treatment period. Clinical images of a control patient after a one-month therapy with the sunscreen are shown in Figure 1b.

An example of comparative RCM imaging is shown in Figure 2. RCM findings are summarised in Table 3a. The presence of scaling, detached corneocytes, and polygonal nucleated cells in the stratum corneum decreased during the treatment with Eryf-AK (p=0.004, p=0.018, and p=0.021, respectively). We also noticed an improvement of the atypical honeycomb pattern and round nucleated cells at the spinous granulose layer (p<0.0005 and p=0.019, respectively). Finally, the mean RCM score for AK improved significantly (~65%) from 0.78 to 0.27 (p=0.002) in patients receiving topical application of Eryf-AK.

| Gender | Male | 12 |
|        | Female | 1 |
| Cutaneous Phototype | I | 1 |
| | II | 7 |
| | III | 3 |
| | IV | 2 |
| Xeroderma Pigmentosum | Yes | 2 |
| Type of predominant sun exposure | Intermittent | 3 |
| | Continuous | 10 |
| Sun burns | Yes | 8 |
| | No | 5 |
| Hair colour | Black | 7 |
| | Brown | 3 |
| | Blond | 2 |
| | Red hair | 1 |
| Eye colour | Black | 4 |
| | Brown | 3 |
| | Green | 1 |
| | Blue-gray | 5 |
| Sun exposure | Before 10 y.o. | 2 |
| | Between 10 y.o. & 18 y.o. | 1 |
| | After 18 y.o. | 1 |
| Use of sunscreens | Before 10 y.o. | 2 |
| | Between 10 y.o. & 18 y.o. | 2 |
| | After 18 y.o. | 2 |
| Actinic damage | Yes | 13 |
| Actinic keratosis | Yes | 13 |
| Actinic cheilitis | Yes | 3 |
| | No | 9 |
| | Unknown | 1 |
| Previous BCC | Yes | 5 |
| | No | 8 |
| | Unknown | 2 |
| Previous SCC | Yes | 3 |
| | No | 3 |
| | Unknown | 7 |
| Presence of solar lentigo | Some | 3 |
| | Multiple | 3 |
| | Many | 5 |
| | No | 0 |
| | Unknown | 2 |
| Previous UVA | Yes | 0 |
| | No | 13 |
| Number of nevi | <50 | 7 |
| | 50-100 | 1 |
| | 100-200 | 0 |
| | >200 | 2 |
| | Unknown | 3 |
| Previous history of cancer | melanoma | 4 |
| Previous familial history of cancer | melanoma | 5 |
| | colon carcinoma | 1 |

Table 2: Baseline characteristics of the studied population-N=13.
Concerning histopathological findings, AK lesions were improved after Eryf-AK use, reaching histological clearance in 4 patients and improvement with focal AK presence associated with inflammation in 3 additional patients.

Immunohistochemistry results are summarised in Table 3b. Interestingly, after 4 weeks of Eryf-AK treatment, a decreased p21 expression in suprabasal layers was observed (p=0.042) and a tendency to a decreased PCNA expression in the basal layer was also found (p=0.076) while the expression of p53, bcl2, p16, ki67, and p27 did not change significantly (Figure 3).

**Discussion**

UV-induced human SCC is a multistep process: normal sun-exposed skin progresses to sun-damaged skin followed by the AK pattern, with atypia of keratinocytes being present in basal and supra-basal layers of the epidermis; then to in situ SCC with atypical keratinocytes in the whole epidermis; and finally to invasive stages of SCC, with basal membrane being destroyed by the tumour. The process occurs simultaneously in all skin areas exposed to the same environmental carcinogen agent (UVB in our study), i.e. the cancerization field. In this cancerization field, all steps of the process may be present at the same time or sequentially and not all steps are necessary to develop an invasive tumour. Cancerization field refers to...
the presence of genetic abnormalities in a tissue chronically exposed to a carcinogen agent [32]. In a recent study, the need to treat not only the visible AK, but also the entire photodamaged surface (field-cancerization targeted therapy), was emphasized in order to reduce the potential risk of invasive carcinoma [33].

In the present study, in addition to the clinical assessment we used biomarkers and biopsies to assess the effect of Eryf-AK to improve the cancerization field of AK, as suggested by Einspahr et al. [17]. In our study, the application of Eryf-AK film-forming medical device containing photolyase and UV filters, twice a day for 4 weeks shows an improvement in the cancerisation field in actinic keratosis patients.

Confocal reflectance microscopy has been reported to be extremely useful in the evaluation of the cancerisation field morphology, with an improvement being shown for most previously described RCM criteria for AK. After 4-weeks, an improved corneocytes coherence and a reduced cytological atypia (less atypical honeycomb pattern, less atypical nucleated cells in the upper layers of the epidermis, and less large bright cells in the stratum granulosum) were observed, as previously described with other therapies effective on field cancerization [34].

In our study, the morphology improvement was also evident in the histopathological evaluation. In a recent study, Ulrich et al. found no improvement of AK at histopathological level after 16 weeks of treatment in a placebo control group while in the current study, the epidermal morphology was no longer consistent with a diagnosis of AK in about 50% of the samples from patients receiving Eryf-AK after only 4 weeks of treatment [35]. Inflammation was present in some biopsies before and after the treatment but, interestingly, the inflammatory profile changed from lympho-plasmocytic to lympho-eosinophytic. Future studies should assess the significance of inflammatory profile changes in the evolution of AK under treatment with Eryf-AK.

In all our samples, a high level of expression of proliferation (Ki67 and PCNA), pro-apoptotic (p53, p21), and anti-apoptotic markers (bcl2) was noted, suggesting a high level of actinic damages in the skin selected for our study [36].

Basal proliferating cell nuclear antigen (PCNA) expression also shows a decreasing trend with Eryf-AK treatment, in agreement with the findings reported by Einspahr et al. [17]. In their study, a lower PCNA expression was detected in sun-damaged skin compared with actinic keratosis, suggesting that Eryf-AK could partially reverse the AK phenotype. On the contrary, in this pilot study the expression of p53 after a one-month treatment did not change, with levels being high both before and after the treatment. Einspahr et al. noted that p53 expression was higher in AK patients as compared with sun-damaged skin, but also failed to find differences between AK in forearms and control skin in the contralateral forearms of the same patients [17]. The expression of p53 is a consequence of the presence of a mutant p53 protein in the cancerisation field that probably might not be directly influenced by the repair of CPDs induced by photolyase. A longer treatment period may be required to replace p53-mutant keratinocytes by wild-type keratinocytes. On the contrary, the expression of p21 and PCNA is related with the down-regulation induced by the reduction of CPDs and not by direct mutations. In the present study, a decreased expression was already observed after one-month treatment. To test this hypothesis, long-term follow-up studies with Eryf-AK treatment, including biopsy assessments at 3 or even 12 months, will be needed, in order to assess the potential delay in p53 expression improvement.

Most immunohistochemistry studies failed to detect a treatment-associated improvement, except for a decreased suprabasal p21 expression. p21 is a protein that plays a critical role in the cellular response to DNA damage, and its over-expression results in cell cycle arrest in response to the p53 checkpoint pathway [37]. Furthermore, Jans et al. demonstrated at a gene expression level that CPDs induce the expression of genes associated with repair and recombinational processing of DNA damage, as well as apoptosis [38]. The decreased p21 expression could therefore be a consequence of a decreased level of CPDs in the cells after a 4-week treatment with Eryf-AK.

We acknowledge the low number of patients as one of the limitations of the study, particularly for the patients receiving the sunscreen as control group. For this reason, pre-treatment versus post-treatment findings were compared in patients receiving Eryf-AK. However, improvement of the same RCM score system used in the present study was shown in another study after the application of 3% diclofenac sodium gel in 2.5% hyaluronic acid, a treatment already approved for AKs treatment [39,40].

In conclusion, our results demonstrate a clinical and subclinical benefit from Eryfotona® AK-NMSC, a medical device containing photolyase and UV filters, in the improvement of the cancerisation field in AK patients, based on clinical, RCM, histopathology and some immunohistochemistry findings. The main limitation of the study was the small number of controls included. Anyhow, the results of this pilot study support its potential use to reduce or improve the subclinical cancerisation field associated with AK and NMSC. Finally, the improvement in patients with xeroderma pigmentosum suggests a further use as a topical treatment that could reduce the number of skin cancers in patients with this rare genetic disorder associated to a very high risk.

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