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Does fluorescence diagnosis have a role in follow up of response to therapy in mycosis fungoides?

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KEYWORDS
Mycosis fungoides;
Fluorescence diagnosis;
Flowcytometry;
CD4+/CD7− T cells

Summary
Background: Monitoring of tumor burden during mycosis fungoides (MF) treatment, is crucial to adjust therapy accordingly. This is usually achieved through combined by clinical assessment with histopathological and immunohistochemical evaluation.
Aim: To assess the validity of fluorescence diagnosis (FD) in the measurement of response to therapy in early MF, using in comparison flow cytometric technique of skin biopsies for CD4+/CD7− malignant T-cell count before and after therapy.
Patients and Methods: Twenty-two patients of histologically proven early MF (stages Ia, Ib, IIa) were subjected to fluorescence diagnosis of their most affected skin lesion before and after 12 weeks of phototherapy with or without combination therapy. In comparison flow cytometric assessment of skin biopsies for CD4+/CD7− malignant T-cell count was evaluated before and after therapy from skin biopsy of the same lesion.
Results: All tested MF lesions showed varying degrees of fluorescence by FD at week zero, with a mean accumulation factor (AF), which is the fluorescence ratio between the tumor tissue and normal skin, of 2.2. After 12 weeks of therapy, the mean AF showed significant reduction to 1.94 ($p = 0.009$). The percent of CD4+/CD7− cells dropped significantly after treatment ($p = 0.029$). No correlation between CD4+/CD7− cell counts and the mean AF could be deduced.
Conclusion: In cases of mycosis fungoides, fluorescence diagnosis can represent an effective tool for evaluating the response to therapy. Changes in accumulation factor values can be used for follow-up of therapy in the same patient, but it should not be used as an absolute value.

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Introduction

Mycosis fungoides (MF) is a clinically indolent neoplastic proliferation of T cells that typically involves the skin and is almost always of T-helper cell lineage. Skin biopsy with routine histology is considered the most important means in establishing the diagnosis of MF. Patients with early stage MF are allocated to a phototherapeutic regimen, with or without combination therapy. Positive response to therapy is assessed clinically by the physician and pathologically by the scarcity of dermal lymphocytes and the absence of atypical cells. Without additional techniques, histologic interpretation of skin biopsy specimens is characterized by low accuracy and reliability [1].

PCR analysis of T-cell receptor genes in skin biopsies is performed in some centers to evaluate the presence of abnormal clones of T-cell in the dermal infiltrate, with a high diagnostic accuracy (92.7%). The finding of a clonal T-cell rearrangement is more closely associated with the histological pattern [2]. However, such an investigation besides being invasive regarding the need to perform repeated biopsies over the indolent disease course, is costly to be implemented for routine follow-up.

Therefore, a search for an objective non-invasive type of investigation allowing for evaluation of malignant infiltrate of mycosis fungoides' atypical cells would be highly beneficial for patients suffering from this chronic relapsing cutaneous T cell lymphoma. The behavior of such T-cells is dominated by their propensity to home to the skin, to be activated and persist in an activated state, and to achieve clonal dominance, thereby accumulating in the skin, lymph nodes, and peripheral blood [3].

Fluorescence diagnosis technique entails the application of 5-aminolevulinic acid (ALA) resulting in increased synthesis of porphyrins, leading to a higher concentration ratio of protoporphyrin IX (PpIX) in tumor tissue, because of their increasing metabolic demands [4]. In dermatology FD has been used previously in diagnosis and follow-up of lesions with keratinocytes hyperproliferation [5,6]. To date its clinical use in the management of tumors of lymphocytic origin has not been readily documented. There is good *in vitro* evidence that lymphocytes accumulate ALA efficiently with subsequent cytotoxic and DNA-damaging effects [7], and that malignant T-Lymphocytes cultured from blood of lymphatic leukemia patients exhibit high sensitivity to the damage invoked by ALA-PDT [8]. In one study [9], application of ALA for 6 h on MF lesions showed moderate fluorescence, and authors were able to sharply identify the lesional margin [9]. FD was investigated in the present work for the follow-up of MF patients under treatment as an alternative to repeated skin biopsies for histopathology and immunohistochemistry, in order to minimize the usage of such an invasive procedure.

Aim of the study

To assess the validity of fluorescence diagnosis (FD) in the measurement of response to therapy in early MF, using in comparison flow cytometric technique of skin biopsies for CD4+/CD7− malignant T-cell count before and after therapy.

Patients

This study was designed as prospective investigational study and conducted at the out-patient clinic of Dermatology Department in Kasr El-Aini, Cairo University Hospitals. Twenty-five patients suffering from early stage MF (stages IA, IB, IIA) were included in this study, after obtaining ethical approval from Dermatology Department Kasr Al-Aini Research Ethics Committee, as well as written consents from patients. From each patient a skin biopsy stained with hematoxylin and eosin was obtained before inclusion in the study to confirm diagnosis of mycosis fungoides. Exclusion criterion was patients with ≥ stages IIB Mycosis Fungoides according to TNM staging classification. Patients were receiving phototherapy with or without combination therapy according to their identified stage.

Methods

Clinical examination

At time of inclusion the following was done: obtaining detailed history, total body clinical examination, determining the site of the most affected lesion to be selected for further clinical follow-up, fluorescence diagnosis and flow cytometric evaluation before and after treatment, one lesion per patient. Selected lesions were documented as patch or plaque, and scored for severity as follows: Color of the skin lesion, scaling, pruritus, and infiltration (Table 1). After 12 weeks of consecutive phototherapy/photochemotherapy with or without combination therapy the degree of improvement of the selected lesion was documented and the clinical response was scored as follows: Complete response (80—100% improvement), partial response (40—79% improvement), poor response (<40% improvement). The side effects of FD were recorded after the procedure.

Table 1 Clinical scoring method for lesion severity.

Item	Characteristic	Score
Color of the skin lesion	Faintly erythematous	+
	Erythematous	++
	Deeply erythematous	+++
Scaling	No scaling	—
	Mild scaling	+
	Moderate scaling	++
	Severe scaling	+++
Pruritus	No pruritus	—
	Mild pruritus	+
	Moderate pruritus	++
	Severe pruritus	+++
Infiltration	No infiltration	—
	Infiltrated	+
	Severe infiltration	++

Treatment using phototherapy/photochemotherapy with or without combination therapy

For NB-UVB therapy: The starting dose was 0.74 J/cm^2/session, according to tables supplied by the device manufacturers. The dose was increased by 20% of the previous dose until the lesions showed mild erythema, fixing the dose thereafter. All patients received three sessions per week. NB-UVB was delivered by a UV cabin (Waldmann GmbH, Germany) with a peak emission at 311 nm.

For PUVA therapy: Patients received 8-methoxypsoralen 0.5—0.7 mg/kg (10 mg/tablet; Neo-Meladinine, Memphis Pharmaceuticals, Egypt) with a meal, 2 h before exposure to UVA. Sessions started at a dose of 1.5 J/cm^2/session, which was increased every second session by 0.5 J/cm^2 until clinical improvement occurred. Dose was then fixed and sessions were administered three times per week, with a maximum allowable dose at 7 J/cm^2/session. The radiation source was a UVA 1000 phototherapy cabin containing 32 lamps emitting UVA with a peak at 365 nm (Waldmann GmbH, Germany).

Combination Therapy: Patients refractory to PUVA received additional low dose IFN-α, 1 million units 3 times per week subcutaneously.

Fluorescence diagnosis (FD) procedure

Amino Levulinic Acid hydrochloride was obtained from Sigma Aldrich Company as purified powder. A cream containing 20% ALA was freshly prepared by the pharmacist. The selected site was marked for a rectangular shape to include normal skin (Fig. 1), it was then cleaned with saline, 5-ALA HCl cream was applied for the whole area and covered by an occlusive dressing. The cream was applied for 3 h, as the mean contrast values peak in tumors in relation to adjacent normal skin after 3 h ALA application [10,11].

Fluorescence intensity on the skin was then recorded using a digital fluorescence imaging system (Dyaderm, Biocam GmbH, Regensburg, Germany), with images taken at a fixed distance of 8 cm. This system consists of a flash light (xenon light source with a custom band pass filter [370—440 nm]) and a 12-bit charged coupled device (CCD) camera combined in one adjustable arm attached to a computer system equipped with custom image capturing software. As PpIX fluorescence emission consists of light in the red spectrum, the red pixels of CCD camera were used to generate a fluorescence image. In this way a normal-colored image and a fluorescence image were processed in real time. Because of the short exposure time to the excitation light, photobleaching of PpIX was minimized. Two pictures of the same spot — one in red channel mode (PpIX fluorescence) and the other in green channel mode (autofluorescence) — were recorded and processed almost simultaneously. The resulting fluorescent image can be false-color coded and superimposed to the normal RGB image. This provides the position and size of the lesion within the clinical image of the skin. The computerized detection system is able to enhance the contrast by image analysis in consideration of autofluorescence. By computing the relative intensities of the PpIX and autofluorescence image, inhomogeneities due to imperfections of the excitation light field are automatically corrected. The resulting image is referred to as 'PpIX filtered'. PpIX-filtered false-color images automatically display (image segmentation) the highest fluorescence value in red and the lowest in blue. Images were recorded in 16-bit gray-scale tagged image file format (tiff).

Analysis of fluorescence images: The Dya-Derm FD system is equipped with digitalized image analysis technique which allows for quantification of the ratio of PpIX fluorescence; as well as pseudo-color images which allow for better visualization of areas of higher porphyrin accumulation by the red fluorescence observed. For each patient, calculation of protoporphyrin IX (PpIX) accumulation factor (AF) was done: AF denotes the fluorescence ratio between tumor tissue and normal skin.

Flow cytometric (FCM) evaluation of abnormal T-lymphocytes in skin biopsies

Although routine H&E biopsy is the standard for evaluating MF response to therapy, it usually yields a rough estimate of the degree of malignant inflammatory infiltrate regression. On the other hand flow cytometric evaluation of malignant T cell infiltration in skin lesions would yield a precise numeric value. Thus, fluorescence diagnosis determination was compared to flow cytometric assessment of 5 mm skin biopsies for CD4+/CD7− malignant T-cell count. It was evaluated before and after therapy from skin biopsy of the same lesion in each patient. CD4+/CD7− T cells were chosen to be assessed, being the most commonly encountered malignant T cell in cases of MF. Touch imprints were prepared from the biopsy, then examined under light microscope after staining with heamatoxylin and eosin to confirm presence of lymphocytes in the specimen. Cells from biopsied skin were dispersed by squeezing the tissue against nylon mesh of pore size 50—70 μm into a glass beaker, adding phosphate buffered saline solutions (PBS) to get single cell suspension, which was centrifuged 4—5 min at 1200—1400 round per minute (RPM). Immunophenotyping staining for FCM analysis were performed according to manufacturer's instructions (Becton Dickinson, Bioscience, USA) using a FACScan flow cytometer.

Statistical method

SPSS version 16 was used as a statistical method. Numerical data were identified as Mean $\pm$ Standard deviation. Non-parametric tests as Mann—Whitney and Wilcoxon tests for paired samples were used to compare between the 2 groups: patients before therapy and patients after therapy: Accumulation Factor (AF), CD4+ve/CD7−ve cells, severity of the disease and degree of improvement of the disease, before and after 12 weeks of therapy. Sub grouping of patients according to the stage of the disease were analyzed by Mann—Whitney's test. For correlation between different parameters the Pearson's test was used. In all tests *P*-value: $\leq$0.05 is considered significant.

Results

Only 22 patients completed the 12 weeks study period. Demographic and clinical data of patients are summarized

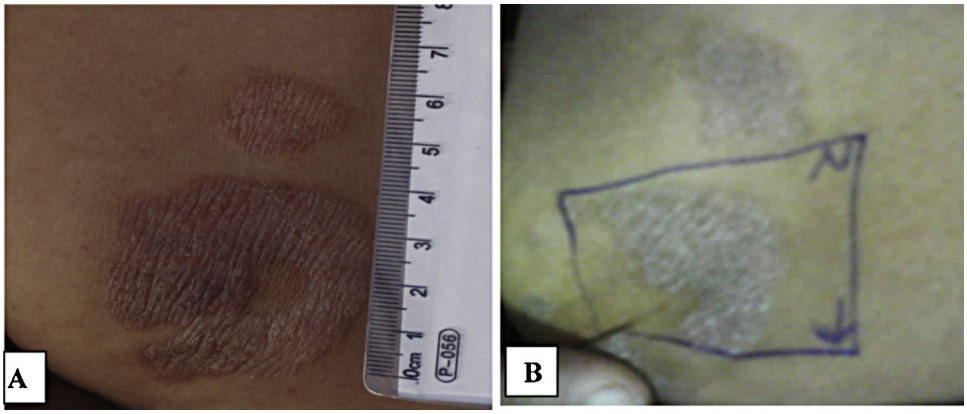

Fig. 1 Clinical picture of the MF lesion in patient No 17. (A) Before treatment, (B) 12 weeks after receiving PUVA therapy. Note clinical improvement as regards decreased erythema, decreased infiltration, however scaling is still evident.

in Table 2. The clinical severity of lesions before inclusion in the study and the degree of clinical response of skin lesions are summarized in Table 3. After 12 weeks of treatment with Nb-UVB or PUVA, the less severe skin lesions showed a better degree of clinical response.

Table 2 Summary of demographic and clinical data of patients: ($n = 22$).

Age	Min: 13 Max: 65 Mean ± SD: 35.18 ± 17.034
Sex	Males: no. = 10 (45.5%) Females: no. = 12 (54.5%)
Extent of the disease	Min. = 10% Max. = 75% Mean ± SD: 42.27% ± 19.86%
Disease stage	Stage IA: no. = 7 (31.8%) Stage IB: no. = 14 (63.63%) Stage IIB: no. = 1 (4.5%)

Min.: minimum; Max.: maximum; SD: standard deviation.

Table 3 Clinical severity before inclusion in study and degree of clinical response in each patient of his/her skin lesion: after 12 weeks (no of patients = 22).

The severity of the skin lesion	Mild lesions no. = 8 (36.4%) Moderate lesions no. = 9 (40.9%) Severe lesions no. = 5 (22.7%)
Degree of clinical response	Lesions showed complete clinical response. No. of lesions = 7 (31.81%) Lesions showed partial response. No. of lesions = 11 (50%) Lesions showed no clinical response. No. of lesions = 4 (18.18%)

Results of fluorescence diagnosis before and after 12 weeks of therapy

Using the FD technique all treated lesions showed varying degrees of fluorescence. For quantitative evaluation of the tumor burden in this study the accumulation factor (AF) for the most severe skin lesion was assessed. It is the fluorescence ratio between the tumor tissue and normal skin. The mean accumulation factor was found at time of start of study to be 2.2. This mean decreased significantly after 12 weeks of therapy to reach 1.94 (Table 3, Fig. 2A, B). The change in AF values before and after treatment is described as AF difference. A positive correlation between the AF difference and the degree of clinical response was obtained after 12 weeks of therapy (Table 5, Fig. 4).

Results flow cytometric (FCM) analysis before and after 12 weeks of therapy

It is a quantitative diagnostic method for the burden of malignant T-cells, having abnormal phenotypic expression, in skin biopsies obtained from the most affected skin lesions. The percent of CD4+/CD7− cells showed a statistically significant decrease after treatment (Table 4, Fig. 3A, B), denoting that the number of abnormally proliferative cells T-cells decreased with therapy in a statistically significant manner. However, the difference between the percentage of abnormal malignant T cells between before and after therapy did not correlate directly with the obtained clinical response (Table 5).

Comparison between FD and FCM results

The decrease in malignant T-cells in the skin could be detected quantitatively by using the fluorescence diagnosis technique as well as by flow cytometric analysis of malignant CD4+ve/CD7−ve cells. However, no correlation was found between the Accumulation Factor (AF) and the percentage of malignant T-cells (CD4+/CD7− cells %) estimated by the flow cytometry (CD4+ve/CD7−ve cells %) before or after therapy (Table 5).

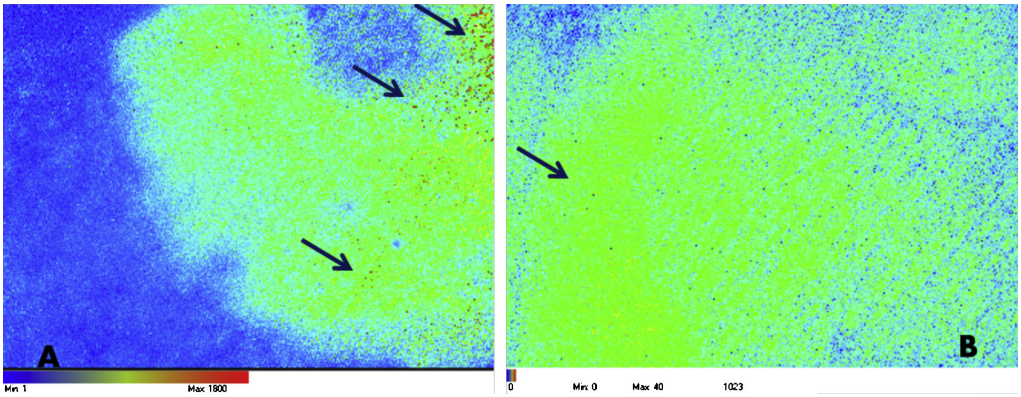

Fig. 2 Fluorescence diagnosis image of patient No 17 from mycosis fungoides skin lesion, (A) before treatment (week 0): note high number of red/orange spots (arrow) denoting high release of PPXI from malignant hyperproliferative cells, compared to the yellow/green color of less active cells. (B) Same lesion after treatment (week 12): denoting decreased number and scattered red spots of malignant cells (arrow).

Correlation of fluorescence results and flow cytometric results to the stage of the disease

The results of FD and FCM were evaluated after categorizing the patients according to MF disease stage, but no significant correlations were detected (Table 4).

Drawbacks of FD

During the occlusion period (3 h), 3 patients complained of a burning sensation at the site ALA application. All patients complained of the long waiting period for 3 h until performing the FD image.

Discussion

The use of FD in evaluating treatment response was investigated in this study in an attempt to find an alternative to the subjective clinical judgment or the pathologic evaluation of repeatedly invasive skin biopsies in MF patients. All treated plaques demonstrated varying degrees of fluorescence after incubation with ALA-HCl for 3 h at time of inclusion in the study, which decreased significantly at the end of treatment period. This denotes that FD was able to detect the clinical changes in the lesions, and the clinical improvement of skin lesions was correlated to the decrease in the amount of malignant T cell hyperproliferation. This was evidenced by the decreased uptake of the photosensitizer by the malignant T-cells present in patches and plaques of MF. After 12 weeks of therapy the mean value of accumulation factor (AF) decreased significantly in response to treatment in the examined lesions. Also the difference in the mean values of AF before and after treatment correlated significantly with the degree of clinical response (p value 0.041).

In a previous prospective study evaluation of treatment of plaque-phase mycosis fungoides with photodynamic therapy (630 nm, 37 J/cm^2; Aktilite) was performed and the results obtained were compared using fluorescence photography. All treated plaques showed positive fluorescence that precisely followed the borders of the covered plaques, and fluorescence diminished in intensity as infiltrates decreased; it was therefore considered that fluorescence positively correlated with clinical assessments [12]. In a more recent study photodynamic therapy showed 75% improvement in localized MF with no difference in response between patches and plaques [13]. It is to be noted that the AF ratio varies from one hyperproliferative condition to another, according to variation in the level of ALA-induced protoporphyrin accumulation. It was found more pronounced in

Table 4 Summary of the fluorescence diagnosis (represented by AF) and flowcytometric analysis (represented by CD4+/CD7− cells) results: (n = 22).

Item		Start of the study (week 0)	End of the study (week 12)	P-value
Accumulation factor (AF)	Minimum	1.23	1.20	0.009*
	Maximum	4.50	3.27	
	Median	2.11	1.70	
	Mean ± SD	2.20 ± 0.68	1.94 ± 0.64	
CD4+ve/CD7−ve cells	Minimum	2.34	4.78	0.029*
	Maximum	60.71	37.39	
	Median	27.84	4.78	
	Mean ± SD	27.46 ± 19.26%	6.97 ± 7.50%	

* P-value < 0.05 = significant.

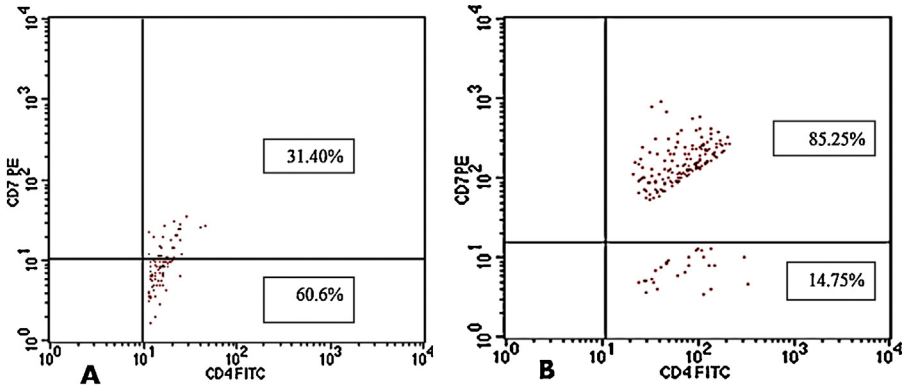

Fig. 3 Representative flowcytometry histogram of patient no. 17. (A) Before treatment (week 0) shows predominately CD4+ve/CD7−ve malignant T-cells, 60.6%, lower right quadrant. (B) Same lesion after treatment (week 12): shows predominately CD4+ve/CD7+ve normal T-cells, 85.25%, upper right quadrant.

psoriasis (1.77) in comparison to cases of actinic keratosis (1.37) [14]. Flow cytometric analysis has proven to be an efficient and sensitive method to detect and enumerate Mycosis Fungoides/Sezary Syndrome cells, as neoplastic T cells frequently have an altered level of expression of various surface T-cell markers compared with normal T cells. These differences were found sufficient to distinguish normal T cells from neoplastic T cells in the same sample [15]. It is used extensively in initial blood staging and monitoring tumor burden during therapy in patients with mycosis fungoides or Sezary syndrome [16].

Although flow cytometry (FCM) is not used routinely in the evaluation of skin biopsy specimens, some studies have used it for the analysis of cells from cutaneous tissues. One study has demonstrated the usefulness of flow cytometry as a diagnostic tool for MF, where among 14 cases histologically and clinically suggestive of MF, flow cytometry identified a T-cell abnormality in 11 of them. Phenotypic abnormalities were defined as: absent CD2, CD5, CD7, or CD26 expression; reduced expression of CD2, CD3, CD4, CD5, or CD8 compared with normal lymphoid cells within the same sample; or expression of CD30 [17]. Loss of CD7 is particularly common in MF. The most common abnormally expressed antigen was CD3, followed by CD7, CD5, and CD2. Except for CD7, abnormally dim or bright antigen expression was more common than deletion [18].

According to previous literature [17,18] we chose to evaluate the amount of T-cells with the CD4+CD7− phenotypic abnormality in skin biopsies in response to therapy. In the current study the mean values for the malignant T-cells detected by FCM decreased in a statistically significant manner after 12 weeks of therapy. The significant decrease in the amount of malignant T-cells detected by FD was also paralleled by a decrease in the number of CD4+CD7− T-cells detected by flow cytometric analysis. In this case FD would be more comprehensive, as malignant cells with all phenotypic expressions will take up protoporphyrin, in contrast to flow cytometry which was used to evaluate the most common single subtype of malignant T cells. In 1998, Edstrom and coworkers reported that treating mycosis fungoides plaques with photodynamic therapy resulted in significant reduction of the lymphocytic infiltrate, particularly of lymphocytes that are positive for CD4 and negative or only weakly positive for CD7. They also used TUNEL staining to demonstrate that the decreased infiltrate did not result from

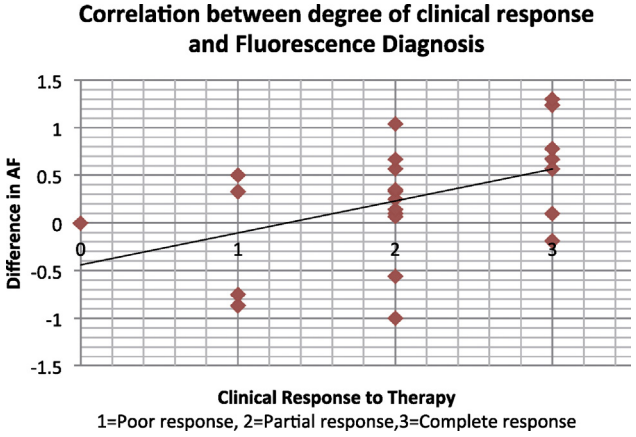

Fig. 4 Correlation between degree of clinical response and fluorescence diagnosis, showing a positive correlation between the accumulation factor difference and the degree of clinical response after 12 weeks of therapy; $r = 0.438$, $P = 0.041$*.

Table 5 Correlation between degree of clinical response and fluorescence diagnosis (as AF difference) and flow cytometric analysis (as CD4+ve/CD7−ve difference): ($n = 22$).

Item	Pearson correlation (r)	P-value
AF difference vs. degree of clinical response	0.438	0.041*
CD4+ve/CD7-ve difference vs. degree of clinical response	−0.159	0.478
AF difference vs. CD4+ve/CD7-ve difference	−0.360	0.100
Stage of the disease vs. Accumulation factor	0.678	0.709
Stage of the disease vs. CD4+ve/CD7−ve cells	3.286	0.193

* P-value < 0.05 = significant.

apoptosis of atypical lymphocytes but rather of a reduction in proliferating cells [19].

The difference in the means of CD4+CD7− cells shown after as compared to before therapy did not correlate with the degree of clinical response. It was also noted that both FD and flow cytometric analysis did not demonstrate a correlation to each other, neither before nor after therapy. This could be explained by the fact that for flowcytometric analysis only CD4+/CD7− cells were evaluated in this study. Deletion of CD7 was observed in only 73% of cases in one study [17], and aberrant expression of CD7 was found in 67% of biopsies in another one [20]. It could be speculated that malignant T-cells in our study group of 22 MF patients had phenotypic abnormalities other than CD4+/CD7−, which we did not investigate for, thus not all malignant T-cells were taken into consideration. Even if other antigenic abnormalities were to be investigated for malignant T-cells in this study by FCM analysis, it would not ensure a positive correlation with the clinical response, as in a recent study the utility of FCM in evaluating skin biopsies for mycosis fungoides was evaluated, to reveal a 78% clinical sensitivity, where abnormal T-cell populations were identified in 14 of 18 patients with histologically and clinically confirmed MF [20]. On the other hand FD evaluates the amount of malignant or hyperproliferating cells, irrespective of their antigenic determinant. Thus, the changes in all malignant T-cells are taken into account in this follow-up method, therefore would be more accurate in reflecting the clinical response. Furthermore, the stage of the disease did correlate neither to level of AF, nor to percentage of malignant CD4+/CD7− cells. This is to be expected since the stage of MF does not include the severity or type of skin lesion only, but also the extent of skin lesions, whether more or less than 10% of skin surface area. In this study FD was performed on a single skin lesion only.

It is to be noted that due to limited power of penetration of ALA into the deep dermis, lesions such as tumors and nodules of MF were supposed not to be readily visualized by the FD technique, therefore excluded from the study.

The drawback of the long waiting period in the clinic for 3 h should be weighed against its advantage of being non-invasive and very practical for young patients. The possibility of evaluating FD results immediately after the image uptake along with follow-up of previous FD images of the same patient in the same session using the digital image analysis system makes it an attractive diagnostic option.

We have for the first time utilized the noninvasive FD technique in the follow up of MF patients response to therapy in 22 cases with quantification of the tumor burden. Further similar studies should be performed to corroborate the value of FD in following treatment response instead of relying solely on clinical judgment, which varies according to physician's expertise or exposing patients to the recurrent traumatizing procedure of obtaining skin biopsies.

Conclusions

Fluorescence imaging is an attractive diagnostic technique for skin tumor assessment, with the potential to come more and more into clinical use, especially for tumors of lymphocytic cell origin. Changes in accumulation factor values can be used for follow-up of therapy in the same patient, as it parallels changes in clinical response. It should not be used as an absolute value.

Recommendations

FD should be implemented in the routine follow-up of therapeutic efficacy for cases of MF.

Further studies should be performed on advanced stages of MF with different application times of photosensitizers.

Further studies should be made to correlate the results of FD to the more accurate PCR for T-cell gene rearrangement, which if correlated; one could use FD safely to decide withdrawal of phototherapy.

Further studies should be performed comparing the results of FD with the results of histopathological evaluation of skin biopsies in response to therapy.

Limitations of the study:

Phenotypic abnormality tested by flowcytometry was restricted to only one type, CD7 deletion, whereas other phenotypic abnormalities may occur in MF.

Conflict of interest

The DyaDerm system was donated to the Dermatology Department, Cairo University by Biocam GmbH, Regensburg, Germany. The firm had otherwise no relation with the study idea, design, method, study results analysis, manuscript preparation or publication decision.

Disclosures

None declared.

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